Characterization of the N-Acetylglucosaminyltransferase Activity Involved in the Biosynthesis of the \textit{Staphylococcus epidermidis} Polysaccharide Intercellular Adhesin*

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Christiane Gerke‡, Angelika Kraft§, Roderich Süßmuth‡, Oliver Schweitzer‡, and Friedrich Götz‡

From the ‡Zentrum Mikrobielle Genetik, Universität Tübingen, Waldhäuser Strasse 70/8, the §Max-Planck-Institut für Entwicklungsbiochemie, Abteilung Biochemie, Spemannstrasse 35, and the ‡Institut für Organische Chemie, Universität Tübingen, Auf der Morgenstelle 18, 72076 Tübingen, Germany

\textbf{The polysaccharide intercellular adhesin (PIA) is an important factor in the colonization of medical devices by Staphylococcus epidermidis. The genes encoding PIA production are organized in the icaADBC (intercellular adhesion) operon. To study the function of the individual genes, we have established an \textit{in vitro} assay with UDP-N-acetylglucosamine, the substrate for PIA biosynthesis, and analyzed the products by thin-layer chromatography and mass spectrometry. IcaA alone exhibited a low N-acetylglucosaminyltransferase activity and represents the catalytic enzyme. Coexpression of icaA with icaD led to a significant increase in activity. The newly identified icaD gene is located between icaA and icaB and overlaps both genes. N-Acetylglucosamine oligomers produced by IcaAD reached a maximal length of 20 residues. Only when icaA and icaD were expressed together with icaC were oligomer chains that react with PIA-specific antiserum synthesized. IcaA and IcaD are located in the cytoplasmic membrane, and IcaC also has all the structural features of an integral membrane protein. These results indicate a close interaction between IcaA, IcaD, and IcaC. Tunicamycin and bacitracin did not affect the \textit{in vitro} synthesis of PIA intermediates or the complete PIA biosynthesis \textit{in vivo}, suggesting that a undecaprenyl phosphate carrier is not involved. IcaAD represents a novel protein combination among \(\beta\)-glycosyltransferases.}

In recent years, \textit{Staphylococcus epidermidis} has emerged as a frequent cause of nosocomial infections in association with indwelling medical devices such as intravascular catheters, cerebrospinal fluid shunts, prosthesis heart valves, prosthetic joints, artificial pacemakers, and chronic ambulatory peritoneal dialysis catheters (reviewed in Refs. 1 and 2). The virulence of \textit{S. epidermidis} in these infections is thought to be based on its ability to colonize medical devices by forming a biofilm composed of multilayered cell clusters embedded in a slime matrix (3). As shown by electron microscopy studies, surface colonization takes place in two steps (4). The first step is primary adhesion of some bacteria, which is followed by proliferation of the cells to multilayered clusters. Factors reported to contribute to primary attachment of \textit{S. epidermidis} cells to a polymer surface include unspecific hydrophobic interactions (5, 6), a capsular polysaccharide/adhesin (PSA) (7, 8), proteinaceous cell-surface antigens (SSP-1 and SSP-2) (9, 10), and the autolysin AtlE identified by our group (11).

A characteristic feature of the second phase of biofilm formation is intercellular adhesion, which results in the formation of large cell clusters by clinical \textit{S. epidermidis} strains. This reaction is associated with the production of the polysaccharide intercellular adhesin (PIA) located at the cell surface (12). PIA consists of two structurally related homoglycans, polysaccharides I and II, composed of at least 130 2-deoxy-2-amino-N-acetylglycopyranosyl residues that are mostly (>80%) N-acetylated. The residues are \(\beta\)-1,6-linked (13), a type of linkage for N-acetylglucosamine polymers that has not been previously described. In addition, a 140-kDa extracellular protein that is missing in an accumulation-negative mutant of \textit{S. epidermidis} RP62A is thought to contribute to intercellular adhesion (14, 15).

