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Staphylococcus aureus Cap5O Has UDP-ManNAc Dehydrogenase Activity and Is Essential for Capsule Expression

MARTA PORTOLÉS,[1] KEVIN B. KISER,[1]† NAVNEET BHASIN,[1]‡
KENNETH H. N. CHAN,[2] AND JEAN C. LEE[1]*

Channing Laboratory, Department of Medicine, Brigham and Women's Hospital and Harvard Medical School, Boston, Massachusetts 02115,[1] and Institute for Biological Science, National Research Council Canada, Ottawa, Canada K1A 0R6[2]

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The *Staphylococcus aureus* serotype 5 capsular polysaccharide (CP5) has a repeating unit composed of (→4)-3-*O*-acetyl-β-D-ManNAcA-(1→4)-α-L-FucNAc (1→3)-β-D-FucNAc-(1→)$_n$. Sixteen chromosomal genes (*cap5A* through *cap5P*) are involved in the synthesis of CP5. We recently demonstrated that Cap5P, a 2-epimerase, catalyzes the conversion of UDP–*N*-acetyl glucosamine (UDP-GlcNAc) to UDP–*N*-acetylmannosamine (UDP-ManNAc). In this study, we show that UDP-ManNAc is oxidized to UDP–*N*-acetylmannosaminuronic acid (UDP-ManNAcA) by a UDP-ManNAc dehydrogenase encoded by *S. aureus cap5O*. We expressed Cap5O in *Escherichia coli* and purified the recombinant protein. The UDP-ManNAc dehydrogenase activity of purified Cap5O was assessed by incubating Cap5P and UDP-GlcNAc (to produce UDP-ManNAc), together with Cap5O, NAD^+, and a reducing agent. Enzymatic activity was quantitated indirectly by measuring the increase in absorbance at 340 nm resulting from NADH formation. The product of the reaction was confirmed as UDP-ManNAcA by gas chromatography-mass spectroscopy. A *cap5O* mutation, created by deletion of 727 bp in the 5′ end of the gene, was introduced by allelic replacement into *S. aureus* Reynolds, rendering it CP5 negative. Mice inoculated intravenously or subcutaneously with the wild-type strain Reynolds had greater numbers of *S. aureus* recovered from their kidneys ($P = 0.019$) or their subcutaneous abscesses ($P = 0.0018$), respectively, than did animals inoculated with the *cap5O* mutant. The results of this study indicate that *S. aureus cap5O* is essential for capsule production and that capsule promotes staphylococcal virulence in mouse models of abscess formation.

Staphylococcus aureus is responsible for a variety of infectious diseases ranging from cutaneous infections, such as abscesses, boils, and wound infections, to life-threatening infections such as bacteremia and endocarditis (23). Because *S. aureus* produces many adhesins, exoenzymes, and exotoxins, the pathogenesis of staphylococcal infections is multifactorial. In addition, the presence of an extracellular polysaccharide capsule promotes staphylococcal virulence in certain animal models of infection (25, 31). Most clinical isolates produce serotype 5 or 8 polysaccharide capsules (CP5 or CP8) (2, 9). These two types of capsule have a similar trisaccharide repeating unit that differs only in the linkages between the sugars and the sites of O acetylation: CP5, (→4)-3-*O*-acetyl-β-D-ManNAcA-(1→4)-α-L-FucNAc-(1→3)-β-D-FucNAc-(1→)$_n$; CP8, (→3)-4-*O*-acetyl-β-D-ManNAcA-(1→3)-α-L-FucNAc-(1→3)-β-D-FucNAc-(1→)$_n$.

Sixteen chromosomal genes (*cap5A* through *cap5P*) are involved in the synthesis of CP5 (29). We recently demonstrated that Cap5P is a 2-epimerase that catalyzes the conversion of UDP–*N*-acetylglucosamine (UDP-GlcNAc) to UDP–*N*-acetylmannosamine (UDP-ManNAc) (14). Adjacent to *cap5P* in the capsule gene region is *cap5O*. The putative amino acid sequence of Cap5O shows homology to *Escherichia coli* RffD, a UDP-ManNAc dehydrogenase involved in the biosynthesis of the enterobacterial common antigen (24). We showed that *S. aureus cap5O* functionally complemented an *rffD* mutation in *E. coli* and restored expression of the enterobacterial common antigen (13). We report here the in vitro enzymatic activity of Cap5O, which catalyzes the oxidation of UDP-ManNAc to UDP–*N*-acetylmannosaminuronic acid (UDP-ManNAcA), the putative donor of ManNAcA residues in CP5.

The deletion of a bacterial gene critical to capsule biosynthesis should yield an unencapsulated phenotype. However, we recently reported that a *cap5P* mutation did not affect *S. aureus* capsule expression. Capsule production by the *cap5P* mutant was explained by the presence on the *S. aureus* chromosome of a second gene (*mnaA*) that encodes a UDP-GlcNAc 2-epimerase (14). In this study, we showed that a *cap5O* mutant of *S. aureus* is unencapsulated and less virulent for mice compared with the parental strain.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth media. The bacterial strains and plasmids used in this study are listed in Table 1. Luria-Bertani medium was used for growth of *E. coli*. *S. aureus* strains were grown in tryptic soy broth or on Columbia agar (Difco Laboratories, Detroit, Mich.) plates supplemented with 2% NaCl. The culture medium contained chloramphenicol (Cm) at 10 μg/ml, erythromycin (Em) at 5 μg/ml, or kanamycin (Km) at 25 μg/ml when required.

