SbnG, a Citrate Synthase in *Staphylococcus aureus*  
*A NEW FOLD ON AN OLD ENZYME*  

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**Background:** *Staphylococcus aureus* contains a second, iron-regulated citrate synthase.  
**Results:** SbnG is a citrate synthase within the phosphoenolpyruvate/pyruvate superfamily.  
**Conclusion:** The structural similarity of the SbnG active site to TCA cycle citrate synthase active sites suggests convergent evolution.  
**Significance:** SbnG is defined as a new structural class of citrate synthase.

In response to iron deprivation, *Staphylococcus aureus* produces staphyloferrin B, a citrate-containing siderophore that delivers iron back to the cell. This bacterium also possesses a second citrate synthase, SbnG, that is necessary for supplying citrate to the staphyloferrin B biosynthetic pathway. We present the structure of SbnG bound to the inhibitor calcium and an active site variant in complex with oxaloacetate. The overall fold of SbnG is structurally distinct from TCA cycle citrate synthases yet similar to metal-dependent class II aldolases. Phylogenetic analyses revealed that SbnG forms a separate clade with homologs from other siderophore biosynthetic gene clusters and is representative of a metal-independent subgroup in the phosphoenolpyruvate/pyruvate domain superfamily. A structural superposition of the SbnG active site to TCA cycle citrate synthases and site-directed mutagenesis suggests a case for convergent evolution toward a conserved catalytic mechanism for citrate production.

*Staphylococcus aureus* is a Gram-positive bacterium that colonizes a variety of mammalian hosts, specifically skin and mucosal membranes of the nasal passage and respiratory tract. In hospital settings, *S. aureus* has a substantial negative impact because of nosocomial cases of pneumonia and surgical wound infections (1). *S. aureus* can infiltrate and colonize many human tissue types, leading to a broad range of diseases from boils and sinusitis to more serious conditions like endocarditis and meningitis. Survival of *S. aureus* depends on the ability to extract iron from the mammalian host. However, iron is actively sequestered as a form of innate immunity against bacterial infection by host proteins such as transferrin, lactoferrin, haptoglobin, and hemopexin (2). In response, *S. aureus* has evolved multiple iron transport systems to maintain a supply of iron from various host sources (3). One of the most commonly employed strategies by bacterial pathogens is the use of siderophore iron acquisition systems. Siderophores are low molecular weight, ferric iron chelators that are synthesized and secreted to scavenge iron from the extracellular environment (for a review, see Ref. 4).

*S. aureus* produces two α-hydroxycarboxylate siderophores termed staphyloferrin A (SA) and staphyloferrin B (SB) (5, 6). Staphyloferrin A is composed of two citrate molecules linked to d-ornithine, assembled by synthetases encoded in the *sfa* gene cluster (7, 8). Staphyloferrin B is synthesized from L-2,3-diaminopropionic acid, citrate, and α-ketoglutarate, assembled by synthetases encoded in the *sbn* gene cluster (9). In contrast to SA, SB is not widespread among staphylococcal spp. and is largely limited to the more pathogenic coagulase-positive staphylococci (10). The biosynthetic gene cluster for SB together with its transport operon (*sirABC*) has only been identified in *S. aureus*, the *Staphylococcus intermedius* group (11), and a select few coagulase-negative staphylococci (e.g. *Staphylococcus arletiae* and *Staphylococcus equorum*). Furthermore, the biosynthetic genes for SB are among the most highly up-regulated under iron restriction and in human blood and serum (12, 13), suggesting an important role for SB in staphylococcal pathogenesis.

Growth of *S. aureus* under iron-restricted conditions results in a global shift in gene expression that is collectively known as the “iron-sparing response” (12). Iron restriction results in the up-regulation of iron uptake systems, including the staphylo-

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<sup>2</sup> The abbreviations used are: SA, staphyloferrin A; SB, staphyloferrin B; DTNB, 5,5′-dithio-bis(2-nitrobenzoic acid); SEC-MALS, size exclusion chromatography multangle static light scattering; r.m.s.d., root mean square deviation; DDGA, 2-dehydro-3-deoxy-galactarate aldolase; MPS, macrophage synthase; PDB, Protein Data Bank.
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ferrin siderophore pathways. Conversely, many metabolic pathways with iron-containing enzymes are down-regulated in an effort to conserve the iron pool necessary for survival. A notable down-regulation is observed of TCA enzymes including the citrate synthase, CitZ (12, 14). Citrate and α-keto-glutarate production are essential for the assembly of SB, leading to an apparent, but recently explained, paradox of how SB production is maintained under iron limitation.

Although TCA cycle activity is essential for the synthesis of SA, SB is freed from this dependence by enzymes encoded in the sbn gene cluster, expressed under iron restriction and during glycolytic growth (15, 16). Recently, SbnA and SbnB were shown to form a metabolic pathway that produces L-2,3-diaminopropionic acid and α-ketoglutarate from O-phospho-L-serine and L-glutamate (15). Both O-phospho-L-serine and L-glutamate can be derived from glycolysis and L-glutamine degradation, pathways independent of the iron sparing response (15). To alleviate the loss of citrate production, a second citrate synthase is encoded by sbnG (17). Both CitZ and SbnG can contribute citrate to the formation of SB; however, only SbnG is up-regulated upon iron starvation (12–14, 16). The precursors to citrate production, oxaloacetate and acetyl-CoA, are also predicted to be available under iron restriction.