Recently, we cloned and sequenced three \textit{S. epidermidis} RP62A genes (icaABC) that are involved in intercellular adhesion and PIA production (16). Transposon insertion mutants in the ica operon of \textit{S. epidermidis} O-47 (class B mutants) (17, 18) are unable to form a biofilm on various surfaces. It has also been shown that the majority of \textit{S. epidermidis} strains isolated from septicemic patients were biofilm-positive, whereas skin and mucosal isolates were not, even though the genome of the biofilm-forming strains contained the ica genes (19).

Here, we report the identification of a fourth gene (icaD) that is located within the ica operon and that is necessary for PIA synthesis. Furthermore, we demonstrate that IcaA and IcaD are located in the membrane and together exhibit N-acetylglucosaminyltransferase activity, in which IcaA represents the catalytic enzyme that requires IcaD for full activity. Finally, we show that in addition to icaA and icaD, icaC is essential for long-chain PIA synthesis.

**EXPERIMENTAL PROCEDURES**

\textbf{Bacterial Strains and Growth Conditions—DNA containing ica was isolated from PIA-producing and biofilm-positive \textit{S. epidermidis} RP62A (ATCC 35984). The \textit{Staphylococcus carnosus} wild-type strain TM300 (20) was used as cloning host for all staphylococcal plasmids and for heterologous expression of ica genes. Cloning in \textit{Escherichia coli} and the production of maltose-binding protein fusions were performed in \textit{E. coli} strain TB1 (ara \Delta [lac proAB]) rpsL (801 lacZΔM15) hsdR obtained from New England Biolabs (Schwalbach, Germany). Staphylococci were...}
prepared in Tryptone (Difco), 0.5% yeast extract (Difco), and 0.5% NaCl, which was also used to cultivate *E. coli*. When appropriate, the media were supplemented with tetracycline (10 μg/ml), chloramphenicol (10 μg/ml), or ampicillin (100 μg/ml).

**DNA Transformation—DNA manipulations, plasmid DNA isolation, and transformation of *E. coli* were performed according to standard procedures (21). To isolate DNA from staphylococci, cells were lysed by adding 0.1 mg/ml lysozyme (Sigma, Deisenhofen, Germany), and the alkaline lysis method was subsequently applied. Chromosomal staphylococcal DNA was prepared according to the procedure of Mar- moun (22). Staphylococcal transformation was determined by the method of Mandel (23). Polymerase chain reaction experiments were performed with Vent DNA polymerase (New England Biolabs) as recommended by the manufacturer. DNA sequences were determined using the Sequenase protocol (Version 2.0, Amersham, Braunschweig, Germany) or a LI-COR DNA sequence analyzer (MWG-Biotech, Ebersberg, Germany).

**Primers and Plasmid Constructions—** In this study, the *ica* genes were amplified by polymerase chain reaction from chromosomal DNA of *S. epidermidis* RPE2A and cloned independently or in various combinations in the inducible staphylococcal expression vectors pTX15 (24) and pCX19, a derivative of pCX15 (25). Gene expression was induced by adding 0.5% xylose (final concentration) to the growth medium (LB medium) at an optical density at 578 nm = 0.4 and further incubation of the strains for 6 h. The restriction sites used for the construction of the following plasmids are indicated in parentheses: pTXicaA carries icaA amplified using primers 4 and 5, cleaved with *BglII* and *MluI* (Hind III); pTXOcarb contains icaA amplified using primers 4, 18, and 5 (see above) with 2 mM UDP-N-acetylglucosamine (UDP-N-acetylglucosamine), which was solubilized as described above using 2% (w/v) Triton X-100 (final Triton X-100 concentration of 0.5% and 50 μm D-cycloserine (26)). For radiolabeling, 10 μM UDP-N-acetyl-1-14C-glucosamine (final radioactive concentration of 84 Bq (2.3 nCi/μl of reaction volume; Amersham) was added). Analysable reactions were carried out in a total volume of 10–50 μl. Reaction mixtures were incubated for 12 h at 20 °C. To test other substrates, UDP-glucose, UDP-galactose, and UDP-glucuronic acid (14C-labeled substrates from Amersham) were used in equimolar amounts. For experiments in the presence of tunicamycin and bacitracin, the antibiotics were added in the above-mentioned concentrations.