Chemicals. Reagents used in enzyme purification and activity assays were obtained from Sigma Chemical Co. (St. Louis, Mo.) unless otherwise noted. Ultrapure reagents used for methanolysis, reduction, and derivatization of sugars were obtained from J. T. Baker, Inc. (Phillipsburg, N.J.); Sigma; or ICN Biomedicals, Inc. (Aurora, Ohio). Alditol acetate derivatives of GlcNAc and ManNAc

* Corresponding author. Mailing address: Channing Laboratory, 181 Longwood Ave., Boston, MA 02115-5899. Phone: (617) 525-2652. Fax: (617) 731-1541. E-mail: jean.lee@channing.harvard.edu.

† Present address: NEN Life Science Products, Inc., Boston, MA 02118.

‡ Present address: Maxwell Finland Laboratory of Infectious Diseases, Boston University School of Medicine, Boston, MA 02118.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype or phenotype	Source or reference
E. coli BL21(DE3)	F^- *ompT lon hsdS*$_B$ (r_B^- m_B^-) DE3	Novagen, Inc.
S. aureus		
Reynolds	CP5 positive	9
JLO22	727-bp deletion in *cap5O* gene of Reynolds, CP5 negative	This study
RN4220	Capsule negative, restriction negative	27
Plasmids		
pET-24a+	*E. coli* expression vector (Km^r)	Novagen, Inc.
pJCL24	9.1-kb *Eco*RI fragment from *S. aureus* Reynolds (*cap5H* to *cap5P*) in pLI50	21
pKBK4	1.3-kb PCR amplicon carrying *cap5O* in pUC19	13
pKBK5	9.1-kb *Eco*RI fragment (*cap5H* to *cap5P*) from pJCL24 in pUC19	This study
pKBK9	4.1-kb *Ava*I-*Eco*RI fragment (*cap5M* to *cap5P*) from pKBK5 in pUC19	This study
pKBK9-2	727-bp *Hpa*I deletion (Δ*cap5O*) of pKBK9	This study
pKBK22	3.4-kb *Bam*HI-*Eco*RI fragment (*cap5M* to *cap5P*; Δ*cap5O*) from pKBK9-2 in pTS1	This study
pKBK24	2.4-kb PCR amplicon carrying *cap5O* and *cap5* flanking sequences in pLI50	This study
pLI50	Shuttle vector (Ap^r Cm^r)	16
pNB1	1.3-kb *Xba*I-*Eco*RI fragment (*cap5O*) from pKBK4 in pET-28a+	This study
pTS1	Temperature-sensitive shuttle vector (Ap^r Cm^r)	26
pUC19	*E. coli* cloning vector (Ap^r)	New England Biolabs, Inc.

(Aldrich Chemical Co., Milwaukee, Wis.) were used as standard sugars in gas chromatography-mass spectroscopy (GC-MS) analysis. Restriction endonucleases and other DNA modification enzymes were obtained from Life Technologies, Inc. (Gaithersburg, Md.), or New England Biolabs, Inc. (Beverly, Mass.).

Subcloning of *cap5O* into the pET-28a+ expression vector. The 1.3-kb *Xba*I-*Eco*RI fragment from pKBK4 comprises a PCR amplicon containing the *cap5O* gene and upstream Shine-Dalgarno sequence; this fragment was ligated into pET-28a+ (Novagen, Inc., Madison, Wis.). In the resultant plasmid, pNB1, the 3′ end of the *cap5O* gene was fused in frame to the His tag sequence of the vector. PCR fidelity was confirmed by DNA sequencing.

Construction of *S. aureus cap5O* mutant. The 9.1-kb *Eco*RI fragment (*cap5H* to *cap5P*) from pJCL24 was subcloned into pUC19 to create pKBK5. Digestion of pKBK5 with *Ava*I released a 5.1-kb fragment containing *cap5H* through *cap5L*. The digested plasmid was treated with Klenow fragment and deoxynucleoside triphosphates and ligated to create pKBK9 which carries a 4.1-kb *Ava*I-*Eco*RI insert (*cap5M* to *cap5P*). A 727-bp *Hpa*I fragment was deleted from pKBK9 to create pKBK9-2. The deletion, encompassing nucleotide positions 9 to 734 of the 1,260-bp *cap5O* gene, resulted in a +1 frameshift. pKBK9-2 was digested with *Eco*RI and *Bam*HI, and the 3.4-kb fragment (*cap5O* deletion and flanking *cap5* sequence) was ligated into pTS1 to create plasmid pKBK22.

pKBK22 was electrotransformed (17) into *S. aureus* RN4220 and then transduced with phage 80α (10) into *S. aureus* type 5 strain Reynolds, in both instances selecting for Cm^r colonies at 30°C. The mutation was introduced into the chromosome by allelic exchange. In brief, plasmid integrants in the chromosome were selected by plating of cells on tryptic soy agar containing Cm (5 μg/ml) at 42°C. Single colonies were then passaged three times at 30°C without antibiotic selection. Cm^s colonies were screened for CP5 production by colony immunoblot as previously described (20) with the use of CP5-specific polyclonal rabbit antiserum. DNA from CP5-negative mutant JLO22 was analyzed by PCR and Southern blot to confirm the presence of the mutation in *cap5O*. Lack of CP5 production by the *cap5O* mutant JLO22 was confirmed by immunodiffusion of capsular extracts with CP5-specific rabbit antiserum.