SbnG can contribute citrate to the formation of SB; however, out of the requirement for citrate to be generated during iron restricted growth. Indeed, SbnG represents a unique citrate synthase by sequence analysis; instead, SbnG was reclassified as a class II aldolase by phylogenetic analysis; it was shown to form a metabolic pathway that produces L-2,3-diaminopropionic acid and α-ketoglutarate from O-phospho-L-serine and L-glutamate (15). Both O-phospho-L-serine and L-glutamate can be derived from glycolysis and L-glutamine degradation, pathways independent of the iron sparing response (15). To alleviate the loss of citrate production, a second citrate synthase is encoded by sbnG (17). Both CitZ and SbnG can contribute citrate to the formation of SB; however, only SbnG is up-regulated upon iron starvation (12–14, 16). The precursors to citrate production, oxaloacetate and acetyl-CoA, are also predicted to be available under iron restriction, through anaplerotic reactions and decarboxylation of pyruvate by the pyruvate dehydrogenase complex (pdhABC), respectively (12, 16). SbnG, therefore, provides an alternate route for citrate to enter the biosynthetic pathway for SB, independent of TCA cycle activity.

Homologs of SbnG have also been identified in the biosynthetic gene clusters that produce the siderophores achromobactin and vibrioferrin, suggesting that a unique group of citrate synthases may have evolved for siderophore biosynthesis out of the requirement for citrate to be generated during iron restricted growth. Indeed, SbnG represents a unique citrate synthase, originally annotated as a metal-dependent class II aldolase by sequence analysis; instead, SbnG was reclassified as a metal-independent citrate synthase that utilizes oxaloacetate and acetyl-CoA (17).

To gain insight into the catalytic mechanism, we report the x-ray crystal structure of SbnG and an active site variant bound to oxaloacetate. Although SbnG is homologous to metal-dependent class II aldolases, we demonstrate through phylogenetic analysis that SbnG is the archetype of a separate metal-independent clade with homologs from other siderophore biosynthetic gene clusters. Furthermore, structural and functional evidence is presented to support a model for convergent evolution in the active site architecture of SbnG and TCA cycle citrate synthases.

**EXPERIMENTAL PROCEDURES**

**SbnG Overexpression and Purification**—The SbnG coding region was cloned into pET28a as previously described (17). SbnG variants (E46Q, H47A, R72A, H96A, E151Q, and D177A) were produced using a modified whole plasmid PCR technique as previously described (18). SbnG variants were introduced into *Escherichia coli* BL21(ADE3), and the mutations were confirmed by DNA sequencing.

His$_6$-SbnG was expressed from *E. coli* BL21(λDE3) cells inoculated from 5-ml overnight cultures into 2× YT medium supplemented with 25 μg/ml kanamycin and grown at 30 °C to an $A_{600}$ of 0.8. Cultures were then induced with 0.3 mM isopropyl β-D-thiogalactopyranoside and incubated for ~16 h at 25 °C with shaking at 200 rpm. Cell pellets were collected by centrifugation at 4400 × g for 10 min, resuspended in 50 mM Tris (pH 8.0), 100 mM NaCl, and lyzed at 10,000 psi with an EmulsiFlex-C5 homogenizer (Avestin). The supernatant was isolated after centrifugation at 39,000 × g for 45 min, and His$_6$-SbnG was purified using a 5-ml HisTrap HP column (GE Healthcare) with a linear imidazole gradient (0–500 mM). Protein fractions were dialyzed into 50 mM Tris (pH 8.0), 100 mM NaCl at 4 °C. The His$_6$ tag was removed by thrombin digestion at a 1:500 mass ratio (thrombin to protein) and incubated over 16 h at 4 °C, followed by dialysis into 50 mM Tris (pH 8.0) for 2 h at 4 °C. SbnG was further purified by anion exchange chromatography using a Source 15Q column (GE Healthcare) equilibrated with 50 mM Tris (pH 8.0) and eluted with a NaCl gradient (0–500 mM). SbnG was dialyzed into 20 mM Tris (pH 8.0), concentrated to 20 mg/ml, and stored at ~80 °C. Selenomethione-incorporated SbnG was produced by methods previously described (19) and purified in a similar manner to native SbnG. All SbnG variants (E46Q, H47A, R72A, H96A, E151Q, and D177A) were also purified in a similar manner to native SbnG.

