Structural Basis for the De-N-acetylation of Poly-β-1,6-N-acetyl-d-glucosamine in Gram-positive Bacteria*

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Exopolysaccharides are required for the development and integrity of biofilms produced by a wide variety of bacteria. In staphylococci, partial de-N-acetylation of the exopolysaccharide poly-β-1,6-N-acetyl-d-glucosamine (PNAG) by the extra-cellular protein IcaB is required for biofilm formation. To understand the molecular basis for PNAG de-N-acetylation, the structure of IcaB from Ammonifex degensii (IcaBAd) has been determined to 1.7 Å resolution. The structure of IcaBAd reveals a (β/α)-heterotetrameric mechanism in comparison to other characterized CE4s with the canonical motifs circularly permuted. The metal dependence of IcaBAd is similar to most CE4s showing the maximum rates of de-N-acetylation with Ni2+, Co2+, and Zn2+. From docking studies with β-1,6-GlCNAc oligomers and structural comparison to PgaB from Escherichia coli, the Gram-negative homologue of IcaB, we identify Arg-45, Tyr-67, and Trp-180 as key residues for PNAG binding during catalysis. The absence of these residues in PgaB provides a rationale for the requirement of a C-terminal domain for efficient deacetylation of PNAG in Gram-negative species. Mutational analysis of conserved active site residues suggests that IcaB uses an altered catalytic mechanism in comparison to other characterized CE4 members. Furthermore, we identified a conserved surface-exposed hydrophobic loop found only in Gram-positive homologues of IcaB. Our data suggest that this loop is required for membrane association and likely anchors IcaB to the membrane during polysaccharide biosynthesis. The work presented herein will help guide the design of IcaB inhibitors to combat biofilm formation by staphylococci.

Staphylococcus epidermidis is a Gram-positive commensal bacteria that is the most prevalent organism found in indwell-ing medical-device-related bacterial infections (1). Nosocomial S. epidermidis infections are often caused by the bacteria forming a biofilm on biomaterials (2, 3). A biofilm is a cluster of microcolonies encapsulated within self-produced extracellular polymeric substances (4, 5). The components of the biofilms can be quite diverse but are generally composed of proteinaceous adhesins, nucleic acids, and exopolysaccharides (6–8). Exopolysaccharides are important for biofilm structure and architecture by allowing the diffusion of nutrients in and waste products out (4, 9). Exopolysaccharides have also been shown to function as adhesins, reduce the diffusion of antibiotics into the biofilm, and provide a barrier against phagocytosis (7, 10).

The conserved exopolysaccharide known as polysaccharide intercellular adh esin was originally identified in the biofilms of S. epidermidis (11) and Staphylococcus aureus (12) and has now been shown to be produced by various Gram-negative bacteria (13–19) and higher eukaryotes (20). Polysaccharide intercellular adhesin is synthesized as a β-1,6-linked poly-N-acetyl-d-glucosamine (PNAG)5 polymer and subsequently modified by partially de-N-acetylation and/or O-succinylation. In S. epidermidis, de-N-acetylated PNAG (dPNAG) production is dependent on the four-gene operon, icaADBC (21). IcaA, which is predicted to contain multiple transmembrane domains and a large cytosolic family 2 glycosyltransferase domain, is thought to be responsible for the production of PNAG and its translo-
acetylation across the membrane, IcaD is a small integral membrane protein that significantly increases PNAG biosynthesis when co-expressed with IcaA and potentially aids in PNAG translocation across the membrane. IcaC is an integral membrane protein that was originally predicted to be responsible for exporting mature long-chain PNAG. However, the proposed function of IcaC has recently been revisited (23). Bioinformatics analysis predicts that IcaC contains 10 transmembrane helices and is a member of a large acetyltransferase family, suggesting it plays a role in the O-modification of PNAG during biosynthesis (23). The presence of succinate groups on PNAG isolated from S. epidermidis (24) and S. aureus (25) supports the role of IcaC as an O-succinyltransferase. IcaB is an extracellular protein that is responsible for the partial de-N-acetylation of PNAG (26), a requirement for surface retention of the polymer. Furthermore, ΔicaB strains of S. epidermidis and S. aureus are unable to de-N-acetylate PNAG, do not form biofilms in vitro, and have highly attenuated virulence in a murine model for an indwelling medical device-related infection and bacteremia, respectively (26, 27). IcaB is a member of the family 4 carbohydrate esterases (CE4s) and has sequence homology (22% identity) to the N-terminal IcaB is a member of the family 4 carbohydrate esterases (CE4s) 

EXPERIMENTAL PROCEDURES

Cloning, Expression, and Purification of IcaB 

Herein we present the first structure of IcaB from Amnonifex deckensi (IcaBαβ). The identification of key active site residues that are conserved within Gram-positive homologues provides a structural rationale for why IcaB, unlike its Gram-negative counterpart PgaB, does not require a C-terminal domain for enzymatic activity (29). Biochemical characterization of IcaBαβ and S. epidermidis IcaB (IcaBse) suggests the extracellular enzymes are membrane-associated and are anchored by a conserved hydrophobic loop. Furthermore, we provide the first mutational analysis of a PNAG deacetylase. The mutagenesis data suggests that the circular permutation of IcaBAd and IcaBαβ domain is inactive (29).

