INFECTION AND IMMUNITY, May 2010, p. 2017–2023 Vol. 78, No. 5
0019-9567/10/$12.00 doi:10.1128/IAI.00016-10

Identification and Characterization of a Glycosyltransferase Involved in *Acinetobacter baumannii* Lipopolysaccharide Core Biosynthesis▽

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Received 8 January 2010/Returned for modification 27 January 2010/Accepted 19 February 2010

Although *Acinetobacter baumannii* has emerged as a significant cause of nosocomial infections worldwide, there have been few investigations describing the factors important for *A. baumannii* persistence and pathogenesis. This paper describes the first reported identification of a glycosyltransferase, LpsB, involved in lipopolysaccharide (LPS) biosynthesis in *A. baumannii*. Mutational, structural, and complementation analyses indicated that LpsB is a core oligosaccharide glycosyl transferase. Using a genetic approach, *lpsB* was compared with the *lpsB* homologues of several *A. baumannii* strains. These analyses indicated that LpsB is highly conserved among *A. baumannii* isolates. Furthermore, we developed a monoclonal antibody, monoclonal antibody 13C11, which reacts to an LPS core epitope expressed by approximately one-third of the *A. baumannii* clinical isolates evaluated to date. Previous studies describing the heterogeneity of *A. baumannii* LPS were limited primarily to structural analyses; therefore, studies evaluating the correlation between these surface glycolipids and pathogenesis were warranted. Our data from an evaluation of LpsB mutant 307::TN17, which expresses a deeply truncated LPS glycoform consisting of only two 3-deoxy-D-manno-octulosonic acid residues and lipid A, suggest that *A. baumannii* LPS is important for resistance to normal human serum and confers a competitive advantage for survival *in vivo*. These results have important implications for the role of LPS in *A. baumannii* infections.

Acinetobacter baumannii, an opportunistic bacterial pathogen whose medical importance is increasing, is a significant cause of nosocomial infections worldwide and has recently emerged as a community-acquired pathogen as well (29). *A. baumannii* has been cultured from moist skin of healthy humans, but increased colonization of skin and the respiratory and gastrointestinal tracts occurs in individuals in long-term care and hospital facilities. Nosocomial colonization or infection typically occurs at surgical sites or via long-lasting invasive medical devices, such as endotracheal breathing tubes (resulting in ventilator-associated pneumonia), indwelling urinary catheters (resulting in urinary tract infections), or ventricular shunts or pressure-monitoring devices (resulting in meningitis or bacteremia). In addition to causing sporadic infections, *A. baumannii* causes epidemic nosocomial outbreaks, as this organism is adept at surviving and persisting for extended periods of time under a wide range of environmental conditions (9, 34). *A. baumannii* has been associated with uncommonly severe, rapidly progressive community-acquired pneumonia (with mortality rates of 40% to 65%), usually in patients with compromised host defenses (4, 17). Furthermore, the incidence of multidrug-resistant *A. baumannii* infections, which are associated with significant morbidity and mortality, is increasing worldwide (for reviews, see references 23 and 29).

The importance of *A. baumannii* infections in war-related injuries is well established. *A. baumannii* was the most common Gram-negative bacillus recovered from traumatic injuries to the lower extremities during the Vietnam War (40). Most recently, *A. baumannii*-associated soft tissue infections, osteomyelitis, pneumonia, and bacteremia have been reported in United States service personnel injured in the Iraq, Kuwait, and Afghanistan regions during Operation Iraqi Freedom and Operation Enduring Freedom (5, 31, 38). *A. baumannii* also emerged as an important pathogen in survivors of the Asian tsunami in 2004 (11, 22). It is the ability of this organism to colonize hospital equipment and to resist typical disinfection methods that perpetuates the outbreak cycle. Moreover, infections caused by this organism are particularly challenging due to its impressive repertoire of intrinsic and acquired antibiotic resistance determinants (30). The changing epidemiology and increasing incidence of infections due to *A. baumannii* demonstrate that the medical importance of this pathogen is increasing.

Compared to the virulence mechanisms of other human pathogens, the virulence mechanisms of *A. baumannii* have not been well characterized (for a review, see reference 29). Thus, there is a significant gap in our knowledge and understanding of the factors involved in *A. baumannii* pathogenesis. However, one of the virulence factors that have been shown to be in-

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▽ Published ahead of print on 1 March 2010.

volved in multiple steps of the disease process for other Gram-negative human pathogens is lipopolysaccharide (LPS).