**SbnG Crystallization and Structure Determination**—Selenomethione-labeled SbnG crystals were grown by hanging drop vapor diffusion at room temperature in 1:1 mixtures of 20 mg/ml SbnG in 20 mM Tris (pH 8.0) with reservoir solution of 0.2 M calcium acetate, 0.1 M imidazole (pH 9.0), and 4–6% (w/v) PEG 8000. Crystals were flash frozen in liquid nitrogen following a brief soak in the same buffer supplemented with 20% (v/v) PEG 400 for cryoprotection. Multiwavelength anomalous diffraction data for selenomethionine protein crystals were collected at the Canadian Light Source on Beamline 08B1. The data were processed and scaled using XDS (20, 21). Crystals grew in the space group P312 with one molecule in the asymmetric unit. Phase determination and model building were done using AutoSol (initial figure of merit 0.73) and AutoBuild (225 of 259 residues built) programs in Phenix (22). Native SbnG was crystallized under similar conditions with the addition of 5 mM citrate to the reservoir, and diffraction data were collected at the Stanford Synchrotron Radiation Laboratory on Beamline 7-1.

SbnG E151Q crystals were grown by sitting drop at room temperature in a 1:1 mixture of 20 mg/ml SbnG E151Q in 5 mM oxaloacetate, 5 mM coenzyme A, and 20 mM Tris (pH 8.0) with reservoir solution of 5% (v/v) tascimate (pH 7.0), 0.1 M HEPES (pH 7.0), 10% (w/v) PEG monomethyl ether 5000 and ~40 mM guanidine hydrochloride. SbnG E151Q crystals were soaked for 10 min in 5 mM oxaloacetate/CoA mixture in the same buffer and supplemented with 20% (v/v) PEG 400 for cryoprotection before data collection with a Rigaku MicroMax 007-HF generator,miniaturized HR optics, and Saturn CCD 944+ detector. The data sets were processed using Mosflm (23) and scaled using SCALA (24). The native structure was solved by molecular replacement using the selenomethionine-labeled coordinates of SbnG.
as the search model in Molrep (25) from the CCP4 program suite (26). Manual building was done using Coot (27), and refinement was performed with Refmac5 (28) using translation libration screw (29) parameters with eight translation libration screw groups. SbnG E151Q was solved by molecular replacement using native SbnG coordinates as a search model in Phaser-MR (30) from Phenix (22). Manual building was done using Coot (27), and refinement was performed with phasix.refine using translation libration screw refinement. Data collection and refinement statistics are shown in Table 1.

| Refinement | SeMet-SbnG | SbnG | SbnG-E151Q |
|------------|------------|------|------------|
| Rwork (Rfree) | 0.187 (0.213) | 0.197 (0.211) | 0.228 (0.272) |
| B factors (Å²) | 27.7 | 56.1 | 28.1 | 56.6 |
| All atoms | 27.7 | 56.1 | 56.6 |
| Protein | 27.7 | 56.1 | 56.6 |
| Ligands | 37.1 | 59.7 | 37.1 | 59.7 |
| Water | 20.4 | 36.3 | 20.4 | 36.3 |
| r.m.s.d. bond length (Å) | 0.009 | 0.004 | 0.009 | 0.004 |

**Ramachandran plot**

- In most favorable region: 95.7 % (95.7 %)
- In disallowed regions: 0.0 % (0.0 %)
- All atoms: 95.7 % (95.7 %)

**PDB accession codes**

- 4TV5
- 4TV6

**a** The values in parentheses represent highest resolution shell.

### X-ray diffraction data collection and refinement statistics

| Data collection | SeMet-SbnG | SbnG | SbnG-E151Q |
|----------------|------------|------|------------|
| Wavelength (Å) | 0.97912 | 0.97929 | 0.97345 |
| Resolution range (Å) | 33.42–2.0 (2.11–2.0) | 33.44–2.0 (2.11–2.0) | 33.41–2.0 (2.11–2.0) |
| Unit cell dimensions (Å) | a: 74.2 | b: 74.2 | c: 77.0 |
| Unique reflections | 16,550 | 16,573 | 16,538 |
| Completeness (%) | 99.8 (100) | 99.8 (100) | 99.8 (100) |
| Mean I/σl | 13.2 (3.0) | 14.1 (3.1) | 13.9 (2.8) |
| Redundancy | 7.5 (7.5) | 7.5 (7.5) | 7.5 (7.5) |
| Rmerge | 0.056 (0.266) | 0.053 (0.254) | 0.054 (0.279) |
| Wilson B (Å²) | 19.9 | 0.089 (0.314) | 0.089 (0.314) |
| Mean | 20.6 | 39.1 | 39.1 |

### SeMet-SbnG

- **Peak Inflection**
- **Remote**

### SbnG

- **PDB accession codes**

### SbnG-E151Q

- **PDB accession codes**

In Vitro Assessment of Citrate Synthase Activity by SbnG Variants—Citrate synthase activity of wild type SbnG and variants was measured using the reagent DTNB to quantify the generation of CoA. The concentration of resulting TNB dianion was monitored at 412 nm on a Varian Cary 50 UV-visible spectrophotometer using an extinction coefficient of 14150 M⁻¹ cm⁻¹. Each reaction contained 50 mM HEPES (pH 8.0), 300 mM NaCl, 0.5 mM oxaloacetate, 0.2 mM acetyl-CoA, and 10 µM protein. Each reaction was incubated for 1 h at 37 °C followed by the addition of 0.1 mM DTNB reagent. Individual blanks for each reaction contained all required components minus protein. Oxaloacetate-independent activity was recorded by performing the reactions in the absence of oxaloacetate. All reactions were performed in triplicate.