Cloning, Expression, and Purification of IcaB Ad Constructs—The plasmid UT032 (32), which contains a codon-optimized version of the icaB gene from S. epidermidis (encoding residues 30–289) in pET16b, was used as a template to subclone icaB into the pET28a expression vector (Novagen). Inverse PCR was used with the forward and reverse primers GGCAATATGGG-GAACCAGAAGAAAACAAAAACGACGGCGATCCAG-ACCATCTAC and GTAGATGGTCTGGTCCTCATGTGC-35908 JOURNAL OF BIOLOGICAL CHEMISTRY

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TTGAAGTTGATCC and CTGGCGCTGAACTACGCCCGAACACCGTGTTACCG, respectively. The hydrophobic loop deletion mutant of IcaBse 30–289 (IcaBse 30–289 Δloop) was generated in three successive steps using the QuikChange lightening site-directed mutagenesis kit with the following modifications; (i) the denaturing and annealing duration steps were 30 s; (ii) the annealing temperature was 55 °C; (iii) the protocol was completed with 25 cycles. The first step deleted residues 54–72 using the forward and reverse primers CCACCGTGTTCGCGCCCGGAAGATGGAATCAA- AAC and GTTTTTGATCTACATTCTGCACGGCAGCAGCA- AACACGGTGTTACCG. The second step inserted residues AAG after Arg-53 using the forward and reverse primers CCACCGTGTTCGCGCCCGGAAGATGGAATCAA- AAC and GTTTTTGATCTACATTCTGCACGGCAGCAGCA- AACACGGTGTTACCG. The third step inserted residues EI to yield AAEIG after Arg-53 using the forward and reverse primers CCACCGTGTTCGCGCCCGGAAGATGGAATCAA- AAC and GTTTTTGATCTACATTCTGCACGGCAGCAGCA- AACACGGTGTTACCG. The resulting construct IcaBse 30–289Δloop contains the mutations K54A, K55A, and K72G with residues 56–69 deleted.

The following protocol was used to express and purify all the IcaBse constructs. E. coli BL21-CodonPlus cells transformed with the appropriate plasmid were grown in 1 liter of Luria-Bertani (LB) medium with 50 μg/ml kanamycin at 37 °C to an absorbance of 0.4–0.5 and moved to 25 °C. When the cultures reached an A600 of 0.6–0.7, protein expression was induced by the addition of isopropyl-D-1-thiogalactopyranoside to a final concentration of 1 mM. The cells were incubated for 3 h at 25 °C, harvested by centrifugation at 5000 × g for 20 min, and frozen on dry ice. Cell pellets were thawed and resuspended in 40 ml of lysis buffer (50 mM HEPES, pH 7.0, 1 M NaCl, 10 mM imidazole, 5% (v/v) glycerol, and one complete mini protease inhibitor mixture tablet (Roche Applied Science)). Resuspended cells were disrupted with three passes through an EmulsiFlex-c3 (Avestin) at 15,000 p.s.i., and cell debris was removed by centrifugation at 31,000 × g for 30 min. The resulting supernatant was passed over a gravity column containing 3 ml of nickel-nitritolactic acid resin (Qia-gen) that was pre-equilibrated with buffer A (20 mM HEPES, pH 7.0, 1 M NaCl, 10 mM imidazole). Bound protein was washed with 10 column volumes of buffer A, 3 column volumes of buffer A with 20 mM imidazole, and eluted with 5 column volumes of buffer A with 250 mM imidazole. The eluted fraction was concentrated using a 10-kDa cut-off Amicon ultrafiltration device (EMD Millipore) and further purified and buffer-exchanged into buffer B (20 mM HEPES, pH 7.0, and 1 M NaCl) by gel filtration chromatography using a HiLoad 16/60 Superdex 200 prep-grade column (GE Healthcare). The purified IcaBse constructs were >95% pure as judged by SDS-PAGE and stable for ~1 week at 4 °C.

Cloning, Expression, and Purification of IcaB Se Constructs—A codon-optimized version of A. deckensi icaB (GeneArt Invitrogen) was used as the template to subclone mature icaB into the pET28a expression vector (Novagen). Inverse PCR was used with the forward and reverse primers GGCAATATGGG-GAACCAGAAGAAAACAAAAACGACGGCGATCCAG-ACCATCTAC and GTAGATGGTCTGGTCCTCATGTGC-35908 JOURNAL OF BIOLOGICAL CHEMISTRY

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Purified IcaBAd loop deletion mutant of IcaBAd residues 23–280. The hydrophobic loop was generated in the same manner as described above for IcaBAd residues 23–280 using the forward and reverse primers CATCGTGTGCACGGCAGCAGCGCTTATATGCAATTAGC and GCTAAATGCAACGCTGCTCGGCAGAACGACGATG. The D114N and H44A mutants of IcaBAd residues 23–280 were generated in the same manner as described above for IcaBAd residues 23–280 using the forward and reverse primers GTTACGATGTTAATATGGTGATCTGAGGC and CGTCGAGATGACCATATGAGGTAACAGTACAGACACGAC and GTCGAGCTACAGACAGAGCACTAAACACCGAAGGACGTCTGCAGG and CTCGCGCAAGAACGAGCGACGATACACAG. All constructs were expressed and purified as described for IcaBAd with the following modifications: (i) cultures were moved prior to and induced at 18°C for 16 h before being harvested; (ii) for the purification of IcaBAd the lysis buffer, buffer A, and buffer B contained 8, 4, and 1 mM CHAPS detergent, respectively; (iii) tris(2-carboxyethyl)phosphine (TCEP) was included in the lysis buffer and buffer A; (iv) 2 mM 2-mercaptoethanol was included in buffer B for fluorescamine assays; (v) buffer C (Tris, pH 7.0, 0.6 M NaCl, and 5 mM DTT) was used during gel-filtration chromatography purification of IcaBAd residues 23–280. All IcaBAd constructs were >95% pure as judged by SDS-PAGE and stable for ~2 weeks at 4°C.