Purification of Cap5O. A 100-ml culture of *E. coli* BL21(DE3) carrying pNB1 was grown at 37°C for 3 h on a platform shaker at 180 rpm. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM, and the culture was incubated for another 3 h at 30°C. All subsequent purification steps were performed at 4°C. The culture was pelleted at 5,000 × *g*, and the bacterial cells were resuspended in 20 mM Tris-HCl buffer (pH 7.9) containing 5 mM imidazole. *E. coli* cells were lysed by three to four cycles through a French pressure cell (Aminco, Urbana, III.) at 800 lb/in^2. The lysate was centrifuged at 39,000 × *g* for 20 min, and the supernatant was loaded onto a Ni^{2+} affinity column (Novagen). The column was washed sequentially with 5 and 60 mM imidazole in 20 mM Tris-HCl buffer, and the protein was eluted with 1 M imidazole in 20 mM Tris-HCl buffer. Fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Fractions containing Cap5O were pooled and dialyzed against 50 mM Tris-HCl buffer (pH 8.5). The protein content of the purified enzyme was determined by the Bradford method (6) (Bio-Rad Laboratories, Hercules, Calif.) with bovine gamma globulin as the standard. Ammonium sulfate was added to the purified protein to a final concentration of 250 mM, and the enzyme solution was stored in aliquots at −70°C.

Dehydrogenase assay. The activity of the purified enzyme was monitored by measuring the increase in absorption at 340 nm resulting from NADH formation. The assay mixture contained 0.5 mM dithiothreitol, 0.5 mM UDP-GlcNAc, 1.5 mM NAD^+, 1.5 to 3 μg of purified Cap5P (14), and ~70 μg of Cap5O in 0.2 ml of 50 mM Tris-HCl buffer (pH 8.5). All the reagents except for NAD^+ were mixed and placed in a dry bath at 37°C. NAD^+ was then added, and the absorbance at 340 nm was read at 0, 10, 20, 30, and 60 min in a Beckman UV-visible spectrophotometer. NADH formation was expressed in nanomoles, determined according to the Lambert-Beer law with an extinction coefficient for NADH of 6,220 M^{-1}.

The effect of EDTA (2, 5, and 10 mM), magnesium (2 mM $MgCl_2$ or $MgSO_4$) and monovalent ions [150 mM KCl, NH_4Cl, or $(NH_4)_2SO_4$] on enzyme activity was determined. These reagents were included in the assay mixture prior to the addition of NAD^+.

Identification of UDP-ManNAcA by GC-MS. Dehydrogenase reaction mixtures were treated with 1 ml of 95% ethanol at 70°C for 10 min to precipitate proteins. UDP-amino sugars in the supernatant were converted to methyl glycosides by methanolysis with 0.5 ml of 1 M methanol containing 35 μl of acetyl chloride for 24 h at 85°C. The methyl glycosides were reduced overnight at room temperature with 3 mg of sodium borodeuteride in 0.3 ml of 95% ethanol. This step reduces the carboxylic acid of ManNAcA to an alcohol group and labels carbonyl carbon-6 with two deuterium atoms. Methyl glycosides were hydrolyzed with 0.5 M trifluoroacetic acid for 12 h at 100°C to remove the UDP moieties. Free sugars were reduced with 3 mg of sodium borodeuteride in 0.3 ml of 1 M ammonium hydroxide. This step converts the sugars into the corresponding alditols and labels the anomeric carbon-1 with one deuterium atom. The alditols were then acetylated with 0.1 ml of acetic anhydride and 0.1 ml of pyridine for 20 min at 100°C. Samples were washed with distilled water, partitioned with 0.5 ml of ethyl acetate, and analyzed by GC-MS (Saturn 2000; Varian, Palo Alto, Calif.) on a DB-17 column (30-m-by-0.25-mm inner diameter by 0.25-μm df). Electron impact ionization was used in all MS methods.

Mouse infection studies. Male ICR and Swiss-Webster mice (6 to 7 weeks old) were obtained from Harlan Sprague-Dawley, Inc. (Indianapolis, Ind.). The mice were housed (up to four animals per cage) in a modified barrier facility under viral antibody-free conditions. Food and water were provided to the mice ad libitum. Animals were handled according to Brigham and Women's Hospital and Harvard Medical School institutional guidelines.

Staphylococci were cultivated on Columbia salt agar plates at 37°C for 24 h, and bacterial colonies were suspended in phosphate-buffered saline (0.01 M phosphate, 0.15 M NaCl; pH 7.2 to 7.4). The optical density of the bacterial suspensions was measured, and the samples were diluted to yield the appropriate numbers of CFU per milliliter.

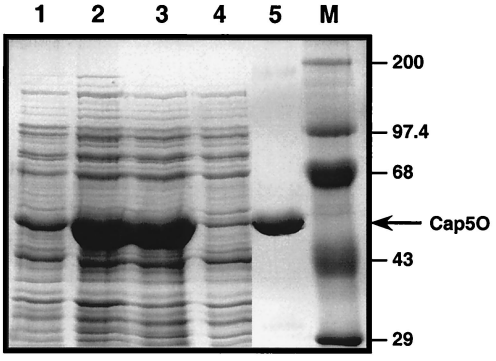

FIG. 1. SDS-PAGE analysis of *S. aureus* Cap5O expression and purification in *E. coli* BL21(DE3). Lane 1, cell lysate from uninduced cells of *E. coli*; lane 2, cell lysate from IPTG-induced cells; lane 3, supernatant resulting from centrifugation at 39,000 × *g* of cell lysate; lane 4, column effluent reflecting unbound proteins; lane 5, purified Cap5O; lane M, molecular mass markers (expressed as kilodaltons).