**Analytical Methods—** For thin-layer chromatography, in each case, 1 μl of *in vitro reaction* assays containing 14C-labeled UDP-N-acetylgalactosamine was applied to NH2-HPTLC plates (Merck, Darmstadt, Germany). PIA was detected with the PIA-specific antiserum (BA83) using the Bio-Dot Microfiltration apparatus (Bio-Rad, München, Germany). PIA was detected with the PIA-specific antiserum (1:5000) (Sigma) and with streptavidin-horseradish peroxidase complex (1:5000; Amersham). Immunoreactive proteins were visualized by enhanced chemiluminescence (ECL, Amerham) with Fuji HR-E30 films. Repробing of a blot with another primary antiserum was performed according to the ECL Western blotting protocol.

**PIA production in vitro** was analyzed in crude extracts and in cell-surface extracts obtained by incubating the cells in the 0.5 μl EDTA, pH 8.0, for 5 min at 100 °C. For production experiments in the presence of antibotics, tunicamycin (5–20 μg/ml) and bacitracin (50–400 μg/ml) were added to cultures of *S. carnosus* pTXADBC at the time of induction. **PIA production in vitro** was determined in the enzymatic assays. Extracts (1 μl each) were applied to a nitrocellulose membrane (BA83) using the Bio-Dot Microfiltration apparatus (Bio-Rad, München, Germany). PIA was detected with the PIA-specific antiserum (1:5000) (16). Bound PIA antibodies were visualized by anti-rabbit immunoglobulin G-alkaline phosphatase conjugate (1:5000; Sigma) and color reaction.

**In Vitro Enzymatic Assay (N-Acetylglucosaminyltransferase Assay)**— *In vitro* reactions to analyze glycosyltransferase activity were performed by incubating crude extracts (see above) with 0.4 μM UDP-N-acetylgalactosamine or crude membranes or Triton-extracted membranes (see above) with 2 mM UDP-N-acetylgalactosamine. To test other substrates, UDP-glucose, UDP-galactose, and UDP-glucuronic acid (14C-labeled substrates from Amersham) were used in equimolar amounts. For experiments in the presence of tunicamycin and bacitracin, the antibiotics were added in the above-mentioned concentrations.

**Analytical Methods—** For thin-layer chromatography, in each case, 1 μl of *in vitro reaction* assays containing 14C-labeled UDP-N-acetylgalactosamine was applied to NH2-HPTLC plates (Merck, Darmstadt, Germany). The chromatogram was developed twice using acetoniitrite/water (60:40 or 65:35, v/v). Radioactive spots were detected by phosphoimaging with a Fuji BAS 1500 (Raytest, Straubenhardt, Germany) or by autoradiography. Fuji HR-E30 films were exposed for 6–10 weeks. As a reference compound, N-acetyl-1-14C-glucosamine (25 Bq) or unlabelled N-acetylgalactosamine (10 μg) was used. If appropriate, 0.005% Triton X-100 was added. The unlabelled standard was visualized by spraying with 0.2% orcinol in H2SO4 and subsequent heating at 100 °C for 15 min.

For HPLC analysis of the *in vitro* synthesized products, the pH of the reaction assays was lowered to 4.5 with phosphoric acid; the samples were heated for 5 min at 100 °C; and the precipitated material was sedimented by centrifugation (10 min, 13,000 g; × g). Acetonitrile was added to the supernatant to a final concentration of 75%. The products...
For detailed studies, the membrane fraction was divided into icaA and icaD deduced polypeptide of fusions of IcaA and IcaD were raised in rabbits. By Western
of IcaA and IcaD, antisera against maltose-binding protein the same direction as the icaABC were expressed in
open reading frame whose relevance was, until then, unclear. In these experiments, evident transferase activity was detected
putative substrate for N-acetylglucosamine oligomer synthesis. When UDP-glucose, UDP-galactose, and UDP-glucuronic acid, which often function as substrates for the biosyn-
and icaA by 37 nucleotides and with the start of icaB by 4 nucleotides. The DNA sequence of the overlapping region of icaA and icaD is shown. The
putative start codon and ribosomal binding site (Shine-Dalgarno (SD)) of icaD are indicated by boldface letters; the stop codon of icaA is underlined. The numbering refers to the sequence of the ica operon available from GenBank under accession number U43366. Relevant restriction sites are indicated. The BseAI site was used to introduce a frameshift deletion mutation in icaD. kb, kilobase.