Crystallographic Data Collection, and Structure Solution—Purified IcaBAd residues 23–280 was concentrated to ~9–10 mg/ml and screened for crystallization conditions at 20°C using hanging-drop vapor diffusion in 48-well VDX plates (Hampton Research) and the MCSG-1 sparse matrix suite (Microlytic). An initial crystallization hit was obtained in condition #72 from the MCSG-1 suite (30% (w/v) PEG 2000 monomethyl ether and 0.1 M potassium thiocyanate). Homemade grid-optimized screens failed to reproduce crystals, so a stock solution of MCSG-1 #72 was purchased from Microlytic. The entire 48-well plates were setup using the stock solution, and on average 3–5 drops per plate produced crystals in 1 month. One irregular shaped crystal (crystal 1) with dimensions of 50 × 30 × 20 μm was suitable for diffraction studies. After four months of incubation of the initial crystallization trials, bipyramidal-shaped crystals with dimensions of 60 × 30 × 30 μm also formed in condition #24 from the MCSG-1 suite (28% (w/v) PEG 2000 monomethyl ether, 0.1 M bis(2-hydroxyethyl)aminotris(hydroxymethyl) methane (Bis-Tris), pH 6.5) and were suitable for diffraction studies (crystal 2). The crystals were cryoprotected for 5 s in reservoir solution supplemented with 20% (v/v) ethylene glycol before vitrification in liquid nitrogen. All data were collected on beam line X29 at the National Synchrotron Light Source (Table 1). A total of 360 images of 1° oscillation were collected for crystal 1 and crystal 2 on an ADSC Quantum-315r detector with a 300- or 260-mm crystal-to-detector distance, respectively, and an exposure time of 0.4 s per image. The data were indexed, integrated, and scaled using HKL2000. The structure was determined for crystal 1 by molecular replacement with PHENIX AutoMR (34) using a truncated version of the N-terminal domain of PgaB42–655 (PDB ID 4F9D) as a search model with residues 43–48, 56–70, 146–160, 191–222, 276–288, and 310–646 removed from the model. The resulting electron density map enabled PHENIX AutoBuild (34) to build ~40% of the protein. The remaining residues were built manually in COOT (35) and refined with reﬁnement using PHENIX.REFINE (34). The model from crystal 1 was then used to solve the structure of crystal 2 using molecular replacement, with manual model building and refinement as described previously. Translation/Libration/Screw (TLS) groups were used during refinement and determined automatically using the TLSMD web server (36, 37). Structure figures were generated using the PyMOL Molecular Graphics System (DeLano Scientific), and quantitative electrostatics were calculated using PDBePQR (38, 39) and APBS (40). Programs used for crystallographic data processing and analysis were accessed through SBGrid (41).

X-ray Absorption Spectroscopy Metal Analysis—X-ray absorption spectroscopy (XAS) was carried out on IcaBAd residues 23–280 on crystal 1 on beam line X29, National Synchrotron Light Source. Two datasets, collected, at wavelengths of 1.2698 and 1.2964 Å, above and below the Zn2+ absorption edge, respectively. The data were collected, indexed, integrated, and scaled as described above. Anomalous electron density maps were generated for both datasets using sfla in the CCP4 suite (42).

Docking Studies—The binding of β-1,6-(GlcNAc)3 and β-1,6-(GlcNAc)4 were investigated using AutoDock Vina 1.1.2 (43). The structure files for β-1,6-(GlcNAc)3 and β-1,6-(GlcNAc)4 were constructed using the web-based Glycam Biomolecule Builder (44). Receptor and ligand Protein Data Bank files were prepared for docking using scripts provided from MGLTools 1.5.6 (45). Docking was conducted with a grid spacing of 0.37 and xyz of 23 × 19 × 18 using a rigid receptor with 10 poses computed. The top productive pose (N-Ac group bound to the zinc ion) for β-1,6-(GlcNAc)3 and β-1,6-(GlcNAc)4 had scores of −7.2 kcal/mol and −6.0 kcal/mol, respectively.

Preparation of PNAG Oligomers—β-1,6-GlcNAc oligomers were synthesized and purified, and their identities were confirmed as outlined previously (46). The β-1,6-GlcNAc oligomers were stored as lyophilized powders at room temperature and dissolved with deionized water for use in assays. Accurate oligomer concentrations were determined by 1H NMR using dimethylformamide as an internal standard.