In the renal abscess model (18), groups of four to six mice were injected in the tail vein with 4×10^6 or 4×10^5 CFU of *S. aureus* in a 0.2-ml inoculum. The number of CFU per milliliter of inoculum was verified by plate counts. Five days after bacterial challenge, mice were euthanized, and the kidneys were excised, weighed, and homogenized in 1 ml of tryptic soy broth. Serial dilutions of the homogenates were plated in duplicate on tryptic soy agar plates, and the results were expressed as the log CFU of *S. aureus*/gram of tissue. The lower limit of detection by culture was 1.1 log CFU/g of tissue. Two separate experiments were performed with each mouse strain.

In the subcutaneous model (7), bacterial suspensions were mixed with equal volumes of dextran beads (Cytodex-1 microcarriers; Sigma) prepared according to the manufacturer's instructions. Groups of three mice were injected subcutaneously in each flank with 0.2 ml of *S. aureus* suspensions ranging from 10^7 to 10^1 CFU. The numbers of CFU per milliliter of inoculum were verified by plate counts. Four days after bacterial challenge, mice were euthanized, and the abscesses were excised and homogenized in 1 ml of tryptic soy broth. Serial dilutions of the homogenates were plated in duplicate on tryptic soy agar plates, and the results were expressed as the log CFU of *S. aureus*/abscess. The lower limit of detection was 1.0 log CFU/abscess. In two separate experiments, Swiss-Webster mice were coinfected with a bacterial suspension containing equal numbers of the parental strain Reynolds and the *cap5O* mutant JLO22 mixed with dextran beads. The number of CP5-positive versus CP5-negative colonies recovered in each abscess was assessed by a colony immunoblot method (20).

Statistical analysis. The results of quantitative renal cultures at each dose were compared with the Welch test, a modification of the unpaired Student's *t* test for comparing Gaussian populations with unequal standard deviations. A semiparametric weighted least-squares method (32) was used to compare the results of quantitative bacterial cultures performed over a range of doses in the subcutaneous abscess model. Data from coinfection experiments were analyzed by the unpaired Student's *t* test.

RESULTS

Purification and properties of Cap5O. Cap5O was overexpressed from pNB1 in *E. coli* BL21(DE3). As shown in Fig. 1, most of the protein was recovered from the 39,000 × *g* supernatant, an indication that the protein was in a soluble form. The hydropathy plot of the deduced amino acid sequence of the protein confirmed its hydrophilic nature (data not shown). The histidine-tagged Cap5O, purified over a Ni^{2+} affinity column, showed a single band by SDS-PAGE, with a mass of ~45.9 kDa (Fig. 1). This result is in agreement with the predicted mass of 45.6 kDa deduced from the nucleotide sequence of the *cap5O* gene. The isoelectric point predicted from the amino acid sequence of Cap5O was 4.8. N-terminal protein sequencing of purified Cap5O yielded the sequence MKLTVVGLGY, which confirmed the translational start site predicted by the nucleotide sequence (29). Purified Cap5O, at a concentration of ~3.5 mg/ml, was stable for at least nine months when stored at −70°C in 50 mM Tris-HCl–250 mM ammonium sulfate (pH 8.5).

Enzymatic function of Cap5O. Because the substrate of Cap5O (UDP-ManNAc) is not commercially available, we produced UDP-ManNAc by incubating purified *S. aureus* Cap5P with its substrate UDP-GlcNAc as previously described (14). Purified Cap5O and the cofactor NAD^+ were added, and the mixture was incubated at 37°C. As an indirect determination of Cap5O activity, we measured the increase in the absorbance at 340 nm resulting from NADH formation. A reaction buffer with a basic pH (ranging from 8 to 9) and a reducing agent (either dithiothreitol or β-mercaptoethanol) were both required for maximal production of NADH (data not shown). Furthermore, no enzymatic activity was detected if NAD^+, Cap5O, Cap5P, or the substrate UDP-GlcNAc was omitted from the assay mixture. No NADH formation was observed if GlcNAc or UDP–*N*-acetylgalactosamine (UDP-GalNAc) was substituted for UDP-GlcNAc as the substrate in the assay