were separated on a Nucleosil 120-7-NH2-HPLC column (4.6 × 250 mm; Bischoff, Leonberg, Germany) and eluted with an acetonitrile gradient in
pure water from 75 to 50% acetonitrile over 45 min at a flow rate of 1 m/min. Subsequently, the column was washed for 10 min with pure water. Elution was monitored by determining A205 nm. Radiolabeled products were detected by measuring 14C using a flow counter (Packard Instrument Co.).

Electrospray mass spectra were recorded on a triple-quadrupole mass spectrometer Model API III (mass range of m/z 10–2400) equipped with a pneumatic supplied electrospray (“ion spray”) interface (Sciex, Thornhill, Ontario, Canada). The mass spectrometer was operated in positive ion mode under unit mass resolution conditions for all determinations. The potential of the spray needle was kept at 4.8 kV; the orifice voltage was +80 V. For mass calibration, a solution of polypropylene glycol was used. Unlabeled IcaAD-dependent products synthesized in vitro were applied to the mass spectrometer by HPLC/MS coupling injecting 100 μl/min into the mass spectrometer.

RESULTS
Identification and Characterization of icaD—To analyze the biosynthesis of the N-acetylgalcosamine polymer PIA, the ica genes were expressed under the control of the xylose-inducible promoter of the pTX15 expression vector (24) independently and in various combinations in S. carnosus. Glycosyltransferase activity was studied by incubating crude extracts or cell fractions of these clones with UDP-N-acetylgalcosamine, the putative substrate for N-acetylgalcosaminyl polymer synthesis. In these experiments, evident transferase activity was detected when icaA and the intergenic region between icaA and icaB were expressed in S. carnosus. This region harbors a small open reading frame whose relevance was, until then, unclear. The gene was designated icaD (Fig. 1).

The icaD gene is 306 nucleotides long and is transcribed in the same direction as the icaABC genes. The start of icaD overlaps with the end of icaA by 37 nucleotides (Fig. 1), and the end of icaD overlaps with the start of icaB by 4 nucleotides. The deduced polypeptide of icaD consists of 101 amino acids with a calculated molecular mass of 11,953 Da and a pI of 9.88. IcaD contains 54.5% hydrophobic amino acids, and two transmembrane helices are predicted. Similarity searches in data bases revealed no sequence similarity of IcaD to known proteins. Inactivation of icaD in a plasmid containing the complete ica operon led to the loss of cell aggregation and PIA production in S. carnosus (data not shown), indicating that, in addition to icaABC, icaD plays an essential role in intercellular adhesion and PIA production in vivo.

Localization of IcaA and IcaD—To determine the localization of IcaA and IcaD, antisera against maltose-binding protein fusions of IcaA and IcaD were raised in rabbits. By Western blot analyses, the presence of IcaA and IcaD was analyzed in various cell fractions from induced S. carnosus pTXicaAD cells. For detailed studies, the membrane fraction was divided into crude membranes (membranes derived from the crude extract by ultracentrifugation) and Triton-extracted membranes (membranes purified by Triton X-100 extraction). The anti-IcaA antiserum reacted with a 38-kDa protein present in the crude extract, the crude membranes, and the Triton-extracted membranes, but not with any protein in the cytosolic fraction or the soluble fraction obtained from Triton X-100 extraction (Fig. 2A). No reaction of the antiserum was observed with Triton-extracted membranes from uninduced S. carnosus pTXicaAD or from S. carnosus pTX16 (24) carrying the vector alone, indicating that the reaction of the antiserum was IcaA-specific. The IcaD-specific antiserum reacted with a 34-kDa protein that was present in the same fractions as IcaA (Fig. 2B). Thus, both IcaA and IcaD are membrane-bound. IcaA and IcaD exhibited very similar apparent molecular masses in the SDS-polyacrylamide gel electrophoresis analyses. We therefore reprobed the blot that was analyzed with the IcaD-specific antiserum with the anti-IcaA antiserum to confirm that IcaA and IcaD are independent proteins. The result (Fig. 2C) clearly showed that the two antisera recognize different proteins.