Fluorescamine Enzyme Activity Assays—The de-N-acetylation activity of various IcaB constructs and mutants on PNAG oligomers was determined by labeling the free amine groups produced during catalysis using a fluorescamine-based assay (47). The assay was performed as described previously (32) with the following modification; BRAND black 96-well plates were used for fluorescence measurements using a SpectraMax M2 plate reader from Molecular Devices (Sunnyvale, CA). For the metal-preference assay, IcaBAd residues 23–280 (30 μM) in buffer B was preincubated in the presence of various divalent metals as chloride salt solutions (30 μM) or metal chelators (1 mM) at room temperature for 30 min. The mixture was then incubated
pellets were washed 3 times and then resuspended with 500 μl Water/Salt Reagent (Thermo Scientific Pierce). Membranes in buffer D. The reactions were incubated for 2 h at 37 °C, and membrane fractions were isolated by centrifugation at 200,000 g. One hundred thousand cells were analyzed by Western blot using anti-IcaB antibodies. Membrane Binding Assays—Staphylococcus carnosus was grown in 1 liter of LB media at 37 °C for 16 h, and cells were harvested at 5000 × g for 20 min. The cell pellet was resuspended and digested with 20 mg/ml lysozyme in 25 ml of buffer D (50 mM HEPES, pH 8.0, 300 mM NaCl, and 2 mM tris(2-carboxyethyl) phosphine) for 90 min at 25 °C. Digested cells were then further disrupted with five passes through an Emulsiflex-c3 at 25,000 p.s.i., and cell debris was removed by centrifugation at 31,000 × g for 30 min. Membranes were then isolated by centrifugation at 200,000 × g for 1 h. Total membranes were weighed and resuspended using a hand press in buffer D with the addition of a protease tablet to a concentration of 75–80 mg/ml. For each reaction a 500 μl volume was used with 10 μM concentrations of protein incubated with ~35 mg of membranes in buffer D. The reactions were incubated for 2 h at 4 °C, and membrane fractions were isolated by centrifugation at 100,000 × g for 45 min. Supernatants were removed, and the pellets were washed 3 times and then resuspended with 500 μl buffer D. The supernatant and resuspended pellet fractions were analyzed by Western blot using α-His6 conjugated to alkaline phosphatase (Sigma) and developed with nitro blue tetrazolium chloride/5-bromo-4-chloro-3’-indolyl phosphate p-toluidine salt reagent (Thermo Scientific Pierce).

RESULTS

Generation of Stable IcaB Constructs—The production and purification of IcaB from Gram-positive bacteria for functional characterization purposes has proven to be challenging. Our group recently reported the generation of the first stable soluble IcaB construct from S. epidermidis (32) using a codon-optimized gene, induction and expression at 10 °C, and with high ionic strength buffers present during the purification process. Despite this success, excessive degradation of the protein occurred during the long induction period, and the protein could not be concentrated, making it unsuitable for structural studies. By reducing the induction period to 2–3 h at 25 °C and using 1 M NaCl in the purification buffers we have been able to reduce protein degradation and concentrate IcaB in a few hours instead of over 24 h. A fluorescence assay using PNAG tetramer and pentamer to detect PNAG de-N-acetylation, fluorescamine assays using PNAG oligomers were completed on all four enzyme constructs. These assays showed no significant differences in activity between IcaB constructs. This search identified IcaB from A. degensii, which has low lysine (4%) and high arginine (8%) content. An IcaB construct lacking the predicted hydrophobic loop was subjected to crystallization trials. Cysteine residues were identified, and the loop was replaced with glycine. The construct was then further purified and used for crystallization. Examination of multiple sequence alignments revealed a predicted hydrophobic loop that was only conserved within Gram-positive homologues (gray box). A survey of IcaB homologues from Bacillus spp. revealed that they all contain high lysine content (8–15%), which is unfavorable for crystallization. To find an IcaB homologue more suitable for crystallization, an in-depth search using the STRING database (48) for bacterial species with the icaABCD operon was performed. This search identified IcaB from A. degensii, which has low lysine (4%) and high arginine (8%) content. An IcaB construct was constructed and found to fractionate with E. coli membranes during purification. Although the protein could be purified when detergent was added to the buffers, it was also recalcitrant to crystallization. Examination of multiple sequence alignments revealed a predicted hydrophobic loop that was only conserved within Gram-positive homologues of IcaB (i.e. not within PgaB) (Fig. 1A). We predicted that the removal of this loop might improve the stability and solubility of the enzymes and in turn make the protein more amenable to crystallization. Deletion of the predicted hydrophobic loop, residues 56–69 in IcaB constructs, and IcaBAd respectively, did not improve the solubility of IcaB constructs. However, deletion of the predicted hydrophobic loop but greatly improved solubility characteristics of IcaBAd. To determine whether the proposed hydrophobic rich loop was involved in de-N-acetylation, fluorescamine assays using PNAG oligomers were completed on all four enzyme constructs. These assays showed no significant differences in activity between IcaB constructs. IcaBAd is a (B/α)7 Barrel with Unique Structural Features—To gain insight into the structure of IcaB, IcaBAd was subjected to crystallization trials. Crystals were obtained from two different crystallization conditions, and diffraction data were collected to 2.25 and 1.7 Å (crystal 1 and 2, respectively, Table 1). The structure of crystal 1 was solved using molecular replacement with E. coli PgaB as the model template (PDB 1D 49FD). Crystal 1 of IcaBAd was solved using molecular replacement with E. coli PgaB as the model template (PDB 1D 49FD).
crystallized in the tetragonal space group I4 with one molecule in the asymmetric unit. Crystal 2 originally indexed in the space group P4₁, and could be solved by molecular replacement using the model from crystal 1. In this space group two IcaB molecules are present in the asymmetric unit. However, density for the second molecule was poor, and the R factors could not be refined below 30%. Patterson analysis revealed significant pseudo-translational symmetry with an off-origin peak 96% of the origin peak located at (x,y,z) = (0.5,0.5,0.5) suggesting the true space group was I4. Re-indexing the dataset in space group I4 produced excellent density maps with one molecule per asymmetric unit and a drop in R factors by ~10%. Refinement of crystal 2 produced a final model with good geometry and R factors of 20.3% (Rwork) and 23.1% (Rfree) (Table 1). Residues 23–30, 278–280, and the hexahistidine tag were not included in the final model as there was no interpretable electron density at those positions.