**Bacterial Strain and Growth Conditions**—All bacterial strains and plasmids employed in this study can be found in Table 2. Unless otherwise indicated, in vivo experiments were performed using S. aureus Newman. For genetic manipulations, E. coli and S. aureus were routinely cultured in Difco Luria-Bertani broth (BD), and Difco tryptic soy broth (BD), respectively. For selection and maintenance of plasmids, medium was supplemented with 100 µg ml⁻¹ of ampicillin for E. coli or 10 µg ml⁻¹ of chloramphenicol for S. aureus. For bacterial growth under iron restriction, RPMI 1640 medium was prepared as directed by the manufacturer (Invitrogen) in sterile polypropylene vessels using water purified with a Milli-Q water filtration system. The nonmetabolizable iron chelator ethylenediamine-di(O-hydroxyphenylacetic acid) (LGC Standards GmbH) was added to the medium, where indicated.
Construction of SbnG Variant Complementation Vectors—To assess the role of residues implicated in SbnG enzymatic function in the production of SB within the cell, whole plasmid mutagenesis was employed to generate complementation vectors expressing sbnG mutated for these residues. Using the previously constructed psbnG vector (a pALC2073 derivative expressing wild type sbnG) as a template, complementary mutagenesis primers were used to amplify plasmids bearing SbnG variants E46Q, H47A, R72A, H96A, E151Q, and D177A. Following amplification, methylated template DNA was inactivated at 80 °C for 5 min, and the resulting reaction mixes were used in the transformation of E. coli DC10B. Plasmids bearing the correct mutation were confirmed through sequencing and introduced into S. aureus Newman ΔcitZ sbnG (H2708) through electroporation. The sbnG variants were constitutively expressed, without induction by the leaky Pxyt/temO promoter of pALC2073 (39).

Assessment of SB Production through Agar Plate Bioassays—Concentrated spent supernatants of the citrate-synthase deficient mutant (ΔcitZ sbnG) complemented in trans by plasmids bearing the sbnG variants, as described above, were prepared from triplicate 10-ml cultures of these strains grown in RPMI 1640, a medium conducive only to the production of SB (16). Following a 36-h incubation with shaking at 37 °C, growth was assessed, and the culture densities normalized to ~2, by A600. Bacterial cells were removed by centrifugation, and the resulting supernatants were lyophilized overnight. Lyophils were resuspended in 1-ml of sterile double distilled H2O, passed through a 0.2-micron syringe filter, and assessed for their ability to promote growth of wild type S. aureus RN6390, as previously described (7, 16). In brief, 10 μl of the reconstituted supernatants were applied to sterile paper disks, which were then placed on Tris minimal succinate agar plates (40) seeded with ~1 × 10^8 colony-forming units ml⁻¹ of RN696 as a reporter strain and containing 10 μM ethylenediamine-di(O-hydroxyphenylacetic acid). The plates were incubated at 37 °C, and growth radius about the disks was measured after 48 h.

In Vivo Assessment of Citrate Synthase-dependent SB Production by SbnG Variants—To assess the role of residues implicated in SbnG enzymatic function in the citrate-dependent production of SB within the cell, sbnG complementation vectors bearing the mutated residues (pE46Q, pH47A, pR72A, pH96A, pE151Q, and pD177A) were assessed for their ability to promote SB production in a previously constructed citrate-synthase deficient strain (H2708; ΔcitZ sbnG) of S. aureus Newman (16). Spent culture supernatants from the complemented ΔcitZ sbnG mutant strains grown in RPMI 1640 for 36 h and 10× concentrated were assessed for the presence of SB using agar plate bioassays, as previously described (7, 16). In brief, S. aureus wild type RN6390 was seeded into Tris minimal succinate agar plates (40), and the above supernatants were applied to sterile paper disks placed onto these plates. SB-dependent growth promotion was assessed by measuring the growth radius around the disks after 48 h of incubation at 37 °C.

RESULTS

Overall Structure of SbnG—The crystal structure of SbnG was solved to a resolution of 1.85 Å with a single subunit in the asymmetric unit. The structure consists of residues 4–258, excluding residues 121–132 that lack clearly defined electron density and form part of an apparent disordered loop. The overall structure of the SbnG protomer revealed an (α/β)₈ barrel fold formed by seven α-helices that pack against an interior β-barrel with an eighth α-helix on the C-terminal end projecting outward (Fig. 1A). Dimers related by a crystallographic 2-fold axis resulted in the domain swapping of the C-terminal α-helices (α8) of each protomer to complete the (α/β)₈ fold. The biological hexamer could be reconstructed through 3-fold crystallographic symmetry (Fig. 1B). Additionally, SbnG (a ~28-kDa subunit) exists primarily as a hexamer in solution with a calculated mass of 171 kDa as determined by multiangle
light scattering (Fig. 2A). The dimer interface includes one π-stacking interaction between equivalent His244 residues from each protomer, as well as extensive hydrophobic interactions between residues Ile24, Leu28, Ile32, Leu234, Ile241, Leu245, and Leu249. The trimeric interface was comprised of a mixture of electrostatic and hydrophobic contacts, including a H-bond between residues His47 and Asp86 and several hydrophobic interactions involving residues Val76, Leu120, Leu123, Leu124, Pro185, and Trp186.