In Vitro Synthesis and Characterization of PIA Oligomers—The glycosyltransferase activity of IcaAD was analyzed in crude extracts and membranes from S. carnosus pTXicaAD. As a negative control, extracts from S. carnosus pTX16 were used. Products synthesized in vitro from UDP-N-acetylgalcosamine (5–25% of the applied substrate was 14C-labeled) were analyzed by HPTLC on NH2-HPTLC plates (Fig. 3). With crude extract, crude membranes, and Triton-extracted membranes from induced S. carnosus pTXicaAD, but not with the cytosolic fraction or the soluble fraction obtained from Triton X-100 extraction, radiolabeled products were formed that were separated in a ladder-like series. This ladder strongly suggested that the products represent oligomers with an increasing number of residues. No such products were synthesized with extracts from S. carnosus pTX16 cells (Fig. 3) or extracts from uninduced S. carnosus pTXicaAD cells (data not shown), indicating that the observed transferase activity was due to icaA and icaD. When UDP-glucose, UDP-galactose, and UDP-glucuronic acid, which often function as substrates for the biosynthesis of exoplymers, were tested, we obtained no ladder of products (data not shown).

The migration rates of the products synthesized with crude membranes and Triton-extracted membranes were different. Since the migration rate of the reference compound N-acetylgalcosamine differed as well after addition of Triton X-100 (0.005%, v/v), as indicated in Fig. 3, the deviation is probably due to the Triton X-100 remaining in the Triton-extracted membrane preparation. These data were supported by mass spectrometric analyses, in which the oligomers synthesized
with Triton-extracted membranes were found to be associated with Triton X-100 molecules (data not shown).

The mass spectrometric analyses were performed to further characterize the in vitro synthesized reaction products. For these analyses, the products were separated by HPLC. The elution profile of the IcaAD-dependent products from Triton-extracted membranes, recorded by analyzing 10 μl of radiolabeled assay mixtures, is shown in Fig. 4. HPLC separation was performed on an NH₂-HPLC column using an acetonitrile gradient. The HPLC separation system was comparable to the NH₂-HPTLC system. By isolating the eluted compounds and analyzing them again by HPTLC, we found that the succession of the eluted IcaAD-dependent products was the same in HPLC and HPTLC analyses (data not shown). The numbers of the spots in the high performance thin-layer chromatogram (Fig. 3) and the peaks in the high performance liquid chromatogram (Fig. 4) correspond to identical substances.

In the electrospray mass spectrometric analyses of nonradioactive products synthesized with Triton-extracted membranes, spectra of the substances eluting as peaks 1–8 were recorded. They revealed molecular ions at m/z 204, 407, 610, 813, 1016, 1219, 1422, and 1625 (Fig. 4). These values correspond to the [M + H]⁺ pseudo-molecular ions of a monomer to octamer of N-acetylglucosamine lacking one water residue/sugar chain since the displayed ions were 18 mass units smaller than the calculated [M + H]⁺ pseudo-molecular ions of the fully hydrated N-acetylglucosamine monomer to octamer (m/z 222, 425, 628, 831, 1034, 1237, 1440, and 1643).