IcaB<sub>Ad</sub>23–280Δloop adopts a (β/α)_7 barrel fold common to CE4s (49) (Fig. 2A). The core of the structure is composed of seven parallel β-strands arranged in a barrel that is surrounded by seven α-helices with one of the helices capping the bottom of the barrel (Fig. 2A). Six loops are present on the top face of the (β/α)_7 barrel (the C termini of the β-strands) and are important for active site architecture. In addition to the (β/α)_7 core, three β-hairpins are located in loops L1, L3, and L4, which contain residues important for the formation of the active site groove (Fig. 2A). The deleted hydrophobic loop in IcaB<sub>Ad</sub>23–280Δloop is located in loop L1 between Ser-49 and Ser-65 (Fig. 2) and would result in a long structural extension not required for folding the (β/α)_7 barrel. Electrostatic potential analysis of IcaB<sub>Ad</sub>23–280Δloop reveals two distinct features; first, is an electronegative active site pocket that is along the top of the (β/α)_7 barrel (Fig. 2B). Second, is an electropositive patch located on one side of the (β/α)_7 barrel near loops L1 and L2, in close proximity to where the deleted hydrophobic loop would extend (Fig. 2B).

IcaB<sub>Ad</sub>23–280Δloop Active Site Metal Analysis Suggests an Ordered Zn<sup>2+</sup>—Examination of IcaB<sub>Ad</sub>23–280Δloop difference electron density maps revealed a large 28ρ peak at the top of the (β/α)_7 barrel, suggesting the presence of a metal ion. The metal ion is coordinated in an octahedral manner by the side chains of Asp-115, His-173, His-178, and three water molecules (Fig. 3A). The arrangement of the Asp-His-His residues is characteristic of the metal coordination found in most CE4s. To determine the identity of the metal, anomalous diffraction data were collected on IcaB<sub>Ad</sub>23–280Δloop crystal 1 above and below the absorption edge for the transition metal Zn<sup>2+</sup>, as it is the most common metal cofactor found in CE4 structures. A significant decrease in anomalous signal was observed for the 28ρ peak for the data collected below the Zn<sup>2+</sup> absorption edge (1.2964 Å) (Fig. 3B), confirming the identity of Zn<sup>2+</sup> as the metal ion.

Previous metal-dependent activity studies on IcaB<sub>Se</sub>23–280 showed a preference for Zn<sup>2+</sup> and Co<sup>2+</sup> in PNGP de-N-acetylation. To determine whether IcaB<sub>Ad</sub>23–280Δloop shows the same metal preference as IcaB<sub>Se</sub>23–280, metal-dependent fluororescence assays were conducted (Fig. 3C). IcaB<sub>Ad</sub>23–280Δloop showed similar levels of activity compared with the as-isolated enzyme in the presence of Zn<sup>2+</sup>, Mn<sup>2+</sup>, and Fe<sup>3+</sup> and increases of ~1.3- and 2.3-fold in the presence of Co<sup>2+</sup> and Ni<sup>2+</sup>, respectively. Enzyme activity was abolished when incubated with 1 mM EDTA or dipicolinic acid.

Structure and Mechanism of IcaB<sub>Ad</sub>

### Summary of data collection and refinement statistics

|                | IcaB<sub>Ad</sub>23–280Δloop Crystal 1 | IcaB<sub>Ad</sub>23–280Δloop Crystal 2 |
|----------------|----------------------------------------|----------------------------------------|
| **Data collection** |
| Beamline      | NSLS X29                               | NSLS X29                               |
| Wavelength (Å) | 1.07                                  | 1.07                                  |
| Space group   | I4                                     | I4                                     |
| Unit-cell parameters (Å) | a = b = c = 84.6, c = 71.4 | a = b = 84.5, c = 71.9 |
| Resolution (Å) | 50.00-2.25 (2.35-2.25) 50.00-1.70 (1.76-1.70) | 47.97-2.20 (2.35-2.20) 47.97-1.70 (1.76-1.70) |
| Total no. of reflections | 137,488                               | 137,488                               |
| No. of unique reflections | 12,020                                 | 12,020                                 |
| Redundancy    | 14.7 (10.6)                            | 14.7 (14.6)                            |
| Completeness (%) | 98.1 (84.7)                             | 98.1 (100)                             |
| Average I/σ(I) | 16.6 (2.8)                             | 16.6 (5.1)                             |
| R<sub:eq</sub> (%) | 17.6 (59.5)                             | 8.8 (57.8)                             |

### Refinement

- **Rmerge**/Rfree: 20.3/23.1
- **No. of atoms**
  - Protein: 1861
  - Chloride: 31.9
  - Water: 44.2
  - Bond lengths (Å): 0.006
  - Bond angles (°): 1.09
  - Ramachandran plot: 96.0%
  - Disallowed (%): 0.9
  - Coefficient Error (Å): 0.22
- **PDB code**: 4WCJ

### Table 1

|                | IcaB<sub>Ad</sub>23–280Δloop                          |
|----------------|------------------------------------------------------|
| **Completeness (%)** | 98.1 (84.7)                                        |
| **Average I/σ(I)**  | 16.6 (2.8)                                          |
| **R<sub:eq</sub> (%)** | 17.6 (59.5)                                        |

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**Structure and Mechanism of IcaB<sub>Ad</sub>**

|                | IcaB<sub>Ad</sub>23–280Δloop | PgaB<sub>Ad</sub>43–309 |
|----------------|-----------------------------|------------------------|
| **Total no. of reflections** | 173,478                    | 411,607                |
| **Resolution (Å)**           | 50.00-2.25                  | 50.00-1.70             |
| **No. of atoms**             | Protein: 1861               | Protein: 1850          |
|                             | Zn<sup>2+</sup>: 1          | Zn<sup>2+</sup>: 1     |
|                             | Chloride: 31.9              | Chloride: 31.7         |
|                             | Water: 44.2                 | Water: 44.2            |
| **Bond angles (°)**          | 1.09                        | 1.09                   |
| **Ramachandran plot (%)**    | 96.0                        | 95.8                   |
| **Disallowed (%)**           | 0.9                         | 0.8                    |
| **R<sub:eq</sub> (%)**       | 17.6 (59.5)                 | 17.6 (59.5)            |