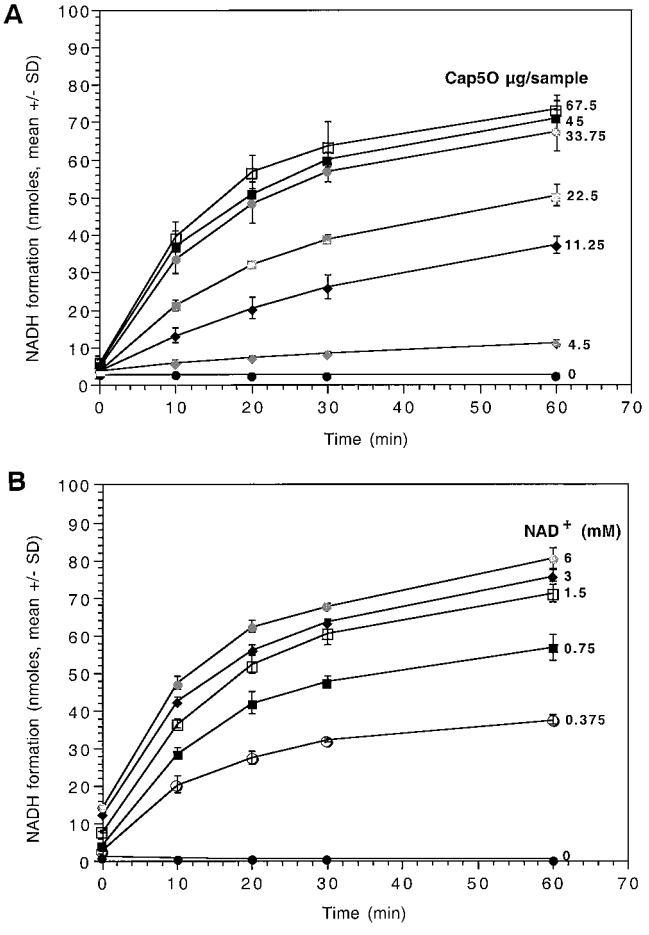

FIG. 2. Time course of NADH formation with different amounts of Cap5O added (A) or with different concentrations of NAD^+ added (B). The results are from three separate experiments. The error bars indicate the standard deviations.

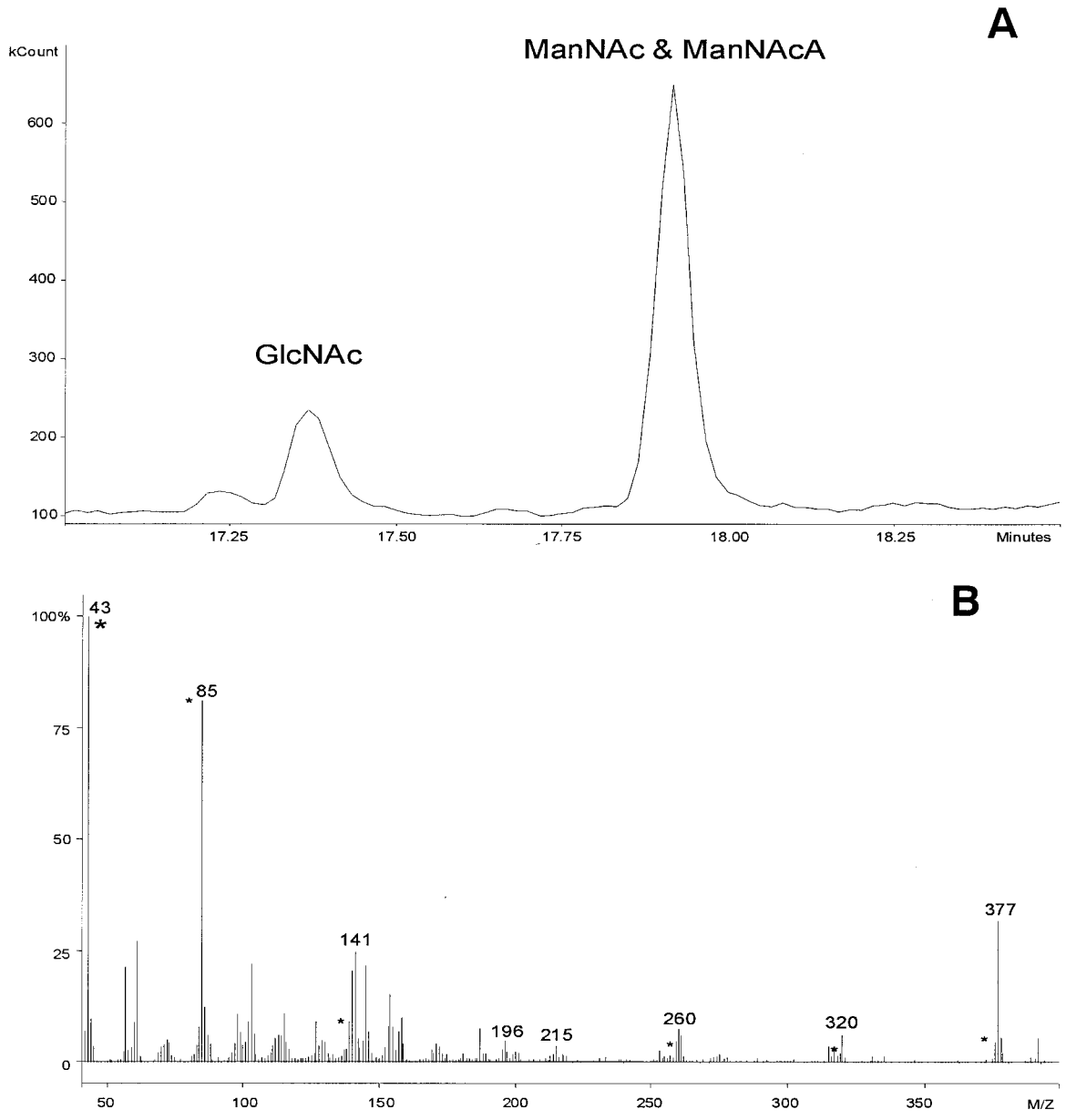

FIG. 3. GC-MS analysis of hydrolyzed and derivatized enzyme assay mixtures. (A) Sample chromatograms showed two peaks at 17.4 and 17.9 min: retention times corresponding to those for derivatives of authentic GlcNAc and ManNAc, respectively. ManNAcA eluted together with ManNAc since the ManNAcA carboxyl group was reduced to an alcohol by sodium borodeuteride. (b) MS analysis at 17.9 min was shown to contain peaks for both ManNAc (marked with an asterisk) and ManNAcA (numbered peaks).