The MS analyses of the in vitro products from S. carnosus induced (unless otherwise stated) cells of IcaD (indicated by arrows) was analyzed in the following extracts from induced (unless otherwise stated) cells of S. carnosus pTixaAD: lane CE, crude extract (1 μg); lane C, cytoplasmic fraction (1 μg); lane M, crude membranes (0.5 μg); lane TM, Triton-extracted membranes (0.5 μg); lane TM⁎, Triton-extracted membranes of uninduced cells (0.5 μg); lane TS, soluble fraction obtained from Triton extraction (same volume as Triton-extracted membrane fraction); lane TM⁎, Triton-extracted membranes heated for 3 min at 100 °C (0.5 μg) before loading on gel (all other samples were not heated); Triton-extracted membranes of S. carnosus pTX16 were applied as a negative control (0.5 μg).

The localization and sizes of the marker proteins are indicated on the left.
crude membranes and Triton-extracted membranes revealed deacetylated N-acetylglucosamine residues, which compose 15–20% of the major polysaccharide I of PIA (13).

Bacterial exopolysaccharides are often synthesized on an undecaprenyl phosphate lipid carrier (27). We therefore investigated whether a lipid carrier is involved in the synthesis of the IcaAD metabolites. Attempts to isolate lipid-linked intermediates in vitro or in vivo according to the method of Bligh and Dyer (28) failed. Furthermore, the antibiotics tunicamycin and bacitracin, which affect undecaprenyl phosphate-mediated syntheses, had no effect on either in vitro oligomer synthesis or in vivo PIA production (data not shown).

Analysis of the Individual Functions of icaA and icaD—To dissect the individual functions of IcaA and IcaD in N-acetylglucosaminyltransferase activity, the corresponding genes were inducibly expressed in S. carnosus pTX-icaAD. Prepurified radiolabeled products were loaded onto a Nucleosil NH2-HPTLC column and eluted with an acetonitrile/water gradient. To specifically detect action products, the elution was followed by measuring 14C using a flow counter. The numbered elution peaks and the substances they represent correspond to the numbered TLC spots in Fig. 3. By HPLC/MS analysis of unlabeled reaction products, ions of the numbered elution peaks corresponding to the [M + H]+ pseudo-molecular ions of the monomer to octamer of N-acetylglucosamine lacking one water residue (M, 18)/oligomer ([M + H]+ of the fully hydrated oligomers: 222, 425, 628, 831, 1004, 1237, 1440, and 1643).

DISCUSSION

In previous work (16), we showed that the ica operon is responsible for the production of PIA. Since PIA consists of a β-1,6-linked homoglycan composed of N-acetylglucosamine (13), we expected activated N-acetylglucosamine to be a substrate for the glycosyltransferase reaction in PIA biosynthesis. Indeed, with UDP-N-acetylglucosamine, we obtained oligomer synthesizing activity in vitro with crude extracts and membranes of S. carnosus clone expressing the ica operon. UDP-glucose, UDP-galactose, and UDP-glucuronic acid each revealed no activity, and nonactivated N-acetylglucosamine and glucosamine also failed to function as substrates in the in vitro assay (data not shown).

To allocate the N-acetylglucosaminyltransferase activity, the ica genes were introduced independently or in various combinations into S. carnosus, and cell extracts were tested for glycosyltransferase activity. We could thus demonstrate that IcaA alone revealed a weak N-acetylglucosaminyltransferase activity (Fig. 5, lane A). Although the activity was very weak, there is no doubt that IcaA represents the actual N-acetylglucosaminyltransferase since no transferase activity was obtained with IcaD, IcaB, and/or IcaC in the absence of IcaA. This is in accordance with the sequence similarity of IcaA to processive glycosyltransferases, which add multiple monosaccharides to a growing sugar chain (29). In the N-acetylglucosaminyl-
Fig. 5. TLC of reaction products of IcaA and/or IcaD. In vitro reactions containing 14C-labeled UDP-N-acetylglucosamine were performed with Triton-extracted membranes from induced S. carnosus carrying pTX16 (lane -), pTXicaA (lane A), pCXicaD (lane D), pTXicaA and pCXicaD (lane AD 2pl.), or pTXicaAD (lane AD 1pl.). In addition, a 1:1 mixture (lane A+D mix) of the Triton-extracted membranes of S. carnosus pTXicaA and S. carnosus pCXicaD was analyzed. The samples were analyzed by NH2-HPTLC. The results were visualized by autoradiography. The arrowhead indicates the origin of TLC. The arrows indicate the products of IcaA alone; their degree of oligomerization is indicated on the right. The position of N-acetylglucosamine (containing 0.005% (w/v) Triton X-100 (GlcNac (T)) is marked by a bar.