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**Crystallographic Comparison of IcaB<sub>Ad</sub> and PgaB Reveals the Basis for PNGP Binding**

- **Structural Alignment**
  - IcaB<sub>Ad</sub>23–280Δloop with the N-terminal domain of PgaB, residues 43–309 (PgaB<sub>Ad</sub>43–309, PDB ID 4F9D), shows strong conservation of the CE4 domain with a root mean square deviation of 1.7 Å over 219 equivalent Ca atoms (Fig. 4A).
  - Two topological differences were found between IcaB<sub>Ad</sub>23–280Δloop and PgaB<sub>Ad</sub>43–309. First, α<sub>7</sub> of IcaB<sub>Ad</sub>23–280Δloop is tilted 105° relative to the equivalent α-helix in PgaB<sub>Ad</sub>43–309 (Fig. 4A). Second, IcaB<sub>Ad</sub>23–280Δloop only has three β-hairpin motifs (βH1–3), and the loops they reside in (L1, L3, and L4) are much shorter than the equivalent loops in PgaB<sub>Ad</sub>43–309 (Fig. 4A). The difference in loop architecture alters the architecture of the active site binding pocket (Fig. 4, B and C). Residues Tyr-67 and Trp-180 narrow the active site pocket to 10 Å in IcaB<sub>Ad</sub>23–280Δloop as compared with 16 Å in PgaB<sub>Ad</sub>43–309 (Met-67 and Gly-191) (Fig. 4, B and C). Furthermore, Arg-45 in IcaB<sub>Ad</sub>23–280Δloop is located at the beginning of the cleft at the ~1 subsite and would be available for hydrogen bonding to PNG. The equivalent residue in PgaB<sub>Ad</sub>43–309 is an asparagine (Fig. 1A) that is buried in the structure by 3 aromatic residues Phe-66, Tyr-117, and Phe-154.
Structure and Mechanism of IcaB

Docking Studies with PNAG Oligomers Suggest Three GlcNAc Binding Sites—Docking studies with β-1,6-(GlcNAc)_3 and β-1,6-(GlcNAc)_5 were conducted using IcaB_{Ad}^{23–280\Delta loop} to gain insight into the determinants for PNAG binding. The predicted affinity for β-1,6-(GlcNAc)_3 (~6.0 kcal/mol) was lower than β-1,6-(GlcNAc)_5 (~7.2 kcal/mol) in the docking studies, and inspection of the poses computed showed that the first and fifth residues of β-1,6-(GlcNAc)_3 (subsites −2 and +2) do not make contacts with IcaB_{Ad}^{23–280\Delta loop}. Furthermore, only the top β-1,6-(GlcNAc)_3 pose showed hydrogen-bonding contacts with all three N-Ac groups to IcaB_{Ad}^{23–280\Delta loop} and thus was used for further analysis. The docked β-1,6-(GlcNAc)_3 binds IcaB_{Ad}^{23–280\Delta loop} along the active site groove occupying subsites −1, 0, and +1 (Fig. 5A). GlcNAc in the −1 subsite has hydrogen bonds between Arg-45 and Glu-149 to the C5 oxygen (3.6 Å) and N-Ac nitrogen (2.9 Å), respectively (Fig. 5B). GlcNAc in the 0 subsite makes contacts with the Zn^{2+} ion via the hydroxyl at C3 (OH3) of the 0 subsite GlcNAc and the N-Ac carbonyl (2.3 Å), Asp-114 and OH3 (2.7 Å), Arg-45 and OH4 (3.6 Å), and stacking interactions with Tyr-67 (Fig. 5B). GlcNAc in the +1 subsite has hydrogen bonds between the backbone carbonyl of Tyr-224 and both the OH3 (3.6 Å) and the N-Ac carbonyl (3.5 Å) and T-shaped stacking interactions with Trp-180 (Fig. 5B). Interestingly, the N-Ac carbonyl and OH3 of the 0 subsite GlcNAc are in close proximity to the crystallographic waters, W2 and W3, with distances of 0.6 and 0.2 Å, respectively (Fig. 5B). This observation reinforces the confidence of the docking studies and the positioning of the 0 subsite GlcNAc during catalysis.

Mutational Analysis of IcaB Suggests an Altered CE4 Catalytic Mechanism—CE4s have been proposed to catalyze the deacetylation of their substrates using a metal-assisted general acid and base mechanism (30). The first conserved aspartate found in CE4 motif 1, TFDD, is considered the catalytic base. The conserved histidine in CE4 motif 5, LXH, is proposed to be the catalytic acid. From structural and mutational studies on the Streptococcus pneumoniae peptidoglycan deacetylase PgdA (30) and the Vibrio cholerae chitin deacetylase CDA (31), we hypothesized that His-50/His-44 and Asp-120/Asp-114 are the catalytic acid and base in IcaB_{Ad}/IcaB_{Se}, respectively. To assess
FIGURE 4. IcaB<sub>Ad</sub> has a narrow active site pocket compared with PgaB<sub>30–289</sub>. A, superposition of IcaB<sub>Ad</sub><sup>23–280</sup>loop (blue) and PgaB<sub>43–309</sub> (orange) shows a strong conservation of the CE4 fold with a root mean square deviation of 1.7 Å over 219 equivalent Ca atoms. B, Hairpin loops for IcaB<sub>Ad</sub> are labeled βH1–3, and the Zn<sup>2+</sup> ion is shown as a sphere colored dark gray. Surface representation of IcaB<sub>Ad</sub><sup>23–280</sup> (β) and PgaB<sub>43–309</sub> (C) is shown in the same representation as panel A with residues contributing to the difference in active site architecture colored magenta and green for IcaB<sub>Ad</sub><sup>23–280</sup>loop and PgaB<sub>43–309</sub>, respectively. The Ni<sup>2+</sup> ion in PgaB is shown as a teal-colored sphere.