(data not shown). The reaction mixture lacking Cap5P was used as a negative control sample for most experiments.

To determine the influence of Cap5O concentration on NADH production, we added increasing amounts of purified Cap5O to the enzyme reaction mixture containing 1.5 mM NAD^+. As shown in Fig. 2A, NADH formation increased in a linear fashion with increasing amounts of Cap5O up to 33.75 μg. Similarly, when we added various concentrations of NAD^+ (0 to 6 mM) to a reaction mixture containing 70 μg of Cap5O, the amount of NADH increased proportionally up to 1.5 mM NAD^+ (Fig. 2B). We used 70 μg of Cap5O and 1.5 mM NAD^+ in our standard assay. Under the standard conditions, the rate of NADH formation was 50 μmol/min/μg of Cap5O.

Cap5O activity was unaffected by the addition of 2, 5, or 10 mM EDTA to the reaction mixture, indicating that exchangeable divalent cations were not required for enzyme activity (data not shown). Similarly, the addition of Mg^{2+} had no significant effect on the kinetics of the assay. The addition of 150 mM ammonium sulfate or ammonium chloride to the assay mixture produced no effect, whereas potassium chloride at the same concentration caused a modest inhibition (~30%) of enzyme activity.

Identification of UDP-ManNAcA. According to our proposed pathway for UDP-ManNAcA biosynthesis, UDP-GlcNAc is epimerized by Cap5P to UDP-ManNAc, which is then oxidized by Cap5O to UDP-ManNAcA. In the reaction products, all three sugars were expected to be present.

The products of the enzyme assay mixtures were reduced with sodium borodeuteride, hydrolyzed, and converted to alditol acetate derivatives. Analysis of gas chromatograms revealed two peaks with retention times of approximately 17.4 and 17.9 min (Fig. 3A), values corresponding to those for alditol acetate derivatives of authentic GlcNAc and ManNAc standards, respectively. Chromatograms from negative control samples with no Cap5P contained only the 17.4-min peak; MS analysis confirmed this peak to represent GlcNAc (data not shown). The peak at 17.9 min includes the derivatives of ManNAc and reduced ManNAcA. During the first reduction step with sodium borodeuteride, the carbon-6 of ManNAcA, but not ManNAc, is labeled with two deuterium atoms. Consequently, ManNAcA is two atomic mass units heavier than ManNAc. Thus, it was possible to distinguish the two enzymatic products accurately by MS analysis. The mass spectrum of the 17.9-min GC peak closely resembles that of a ManNAcA standard (peaks at (43, 85, 141, 196, 215, 260, 320, and 377) *m*/*z*; Fig. 3B). However, superimposed on the spectrum are peaks associated with ManNAc (peaks (43, 85, 139, 258, 318, and 375) *m*/*z*). By integration of the mass associated with the products of the reaction, the ratio of GlcNAc to ManNAc to ManNAcA was determined to be 1:3:22.

Essential role of the *cap5O* gene in CP5 expression. A *cap5O* mutation was created by deletion of 727 bp in the 5′ end of the *cap5O* gene and subcloning the mutated fragment in a temperature-sensitive shuttle vector. The *cap5O* deletion was introduced by allelic exchange into the chromosome of *S. aureus* serotype 5 strain Reynolds, yielding mutant JLO22. To confirm the deletion in the chromosomal copy of *cap5O* in the mutant, genomic DNA from Reynolds and JLO22 was digested with *Hin*dIII, electrophoresed in an agarose gel, and analyzed by Southern blotting. Labeled pKBK4 (*cap5O* gene in pUC19) hybridized to 6.2- and 1.4-kb DNA bands from Reynolds and a single 6.9-kb DNA band from JLO22. The band sizes reflect the deletion of the 727-bp *HpaI* fragment (including an internal *Hin*dIII site) from the *cap5O* gene in the mutant JLO22. Mutant JLO22 was negative for CP5 production as determined by immunodiffusion and colony immunoblots with CP5-specific antiserum. Its growth rate *in vitro* was identical to that of the parental strain (data not shown).

Effect of *cap5O* deletion on staphylococcal virulence. Two different strains of outbred mice were challenged intravenously with either *S. aureus* Reynolds or JLO22 to compare their virulence in the renal abscess model of infection. Swiss-Webster mice challenged with 4×10^5 CFU strain Reynolds had significantly ($P = 0.019$) higher numbers of CFU recovered per gram of kidney than mice challenged with mutant JLO22 (Table 2). However, this difference in infectivity could be overcome by increasing the inoculum to 4×10^6 CFU/mouse, in which case both groups of animals had similar numbers of staphylococci recovered from the kidney (data not shown). Significant differences in virulence were not seen at either inoculum when similar experiments were performed with ICR mice (Table 2).

We challenged Swiss-Webster mice subcutaneously with *S. aureus* inocula ranging from 10^7 to 10^1 CFU. As shown in Fig. 4, animals challenged with the large inocula (10^7 or 10^5 CFU) showed similar bacterial densities ($\sim 10^7$ CFU/abscess) independent of the challenge strain. However, mice inoculated with 10^3, 10^2, or 10^1 CFU of the *cap5O* mutant JLO22 had significantly fewer CFU per abscess than mice inoculated with the wild-type strain Reynolds ($P = 0.0018$; Fig. 4). Significant differences between the strains were not observed when ICR mice were challenged with 10^7, 10^5, or 10^3 CFU *S. aureus* (data not shown).