SbnG Active Site—Based on homology to class II aldolases, the SbnG active site was hypothesized to be situated within a groove at the opening of the αβ8 barrel near the 3-fold interface shared with an adjacent SbnG protomer (Fig. 1C). The putative active site was highly polar and is composed in part of two histidine (His47 and His96), two glutamate (Glu46 and Glu151), aspartate (Asp177), and arginine (Arg72) residues. In the center of this site, density for a metal ion was identified residing within an acidic patch on the protein surface (Fig. 1D). Because of the high calcium acetate concentration (0.2 M) from which crystals were formed, the density was modeled as a Ca2+ ion, a known inhibitor of SbnG (17). The Ca2+ is bound by Glu151-O1 (2.4 Å) and Asp177-O62 (2.2 Å), as well as four ordered water molecules (2.3–2.5 Å) that complete the octahedral coordination sphere (Fig. 1C). The coordinated water molecules participate in a H-bond network involving the side chains of residues Glu46, His47, Arg72, and His96 based on H-bond distances under 3.0 Å. Because of the hexameric oligomerization of SbnG, an adjacent protomer sits atop the active site groove at the opening of the αβ8 barrel. A single serine residue (Ser119) from a neighboring protomer adopts two different conformations and also participates in the Ca2+-binding site and within the same protomer lies an extended channel lined with intermittent patches of positive (Arg72, Arg189, Arg216, Arg238, and Arg243) and near neutral electrostatic potential (Fig. 1D).

Crystal Structure of SbnGE151Q Variant Bound to Oxaloacetate—Previously, citrate synthase activity of SbnG was shown to be inhibited by low millimolar concentrations of Mg2+ and Ca2+ and not inhibited by the addition of EDTA (17). These observations were in stark contrast to all characterized class II aldolases, which require Mg2+ for activity. Attempts to
cococrystallize SbnG in the presence of either acetyl-CoA or oxa-
loacetate using crystallization conditions lacking calcium ace-
tate proved unsuccessful. Furthermore, soaking experiments of
wild type SbnG crystals failed to produce substrate-containing
structures, leading to the hypothesis that calcium sterically
inhibits substrate binding. An SbnG variant (E151Q) was
created to prevent divalent metal ion inhibition and thus
favor substrate binding under buffer conditions suitable for
crystallization.

SbnG E151Q crystals soaked in the presence of oxaloacetate,
and CoA diffracted to a resolution of 2.6 Å. Oxaloacetate was
modeled in at 80% occupancy into a patch of difference elec-
tron density discovered within the proposed active site (Fig.
3A). Oxaloacetate was oriented such that the two carboxylate
groups formed H-bonds to residues Glu46, His47, Arg72, His96,
and Gln151, while exposing the carbonyl group to the solvent.
Ser119, a residue from an adjacent protomer, formed a H-bond
to the carbonyl group of oxaloacetate. The E151Q variant also
existed as a hexamer in solution with a mass of 169 kDa as
determined by SEC-MALS (Fig. 2B).

The wild type SbnG and the substrate bound E151Q variant
overlay with a root mean square deviation (r.m.s.d.) of 0.86 Å
over all atoms. The largest main chain differences were dis-
placement of two α-helices that contained Glu/Gln151 and
Asp177. This displacement likely resulted from the loss of Ca2+
coordination and binding of oxaloacetate. Within the active
site, Ca2+ present in the wild type SbnG structure occupied an
equivalent position to the oxaloacetate observed in SbnG
E151Q (Fig. 3B). The amino acid side chain conformations in
the active site were similar with the exception of Asp177, in
which the side chain is rotated ~45° about χ2 (Fig. 3B).

**Structural Comparison of SbnG to the Phosphoenolpyruvate/
Pyruvate Domain Superfamily**—A search for similar structures
of SbnG in the Protein Data Bank using the DaliLite server (33)
identified several members of the metal-dependent class II
aldolase (top seven unique entries with 2.4 Å r.m.s.d. over
240 Cα atoms). Examples are the well characterized 2-de-
hydro-3-deoxy-galactarate aldolase (DDGA) (41) and macro-
phomate synthase (MPS) (42). These class II aldolases also form
hexamers that are assembled by domain swapped dimers. More
distantly related but significant matches in the DaliLite search
were many structures of the phosphoenolpyruvate/pyruvate
domain superfamily as defined in the SCOP database (43). Of
these, the most similar were pyruvate kinase and the
β-subunit of citrate lyase. A list of selected structural superpositions with
supporting statistics is presented in Table 3.

To compare active site architectures among SbnG structural
homologs, a total of four superpositions were assembled with
DDGA (41), MPS (42), the citrate lyase β-subunit (CitE) (44),
and malate synthase G (GlcB) (45). The four superpositions
with SbnG revealed common active site features. Notably, all
these structurally characterized homologs contained an Mg2+
in an equivalent position to the Ca2+ present in wild type SbnG
(Fig. 4, A and B). To the best of our knowledge, all other mem-

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**Figure 2.** SbnG is a hexamer in solution as determined by SEC-MALS. Molar mass was determined for wild type SbnG (A) and SbnG E151Q (B). The Rayleigh ratio is shown as a **black line**, and the calculated molar mass is shown as a **blue line**.