FIGURE 5. Docking of PNAG oligomers. A, IcaB<sub>Ad</sub><sup>23–280</sup>loop (blue) with a β-1,6-(GlcNAc<sub>n</sub>) (yellow) docked in the active site shown in surface and stick representations, respectively. The model predicts that Arg-45, Tyr-67, and Trp-180 (magenta) are important for the binding of β-1,6-(GlcNAc<sub>n</sub>), B, active site close-up of the docked β-1,6-(GlcNAc<sub>n</sub>), C, active site close-up of the docked β-1,6-(GlcNAc<sub>n</sub>), showing the central sugar binds to the Zn<sup>2+</sup> ion and the crystallographic waters (W2 and W3, green spheres) overlap with the N-Ac carbonyl and OH-3 oxygens. The Zn<sup>2+</sup> ion is shown as a dark gray colored sphere, and the GlcNAc subsites are labeled from the non-reducing to reducing end as −1, 0, and +1 in both panels.

The role of these residues in the catalytic mechanism H50A/H44A and D120N/D114N variants were assayed for de-N-acetylation activity. The histidine variants were completely inactive (Fig. 6A), suggesting this conserved residue plays a key role in catalysis. However, the IcaB<sub>Se</sub> and IcaB<sub>Ad</sub> aspartate variants displayed 53 and 56% of wild type activity, respectively (Fig. 6A), suggesting these residues do not serve essential roles in the catalytic mechanism. Asp-120/Asp-114 may actually be involved in tuning the electrostatics of the active site as the asparagine variants lose their pH activation profile (Fig. 6B). To identify a residue in the active site pocket that could potentially serve as the base in the reaction, we used the p<sub>K<sub>a</sub></sub> prediction server PROPKA3 (50). The predicted p<sub>K<sub>a</sub></sub> of His-50/His-44 is ~3, suggesting it is basic under the assay conditions and could potentially have a bifunctional role in catalysis (Fig. 6C).

The Conserved Hydrophobic Loop of IcaB Is Involved in Membrane Association—The presence of a solvent-accessible hydrophobic loop in Gram-positive homologues of IcaB suggests it may have a functional importance. Given that these proteins are found on the extracellular surface of the bacteria, we hypothesized that the loop may play a role in membrane localization. IcaB<sub>Se</sub><sup>30–289</sup>, IcaB<sub>Se</sub><sup>30–289</sup>loop, IcaB<sub>Ad</sub><sup>23–280</sup>, and IcaB<sub>Ad</sub><sup>23–280</sup>loop were, therefore, assayed for their ability to bind and associate with staphylococcal membranes. Using a membrane pulldown assay, IcaB<sub>Se</sub><sup>30–289</sup> and IcaB<sub>Ad</sub><sup>23–280</sup> were co-fractionated into the pellet when incubated with staphylococcal membranes (Fig. 7A). IcaB<sub>Ad</sub><sup>23–280</sup>loop was only present in the soluble fraction, and IcaB<sub>Se</sub><sup>30–289</sup>loop had significantly reduced quantities found in the insoluble fraction (Fig. 7A). These data suggest that the hydrophobic loop plays a role in binding and associating with the membrane. Structural modeling of the hydrophobic loop in IcaB<sub>Se</sub><sup>30–289</sup> using Phyre<sup>2</sup> (51) predicts an α-helix spanning residues 59–66 that is amphipathic, with Phe-61, Ile-62, Leu-64, and Leu-65 lying on the solvent exposed face (Fig. 7B). A similar result was also seen for Trp-54, Gly-55, Leu-57, and Phe-58 in IcaB<sub>Ad</sub><sup>23–280</sup>.

DISCUSSION

IcaB has been identified as an attractive target for the design of inhibitors to combat biofilm-related infections by S. epidermidis and S. aureus for two reasons. First, it is an extracellular protein (26), which eliminates the need for membrane permeable drugs. Second, it is responsible for PNAG de-N-acetylation, which is required for biofilm formation and virulence in animal models (26, 27). Our recent study of IcaB<sub>Se</sub><sup>30–289</sup> was the first functional characterization of its metal- and length-dependent de-N-acetylation activity (32). IcaB<sub>Se</sub> and its Gram
negative homologue PgaB have the same enzymatic function but displayed different metal- and length-dependent activity on PNAG oligomers (28, 32). Additionally, the domain architecture and localization of the two proteins are drastically different. IcaBSe is a single-domain extracellular protein, whereas PgaB is a two-domain periplasmic lipoprotein (52, 53).