The glycosyltransferase NodC of rhizobia (30, 31), the hyaluronan synthase HasA of Streptococcus pyogenes (32), bacterial cellulose synthases (33), chitin synthases of fungi (34), and the DG42 protein of Xenopus laevis, which synthesizes Nod-like oligomers during embryogenesis (35), five amino acids are conserved that are thought to function as catalytic residues (36). These amino acids are also conserved in IcaA (Asp134, Asp227, Gln273, Arg276, and Trp277) as shown in Fig. 7.

Fig. 6. Analysis of the influences of IcaB and IcaC on the degree of polymerization of the IcaAD-dependent products. The in vitro effect of IcaB and IcaC was analyzed using crude membranes of induced S. carnosus harboring pTXicaAD (lane AD), pTXicaADB (lane ADB), pTXicaADBC (lane ADBC), pTXicaBC (lane BC), or the negative control plasmid pTX16 (lane -) and, in addition, a mixture of crude membranes from S. carnosus pTXicaAD and S. carnosus pTXicaBC (lane AD+BC). The assays performed with 14C-labeled substrate were analyzed by NH2-HPTLC. N-Acetyl-β-[1-14C]glucosamine and UDP-N-acetyl-[U-14C]glucosamine (25 Bq each) were used as reference compounds. The results were visualized by autoradiography. The monomer to octamer of N-acetylglucosamine as determined by mass spectrometry are marked. The unreacted UDP-N-acetylglucosaminyltransferase of the reaction assays did not correspond in its migration to that of the pure substance, but it was contained in the spot smearing directly above the origin. The arrowhead indicates the origin of TLC.

protein could also correspond to a trimmer of the 12-kDa monomer. IcaA and IcaD are both membrane-bound (Fig. 2), which correlates with the finding that essentially membrane fractions showed transferase activity (Fig. 3). The membrane location was not unexpected since the hydrophobicity plots suggested the presence of four transmembrane helices in IcaA (one located at the N terminus and three at the C terminus) (16) and two transmembrane domains in IcaD.

To exhibit its function, IcaD probably has to interact with IcaA prior to membrane integration since mixing of extracts from an S. carnosus clone expressing IcaA and a clone expressing icaD did not increase the N-acetylglucosaminyltransferase activity over that of IcaA alone (Fig. 5, lane A+D mix), whereas in trans expression of icaA and icaD did (lane AD 2pl.). The lower activity obtained with icaA and icaD expressed in trans compared with icaAD expressed in cis probably resulted from the different copy numbers of the vectors used (high copy pTX-icaA and medium copy pCXicaD).

The concrete function of IcaD is not yet known. The interaction of IcaA and IcaD might be necessary due to the unusual β-1,6-linkage of the N-acetylglucosamine residues in PIA. Sugar chains composed of β-1,6-linked monomers differ greatly in their three-dimensional structure from the straight β-1,4-sugar chains synthesized by most of the processive glycosyltransferases. Another possibility is that the IcaA protein needs IcaD to obtain an active conformation. Since IcaD is also a...
membrane-bound protein, it might act as a chaperone that directs the membrane insertion of IcaA.

The combination of two proteins to achieve optimal activity is new among the β-glycosyltransferases. No IcaD-like protein appears to be involved in the transferase activities of homologous enzymes. NodC (424 amino acids) and HasA (395 amino acids), with a length similar to that of IcaA (412 amino acids), as well as the bacterial cellulose synthase AcscA (754 amino acids), the eukaryotic chitin synthases (>1000 amino acids), and the DG42 protein (588 amino acids) do not contain a motif similar to IcaD.