**Figure 3.** Oxaloacetate bound to SbnG E151Q. A, a stereo view of the active site of SbnG E151Q variant bound to oxaloacetate. The active site SbnG E151Q is represented as **sticks** with the active site protomer colored **gray** and the adjacent protomer colored **black**. Oxaloacetate (OAA) is colored to match its corresponding protomer active site. Omit difference electron density is shown as **gray mesh** contoured at 2.0σ. B, an overlay of wild type SbnG (**blue**) and E151Q variant (**gray**) structures. The Ca2+ from wild type SbnG is shown as a **yellow sphere**. Oxaloacetate from SbnG E151Q is shown as **sticks** and is colored to match its respective active site residues.
bers of the superfamily employ either \( \text{Mg}^{2+} \) or \( \text{Mn}^{2+} \) as a cofactor. Also, residues equivalent to Glu\(_{151}\) and Asp\(_{177}\) directly coordinate to a divalent metal ion, and these two residues are conserved in the phosphoenolpyruvate/pyruvate domain superfamily. Glu\(_{46}\) and Arg\(_{72}\) of SbnG are also conserved in the phosphoenolpyruvate/pyruvate domain superfamily and have been suggested to play a role in ordering water molecules in the active site or in substrate binding (41, 46–48). Interestingly, amino acid residues equivalent to SbnG His\(_{47}\) and His\(_{96}\) show variation across the homologs.

**TABLE 3**

Superposition statistics of wild type SbnG to structurally characterized homologs determined from DaliLite

| Protein | Organism   | PDB entry code | Sequence identity | r.m.s.d. value | Aligned residues/259 |
|---------|------------|----------------|-------------------|----------------|----------------------|
| DDGA    | *E. coli*  | 1DXE           | 28.9              | 1.77           | 232                  |
| YfaU    | *E. coli*  | 2VWS           | 28.2              | 1.77           | 227                  |
| B8FRX2  | *Desulfitobacterium hafniense* | 3QZ6 | 27.1 | 1.44 | 240 |
| MPS     | *M. commeliniae* | 1IZC | 27.1 | 1.72 | 236 |
| HcpH    | *E. coli*  | 2V5J           | 25.0              | 1.64           | 236                  |
| CitE    | *M. tuberculosis* | 1Z6K | 20.5 | 2.07 | 171 |
| CitE2   | *Yersinia pestis* | 3QLL | 17.0 | 2.21 | 159 |
| PtsA    | *E. coli*  | 2HWG           | 15.6              | 2.51           | 179                  |
| PtsI    | *S. aureus* | 2WQD | 15.4 | 2.44 | 175 |
| PykF    | *E. coli*  | 1PKY           | 15.0              | 2.14           | 173                  |
| CitE3   | *Cupriavidus necator* | 3QQW | 14.4 | 2.34 | 181 |
| GlcB    | *E. coli*  | 1D8C           | 11.8              | 2.43           | 186                  |
| GlkB    | *M. tuberculosis* | 1NW8 | 10.2 | 2.46 | 186 |

**FIGURE 4. Conservation of active site structure in SbnG homologs.** A and B, superposition of active site residues from SbnG (blue) with those of *E. coli* DDGA (PDB code 1DXE) (light gray) and *Macrophoma commeliniae* MPS (PDB code 1IZC) (green) (A) and *Mycobacterium tuberculosis* CitE (PDB code 1Z6K) (dark gray) and *M. tuberculosis* GlcB (PDB code 1NW8) (tan) (B). Active site residues are shown as sticks with oxygen and nitrogen colored as red and blue, respectively. \( \text{Mg}^{2+} \) and \( \text{Ca}^{2+} \) are depicted as spheres and colored to match their respective active site residues. C, unrooted phylogenetic tree of SbnG and homologs. Bootstrap values are presented as percentages at key branch points. Each protein is labeled by the genus, species, and protein name. The dashed box represents a novel clade with the phosphoenolpyruvate/pyruvate domain superfamily for SbnG-like citrate synthases identified in other siderophore biosynthetic gene clusters.
SbnG, a New Fold for Citrate Synthase

SbnG Represents a New Family of Metal-independent Enzymes within the Class II Aldolase Superfamily—To further classify SbnG within the class II aldolase family, a multiple sequence alignment was constructed and used to derive a phylogenetic tree (Fig. 4C). Included in the analysis were functionally uncharacterized homologs identified in achromobactin and vibrioferrin biosynthesis gene clusters (17), in addition to the structurally established homologs described above. Inspection of the tree revealed that SbnG and homologs from other siderophore biosynthetic pathways group together to form a separate clade (91% bootstrap value) from other class II aldolases. Homologs outside the SbnG clade are all known to require divalent metal for activity.