IcaB contains a hydrophobic loop that is conserved only in Gram-positive homologues (Fig. 1A). This hydrophobic loop is not required for de-N-acetylation of PNAG oligomers (Fig. 1B) but likely contains an amphipathic α-helix that can bind to staphylococcal membranes (Fig. 7). This type of amphipathic α-helix has been found in other integral or associated membrane proteins (54, 55) and is likely, at least in part, responsible for the retention of IcaB to the cell surface. Furthermore, the electropositive patch found on the structure of IcaBAd 23–280 contains an Asp-His-His triad that coordinates an ordered Zn²⁺ ion in the structure (Fig. 3B). IcaBAd 23–280 displays metal-dependent de-N-acetylation of PNAG oligomers with a preference for Ni²⁺, Co²⁺, and Zn²⁺ (Fig. 3C). PgaB displayed the highest activity with Co²⁺ and Ni²⁺ cofactors (28), whereas IcaBSe 30–289 had the highest activity with Co²⁺ and Zn²⁺ (32). The metal ion promiscuity in IcaB appears to be a continuing trend for the CE4 family. IcaBAd 23–280 shows structural similarity to PgaB 43–309 and contains the same circularly permuted arrangement of the CE4 motifs, a feature unique to these CE4 members (28, 56). Despite the high structural similarity and metal ion preference between IcaB and PgaB 43–309, only IcaB is enzymatically active on PNAG; PgaB requires both N- and C-terminal domains for de-N-acetylation activity (29). Structural studies on CDA and comparison to known CE4 structures reveals that loop architecture plays a major role in substrate binding and the enzymatic mechanism (31). The loop architecture of IcaBAd 23–280 is similar to other CE4s, like PgdA (30), which has a narrow binding pocket. Additionally, the conserved residues Arg-45, Tyr-67, and Trp-180 make important interactions with the three GlcNAc moieties at -1, 0, and +1 subsites, respectively, in the docking studies (Fig. 5). PgaB 43–309 does not contain any of
these conserved elements (Fig. 4, A and C), likely leading to loss of PNAG binding and thus rendering the isolated domain inactive. Previous docking and simulation studies have suggested that conserved elements, including a conserved arginine and tryptophan, on PgaB<sup>310–672</sup> are required for PNAG binding and catalysis in the <i>E. coli</i> enzyme (29). The cleft formed between the two domains of PgaB also creates a long extended active site (29). This correlates with the enzymatic data that shows length-dependent de-N-acetylation of the oligosaccharides up to β-1,6-(GlcNAc)<sub>3</sub> with preference for the central GlcNAc (28). The structure of IcaB<sub>Ad</sub><sup>23–280</sup>Δloop suggests only three GlcNAc subsites within the active site pocket (Fig. 5). This is supported by previous studies with IcaB<sub>Se</sub><sup>301–289</sup> that showed similar de-N-acetylation of β-1,6-(GlcNAc)<sub>3</sub> to β-1,6-(GlcNAc)<sub>6</sub> and displayed deacetylation preference at the second GlcNAc from the reducing end (32). The involvement of PgaB<sup>310–672</sup> during de-N-acetylation appears to be part of a mechanism for PgaB to continuously associate with the dPNAG polymer (29). This type of processive mechanism would produce an energetically favorable extended state of dPNAG to allow for efficient periplasmic translocation and hand-off to the export machinery (29). Because IcaB is an extra-cellular protein it does not need an auxiliary domain for continual processing and export of dPNAG. It is probable that IcaB will interact with dPNAG multiple times as they both are present in the extracellular matrix. This is consistent with the higher levels of deacetylation observed for <i>S. epidermidis</i> (~15–20%) over <i>E. coli</i> (~3–5%) in dPNAG isolated from source even though IcaB displays lower levels of activity than PgaB in vitro. The circular permutation of the CE4 motifs in IcaB and PgaB must play a role other than substrate binding during catalysis. PgaB has been proposed to operate through a similar enzymatic mechanism to other CE4 members (57) but is lacking a key catalytic residue that has been proposed to attenuate its catalytic efficiency (28). Our preliminary mutational analysis suggests Asp-120/Asp-114 is not the catalytic base as the equivalent D275N variant in PgdA inactivates the enzyme. In the IcaB<sub>Ad</sub><sup>23–280</sup>Δloop structure Asp-114 is in a bi-dentate salt bridge with Arg-261 (Asp-120 and Arg-267 in IcaB<sub>Se</sub>), which would prevent a free carboxylate group that could serve as the base. The equivalent salt bridge in both PgdA and CDA are not bi-dentate as the conserved arginine spans the active site in a different orientation. This would free the aspartate carboxylate group to abstract a proton from the nucleophilic water. Thus, we propose His-50/His-44 to be a bifunctional acid catalyst in the enzymatic mechanism (Fig. 6C). In our proposed scheme, hydrolysis of the 0 subsite GlcNAc N-Ac group would be initiated by the Zn<sup>2+</sup>-coordinated water molecule. Deprotonation of the water by His-50/His-44 would lead to nucleophilic attack of the N-Ac carbonyl group to produce a tetrahedral oxanyan intermediate that would be stabilized by the Zn<sup>2+</sup> ion and the backbone amide of Tyr-229/Tyr-224. The intermediate would then be protonated on the N-Ac nitrogen by the imidazolium of His-50/His-44. This would generate a free amine leaving group (dPNAG) and the acetic acid byproduct. Diffusion of acetic acid out and water into the active site would regenerate the enzyme and lead to another round of de-N-acetylation on the dPNAG polymer. Similar bifunctional roles have been proposed for a glutamic acid in the zinc metalloamidases LpxC (58, 59) and carboxypeptidase A (60, 61). Together, these results suggest that the circular permutation of the CE4 motifs in IcaB and its Gram-negative homologue PgaB may result in an altered CE4 enzymatic mechanism.

The structure, functional characterization, and membrane association of IcaB reported here will help facilitate additional studies that probe our proposed model for the biosynthesis and modification of PNAG in Gram-positive bacteria (Fig. 7C) and will help guide the development of novel inhibitors to prevent biofilm-related infection caused by <i>S. epidermidis</i> and <i>S. aureus</i>.

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