TABLE 2. Results of quantitative kidney cultures from Swiss-Webster mice inoculated intravenously with 4×10^5 CFU *S. aureus*

Mouse strain (*n*)	Log CFU/g of kidney (mean ± SEM)		*P*[a]
	Reynolds	JLO22	
ICR (9)	3.55 ± 0.87	2.13 ± 0.33	0.1582
Swiss-Webster (10–11)	4.33 ± 0.60	2.58 ± 0.25	0.0190

[a] Welch test, a modification of the unpaired Student's *t* test.

We challenged an additional group of 10 mice with a mixed inoculum containing equal numbers of strain Reynolds and JLO22 (either 10^3 or 10^2 total CFU). As shown in Table 3, between 74 and 81% of the bacteria recovered from the abscesses on day 4 were capsule positive. These data suggest that encapsulation promotes bacterial growth and/or survival within the abscess and confirm the results depicted in Fig. 4.

DISCUSSION

DNA sequence analysis of the *S. aureus cap5* and *cap8* genes revealed that 16 genes [*cap5(8)A* through *cap5(8)P*], clustered on the bacterial chromosome, are involved in capsule biosynthesis (29). This information allowed us to compare the predicted amino acid sequences of *cap5* and *cap8* with sequences in the public databases and to assign putative functions to most of the genes (19, 29). The *cap5* and *cap8* gene clusters are almost identical in their flanking sequences (*capA* through *capG* and *capL* through *capP*), but they differ in the central serotype-specific gene region (*capH* through *capK*) (29). The function of only a few of the biosynthetic genes has been proven. We showed that the gene product of *cap5H*, one of the CP5-specific genes, O acetylates the third carbon on the ManNAcA residues of CP5 (5). In addition, we showed that the purified product of the *cap5P* gene is a UDP-GlcNAc 2-epimerase that catalyzes the conversion of UDP-GlcNAc to UDP-ManNAc (14). In this study, we characterized the *S. aureus cap5O* gene product as a UDP-ManNAc dehydrogenase.

The enzymatic activity of Cap5O was quantitated indirectly by mixing Cap5O with Cap5P and UDP-GlcNAc and measuring NADH production. The oxidation product was confirmed to be a UDP-ManNAcA by reduction of the reaction products with sodium borodeuteride and analysis of the derivatized products by GC-MS. Thus, we propose that the synthesis of UDP-ManNAcA in *S. aureus* occurs as presented in Fig. 5.

GC-MS analysis of some of the coupled reaction mixtures containing both Cap5P and Cap5O revealed that all of the UDP-GlcNAc substrate was converted into UDP-ManNAc and UDP-ManNAcA. This is in contrast to the reversible Cap5P-mediated UDP-GlcNAc 2-epimerase reaction in which only ~10% of the substrate was converted to UDP-ManNAc (14). It is likely that coupling the 2-epimerase reaction to the dehydrogenase reaction depletes the intermediate product

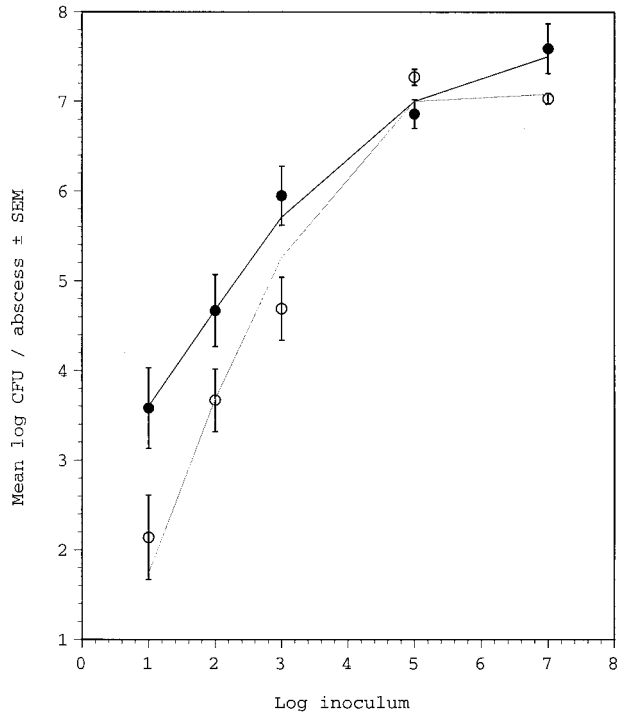

FIG. 4. Results of quantitative abscess cultures from groups of three to seven Swiss-Webster mice challenged subcutaneously with *S. aureus* Reynolds (solid symbols) or mutant JLO22 (open symbols). Curve fitting according to the statistical analysis for comparison of Reynolds and JLO22 groups is shown.

UDP-ManNAc. As a result, the reversible 2-epimerase reaction is driven toward the formation of more UDP-ManNAc, the substrate for Cap5O.

The biochemical properties of *S. aureus* Cap5O are similar to those described for other microbial dehydrogenases. For example, both *S. aureus* Cap5O and an *E. coli* UDP-ManNAc dehydrogenase require a basic pH and a reducing agent to be present for maximal activity (12). Like other dehydrogenases with specificity for UDP-ManNAc (11, 22) or UDP-glucose (3), *S. aureus* Cap5O was unaffected by EDTA or divalent cations. Moreover, *S. aureus* UDP-ManNAc dehydrogenase contains the N-terminal NAD-binding domain (GXGXXG) typical of other dehydrogenases (33) requiring NAD^+ as a cofactor.