SbnG Active Site Variants Show Reduced SB Production in a Citrate Synthase-deficient S. aureus Strain—Based on the interaction of Glu46, His47, Arg72, His96, and Asp177 with oxaloacetate in the E151Q crystal structure, variants of these putative active site residues were generated, in addition to E151Q, to evaluate their role in citrate synthase activity. SbnG variants E46Q, H47A, R72A, H96A, and D177A were all recombinantly expressed in E. coli BL21 and successfully purified. Production of CoA from acetyl-CoA by wild type and variant forms of SbnG was monitored using DTNB in the presence and absence of divalent metal for activity. The activity by this assay was modest (5 nmol CoA released per mg of SbnG) as observed previously (17). The activity of the variants did not differ substantially from wild type enzyme, likely because of the low wild type activity. We hypothesized that physiologically relevant activity of SbnG type enzyme, likely because of the low wild type activity. We hypothesized that physiologically relevant activity of SbnG type enzyme, likely because of the low wild type activity.

To circumvent the above issue of poor enzyme performance in vitro, SbnG activity was assayed by measuring SB production from a strain of S. aureus in which both citZ and sbnG are inactivated (16). Expression of wild type SbnG from a plasmid in this deletion strain restores SB production as quantified using a disk diffusion assay (Fig. 5). SB production from S. aureus strains expressing each of the six variants was diminished in comparison to wild type SbnG. Notably, the assay indicates that the D177A variant is unable to produce sufficient citrate for SB production to be detected.

DISCUSSION

SbnG is a citrate synthase that functions to provide citrate for SB biosynthesis (17). Previously, SbnG was hypothesized to be an aldolase based on sequence alignments to characterized homologs DDGA and YfaU. These alignments revealed a conserved pair of amino acids (Glu151 and Asp177 in SbnG) that in the homologs coordinate the active site Mg2+ required for catalysis (41, 47). Thus, SbnG was suggested to have metal-dependent aldolase activity and to possibly participate in SB degradation for iron release (17). Furthermore, a homolog found in the biosynthetic operon for the siderophore achromobactin, AcsB, was speculated to play a role in converting an intermediate into pyruvate and an aldehyde, which would eventually feed into siderophore biosynthesis (49). However, recent work by Cheung et al. (17) has revealed SbnG to exhibit citrate synthase activity in the absence of divalent metals like Ca2+ and Mg2+. The crystal structure of SbnG contained electron density for a Ca2+ directly coordinated to Glu151 and Asp177 in the active site. Ca2+ was modeled instead of Mg2+ because the observed bond lengths were not consistent with Mg2+ coordination, which are typically ~2.1 Å (50). Furthermore, 0.2 mM calcium acetate was used in the crystallization condition, a sufficiently high enough concentration for full occupancy of bound Ca2+. In the case of DDGA, the closest structurally characterized homolog to SbnG, Mg2+, Co2+, or Mn2+ can be used as cofactors for catalysis (41, 51). To the best of our knowledge, no example exists in the literature where Ca2+ serves as a functional cofactor in class II aldolases. Thus, the SbnG-Ca2+ complex is likely an inhibited form of the enzyme, and the binding of Ca2+ may stabilize crystal packing.

Binding sites for oxaloacetate and acetyl-CoA to SbnG were inferred by analysis of the E151Q variant and by comparison to the homolog malate synthase G, respectively. Malate synthase G catalyzes a similar Claisen condensation reaction as citrate synthases using substrates glyoxylate and acetyl-CoA to produce malate (52). Crystals of the SbnG E151Q variant produced in the absence of Ca2+ were soaked in a substrate solution revealing density for oxaloacetate. The possibility that the binding mode of oxaloacetate is altered by the amino acid substitution of Glu151 cannot be excluded. Nonetheless, the overlay of the oxaloacetate and Ca2+-binding sites suggests that Ca2+ may be a competitive inhibitor with this substrate. Density for CoA was not identified in the E151Q variant electron density map. Inspection of the structure does reveal a large positively charged channel that lies next to the oxaloacetate binding site that could serve as a binding site for acetyl-CoA, analogous to that observed in the distantly related homolog malate synthase G (45), although this channel is on the opposite side of the active site. Additionally, a disordered loop (residues 121–132) from an adjacent protomer lies directly above the active site and

![Figure 5. SbnG variants are impaired for citrate-dependent production of SB. Agar plate bioassays were performed using supernatant extracts prepared from a citrate synthase-deficient strain of S. aureus Newman (ΔcitZ ΔsbnG) complemented with vectors, as indicated, expressing wild type SbnG, SbnG variants E46Q, H47A, R72A, H96A, E151Q, and D177A or a blank pALC2073 vector control (vehicle). Strains were grown for 36 h in RPMI 1640, a medium conducive to the production of SB (and not SA), and supernatants were assessed for SB through their ability to promote growth of S. aureus RN6390 seeded in iron-restricted Tris minimal succinate agar plates. Growth radius about paper disks, to which the supernatants were applied, was measured after 48 h and reflects the average of three biological replicates for each strain.](image-url)

FIGURE 5. SbnG variants are impaired for citrate-dependent production of SB. Agar plate bioassays were performed using supernatant extracts prepared from a citrate synthase-deficient strain of S. aureus Newman (ΔcitZ ΔsbnG) complemented with vectors, as indicated, expressing wild type SbnG, SbnG variants E46Q, H47A, R72A, H96A, E151Q, and D177A or a blank pALC2073 vector control (vehicle). Strains were grown for 36 h in RPMI 1640, a medium conducive to the production of SB (and not SA), and supernatants were assessed for SB through their ability to promote growth of S. aureus RN6390 seeded in iron-restricted Tris minimal succinate agar plates. Growth radius about paper disks, to which the supernatants were applied, was measured after 48 h and reflects the average of three biological replicates for each strain.
could play a role in catalysis by acting as the lid for the reaction. Unlike in SbnG, this loop is well ordered in all structurally characterized homologs and faces away from the adjacent active site.