An undecaprenyl phosphate lipid carrier that is often involved in the biosynthesis of bacterial exopolysaccharides (27) does not appear to participate in the activity of IcaAD. First, we never detected lipid-linked intermediates in vitro or in vivo. And second, the antibiotics tunicamycin and bacitracin had no effect on the synthesis of IcaAD-dependent oligomers or PIA in vitro and in vivo. The homologous enzymes NodC, HasA, the chitin synthases, and the cellulose synthase of Acetobacter xylinum also produced no detectable lipid-linked intermediates, and tunicamycin and bacitracin had no effect on their activities (37–40).

The products synthesized from IcaAD in vitro were analyzed in detail by HPLC separation combined with mass spectrometry. The analysis of the products synthesized with Triton-extracted membranes revealed products that correspond to N-acetylglucosamine oligomers lacking one water residue/chain. The putative anhydro oligomers were also formed with crude membranes. In addition, two further types of oligomers were obtained with crude membranes that correspond to (i) fully hydrated N-acetylglucosamine oligomers and (ii) N-acetylgalactosamine oligomers carrying an as yet unidentified modification that is currently under investigation. The water elimination that produces the anhydro oligomers might result from an intramolecular reaction that releases the oligosaccharide from the synthesizing enzyme. Since an anhydro bond can be considered to store the energy of a glycosidic bond, it could be important for a further elongation of the oligomers without a new activation. Anhydro bonds, for example, also exist in the peptidoglycan of E. coli, in which the sugar chains do not contain a reducing end, but carry 1,6-anhydromuramic acid (41).

No oligomers containing N-unsubstituted residues, which compose 15–20% of the major polysaccharide I of PIA in vivo (13), were detected by MS analysis. Since the known pathways for the synthesis of amino sugar-containing polysaccharides utilize the nucleotide-linked N-acetylamino sugars as precursors, it seems most likely that the N-unsubstituted amino sugar residues of PIA are produced through enzymatic deacetylation subsequent to the formation of the polymer chain. The MS analyses were only performed with products synthesized with membranes, and therefore, the putative deacetylation activity may have escaped if it is located in another cell fraction. Known deacetylating enzymes are localized in different cell fractions (42–44). However, it is also possible that the percentage of deacetylated IcaAD-dependent oligomers was too low to be detected or that only full-length PIA is a substrate for deacetylation. It was not possible to test whether the N-unsubstituted residues in PIA might be derived from the direct incorporation of glucosamine because UDP-glucosamine is not commercially available.

IcaAD synthesized oligomers of a maximal length of 20 residues as determined by TLC analysis, but did not form polymers comprising full-length PIA, which was shown to be at least 130 residues long (13). By expressing icaADC in S. carnosus, products were synthesized that did not migrate on TLC plates and that reacted with the PIA-specific antiserum. We therefore assume that the nonmigrating products represent longer oligosaccharide chains. We cannot rule out that these products might represent modified or protein-bound oligomers. However, an S. carnosus clone expressing icaADC is capable of forming cell aggregates. This speaks against the possibility that the IcaAD-dependent products simply represent PIA precursors and suggests that the genes icaA, icaD, and icaC are sufficient to direct the biosynthesis of PIA. The genes icaA, icaD, and icaC must, however, be coexpressed in one strain to obtain synthesis of the longer oligomers (Fig. 6). Thus, IcaA, IcaD, and IcaC, which is predicted to be an integral membrane protein (16), probably form a complex and assemble in the membrane in a coordinated manner. This would explain why mixing of crude extracts from strains expressing icaAD and icaC, respectively, did not lead to PIA biosynthesis.

The IcaB protein, predicted to be a secreted protein (16), does not seem to be involved in the biosynthesis of the PIA sugar chain; it had no detectable effect in the in vitro assays. Its function in the PIA-mediated cell aggregation is being investigated.

The data presented here show that IcaA, IcaD, and IcaC are involved in the biosynthesis of the sugar backbone of PIA. IcaAD constitutes N-acetylglucosaminyltransferase activity in which IcaA represents the actual transferase that requires IcaD for full activity. This is the first enzymatic activity identified to be involved in intercellular adhesion in S. epidermidis.

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