The deletion of *cap5O* in *S. aureus* Reynolds yielded a CP5-negative mutant. Similarly, Sau et al. (30) showed that the serotype 8 strain Becker with a chemically induced mutation in *cap8O* was negative for CP8 production. Recombinant plasmids containing intact *cap8O* complemented the function of the mutated *cap8O* gene *in trans*. Similarly, we showed that introduction of the wild-type *cap5O* gene on a plasmid restored CP5 expression to a *cap5O* mutant of strain Newman (28).

The role of the *S. aureus* capsule in the pathogenesis of staphylococcal infections has been examined in a number of test systems. Serotype 5 and 8 strains of *S. aureus* were shown to resist opsonophagocytic killing by human polymorphonuclear leukocytes (8, 31). In addition, CP5 enhanced virulence in mouse models of lethality (31), bacteremia (31), and septic arthritis (25) and promoted long-term nasal colonization by *S. aureus* in mice (15). In contrast, both CP5 and CP8 attenuated staphylococcal virulence in a rat model of catheter-induced endocarditis (4). In 1991, we challenged inbred C57BL/6J mice intravenously with $\sim 5 \times 10^6$ CFU of the wild-type strain Reynolds, a capsule-deficient mutant created by transposon mutagenesis, or a chemically induced capsule-negative mutant (1). Because all of the mice developed renal abscesses and the numbers of bacteria recovered from the kidneys were similar, we concluded that CP5 did not influence renal abscess formation. The staphylococci in that study were harvested from logarithmic-phase broth cultures, in which little capsule is expressed (28, 31). In this study, we reexamined the role of capsule in renal abscess formation by challenging two different strains of mice with *S. aureus* cultivated under conditions known to optimize capsule expression (31). At a challenge inoculum of 4×10^5 CFU, significantly greater numbers of the parental strain Reynolds were recovered from the kidneys of Swiss-Webster mice compared with those challenged with the CP5-negative mutant JLO22. This effect on virulence was modest, however, since no differences between the two groups of animals were observed at a 10-fold-greater inoculum. Similar experiments carried out in ICR mice revealed no differences in virulence at either challenge dose. Mice are highly resistant to *S. aureus* infection and, in this model, an inoculum $>10^5$ CFU is essential for infectivity.

The subcutaneous abscess model proved to be a more sensitive model of infection since inocula as low as 10 CFU could provoke an infection by the wild-type *S. aureus* strain. Significantly fewer organisms were recovered from the subcutaneous abscesses of Swiss-Webster mice challenged with $\leq 10^3$ CFU mutant JLO22 compared with the wild-type strain. Moreover, in coinfection experiments, 75 to 80% of the organisms recovered after 4 days were CP5 positive. This result is in agreement with our findings that the encapsulated wild-type strain is more virulent than the acapsular mutant in this model. However, no differences in virulence were observed when ICR mice were challenged subcutaneously with 10^7, 10^5, or 10^3 CFU. Taken together, these results suggest that the role of the capsule in the pathogenesis of staphylococcal infections is dependent not only on the bacterial growth conditions and inoculum size but also on the genetic background of the host.

The pathogenesis of the *S. aureus* subcutaneous infection model is clearly different from that of the renal abscess model. To induce subcutaneous abscesses, the bacteria are injected directly into the subcutaneous tissue in the presence of a foreign body (cytodex beads). It is likely that encapsulated staphylococci avoid uptake by phagocytes recruited to the site of infection, and thus capsule-positive *S. aureus* have a survival and growth advantage over staphylococci lacking a capsule. In contrast, a bolus dose of staphylococci is delivered intravenously to mice to provoke renal abscesses. The liver and spleen clear the majority of organisms, and only a small number of blood-borne organisms seed the kidney. We did not observe a significant difference in the number of parental or mutant *S.*

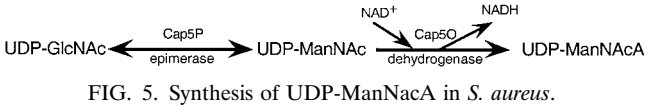

FIG. 5. Synthesis of UDP-ManNacA in *S. aureus*.

TABLE 3. Results of coinfection experiments in which Swiss-Webster mice were challenged subcutaneously with equal numbers of the strains Reynolds and JLO22

Log inoculum (CFU/mouse)	*n*	Log CFU/abscess (mean ± SEM)	Amt (%) of each strain recovered after 4 days[a] (mean ± SEM)		*P*[b]
			Reynolds	JLO22	
2.0	5	4.8 ± 1.1	81 ± 8	19 ± 8	0.0007
3.0	5	6.5 ± 0.1	74 ± 6	26 ± 6	0.0003

[a] CP5-positive and CP5-negative colonies were scored by the colony immunoblot method (20).
[b] Unpaired Student's *t* test.

aureus cells recovered from the kidney 24 h after bacterial challenge (unpublished observations). However, an *S. aureus cap5O* mutant lacking CP5 expression showed greater adherence to endothelial cells in vitro compared with the parental strain (28). It is likely that capsule expression augments staphylococcal survival within the kidney by enhancing its resistance to phagocytic uptake and killing.

In conclusion, we have purified the *S. aureus cap5O* gene product and demonstrated that it has UDP-ManNAc dehydrogenase activity. Cap5O is essential for CP5 expression, and a mutant lacking this enzyme is less virulent in two Swiss-Webster mouse models of abscess formation. Our findings are consistent with the observation that antibodies that neutralize the *S. aureus* capsule show some protection against *S. aureus* infections.

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