SbnG shares a similar overall fold with the phosphoenolpyruvate/pyruvate domain superfamily, and features of the active site architecture across this family of proteins are remarkably conserved despite the differences in metal binding and catalytic function. In addition to the polypeptide backbone, the amino acids at the equivalent positions to Glu\(^{46}\), Arg\(^{72}\), Glu\(^{151}\), and Asp\(^{177}\) are all conserved across this family. In contrast, His\(^{57}\) and His\(^{84}\) of SbnG show variation among homologs. Interestingly, in MPS these two residues are also histidines, and both enzymes have specificity for oxaloacetate as a substrate (42, 53).

A multiple sequence alignment of SbnG and select class II aldolase homologs (25–70% sequence identity; Tables 3 and 4) revealed two residues in close proximity to the metal binding site that are differently conserved between homologs within the SbnG clade and the class II aldolases (Fig. 6). The first residue is Met\(^{49}\), which is a glutamine in all representative class II aldolases (Fig. 6). In the MPS crystal structure, this glutamine residue is within H-bonding distance to the pyruvate product and the active site arginine residue (Fig. 7A). The second residue is Ala\(^{175}\), which is a proline residue in all representative class II aldolases (Fig. 7B). These substitutions may account for differences in metal binding under physiological conditions.

SbnG was shown to catalyze an identical reaction to TCA cycle citrate synthases (17). However, the fold of both proteins are different as SbnG forms an (\(\alpha/\beta\)_)\(^8\) barrel, whereas citrate synthase types I and II are \(\alpha\)-helical. Two histidines and an aspartic acid participate in catalysis by TCA cycle citrate synthase by acting as general acids and general bases during catalysis. The active site of SbnG contains the same configuration of these three residues: His\(^{47}\), His\(^{96}\), and Asp\(^{177}\). The side chains of His\(^{47}\), His\(^{96}\), and Asp\(^{177}\) (15 atoms total) of the oxaloacetate bound SbnG E151Q structure were superimposed on the equivalent residues of porcine heart citrate synthase (type I) and of Acetobacter aceti citrate synthase (type II) (54, 55). The overlays clearly demonstrate a conserved spatial distribution of the three catalytic residues with r.m.s.d. values of 2.2 and 2.3 Å with...
citrate synthases types I and II, respectively (Fig. 8). In SbnG E151Q and the structures of either citrate synthase types I and II, the catalytic Asp residues are not observed to H-bond to oxaloacetate. Additionally, Arg72 of SbnG E151Q roughly overlays with arginine residues of the two citrate synthases that also interact with bound substrates. However, the orientation of Arg72 relative to the TCA cycle citrate synthases are ~90° apart. The conformation of this arginine may determine the orientation of oxaloacetate binding in the active site, which is also rotated by ~90° (Fig. 8). The acetyl CoA analog (carboxymethylthio-CoA) bound to citrate synthase from A. aceti does not overlay with the proposed binding site in SbnG. Rather, a similar ~90° rotation is required to align the acetyl CoA with the positively charged groove with respect to the oxaloacetate molecule.

The structural similarity between the active sites of SbnG and TCA citrate synthases suggests convergent evolution of a clade of the phosphoenolpyruvate/pyruvate domain superfamily. Expression of SbnG active site variants, including the residues His47, His96, and Asp177, had significant reductions in SB activity. Expression of SbnG active site variants, including the residues Arg72, Arg401, and Arg398, had significant reductions in SB activity. However, the orientation of Arg72 relative to the TCA cycle citrate synthases are similar to that of TCA cycle citrate synthases. Analysis of oxaloacetate binding in the SbnG E151Q structure did not readily identify the specific catalytic role of His47 and His96. The position of oxaloacetate in the active site may differ in the wild type structure or in presence of acetyl-CoA. Nonetheless, Asp177 is proposed to act as a general acid and either His47 or His96 as a general base to afford the enol of acetyl-CoA. A condensation reaction between the enol and oxaloacetate could be assisted by either His47 or His96 acting as a general acid to yield a citryl-CoA intermediate. Hydrolysis gives the products citrate and CoA.

In summary, we have defined SbnG as a new structural class of citrate synthase from S. aureus. Therefore, we suggest reclassifying SbnG as a type III citrate synthase. Though SbnG is formally part of the metal-dependent class II aldolase family, we have demonstrated that it has lost the requirement for a metal cofactor and, together with homologs identified in other siderophore biosynthetic gene clusters, forms a new metal-independent category of class II aldolases.

Acknowledgment—We thank Angelé Arrieta for technical assistance.

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