LPS typically consists of a hydrophobic anchor domain termed lipid A (or endotoxin) that makes up the outer leaflet of the Gram-negative bacterial outer membrane, a nonrepeated core oligosaccharide structure, which can be divided into "inner core" (lipid A proximal) and "outer core" regions, and a distal polysaccharide comprised of repeat-unit structures having variable lengths termed the O antigen (OAg). In general, the lipid A region is considered the most toxic or inflammatory region of LPS, although the polysaccharide portion of the molecule also has potent immunomodulating and immunostimulating properties (24, 33). LPS has been shown to contribute both to bacterial evasion of host immune responses, affecting both innate and acquired host responses to infection, and to initiation of an overwhelming host inflammatory response that significantly correlates with the morbidity and mortality of infected patients (15, 24, 32). Moreover, the cell surface location of LPS contributes to the interaction between the bacterium and its environment. To begin to understand how *A. baumannii* LPS impacts virulence and immune responses to this pathogen, insight into the chemical structure and biologic activities of LPS is essential.

The LPS structures expressed by a variety of *A. baumannii* isolates have been characterized in previous studies. These studies demonstrated that the *A. baumannii* OAg region is highly heterogeneous as numerous LPS glycoforms have been defined using both structural and antibody-based studies (21, 26–28, 41–44). Although the structural analyses of *A. baumannii* LPS determined that this organism expresses an S-form LPS, phenotypic analyses of the molecules has been challenging because the OAg cannot be visualized following gel electrophoresis. This problem appears to be a result of the absence of unsubstituted vicinal OH groups, which makes the polysaccharides nonoxidizable for detection (41). Recent biologic studies indicated that *A. baumannii* LPS may be an important immunostimulatory molecule involved in Toll-like receptor 4 (TLR4) signaling in a mouse pneumonia model (14). This immunostimulatory capacity of LPS was substantiated using human cells *in vitro*, although the latter study also identified TLR2 as an important signaling factor (8). Despite these elegant structural and biologic studies, there is currently no definitive link between *A. baumannii* LPS and virulence. In addition, there have not been any reports defining any of the genes involved in the biosynthesis of the *A. baumannii* LPS molecule. Thus, detailed studies evaluating the correlation between the principal surface glycolipids and pathogenesis are warranted.

In this paper, we describe isolation and characterization of an *A. baumannii* LPS mutant, 307::TN17, that was identified using transposon mutagenesis and an antibody specific to a core LPS glycoform expressed by *A. baumannii* strain 307-0294. Our data indicate that LpsB, an LPS glycosyl transferase involved in biosynthesis of the LPS core, is highly conserved among clinical isolates. Furthermore, our studies comparing an *lpsB* mutant to the wild-type strain demonstrated that *A. baumannii* LPS is important for serum resistance *in vitro* and for survival *in vivo*.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *A. baumannii* strain 307-0294, a blood isolate from a patient hospitalized at Erie County Medical Center, Buffalo, NY, is classified as a sequence type 15, clonal group 1 strain (7, 18, 35, 36). *A. baumannii* strains ATCC 19606, ATCC 15308, and ATCC 17978 were purchased from the American Type Culture Collection (Manassas, VA). In addition, we used 74 *A. baumannii* clinical strains isolated from infected military personnel serving in Iraq and Afghanistan (kindly provided by David Craft and Paul Scott of the Walter Reed Army Medical Center). All strains were routinely cultured using Mueller-Hinton (MH) medium or agar supplemented with antibiotics when appropriate (50 μg/ml kanamycin, 200 μg/ml carbenicillin, 5 μg/ml tetracycline). *Escherichia coli* XLI-Blue was used for plasmid cloning and was cultured at 37°C on Luria-Bertani agar plates or in Luria-Bertani broth in the presence of antibiotics when they were required.

Nucleic acid manipulations. Standard molecular biology reagents were obtained from New England Biolabs, Inc. (Beverly, MA). Plasmid isolation and amplicon purification were performed using kits manufactured by Qiagen (Santa Clarita, CA). Chromosomal DNA was isolated using standard methods. PCR amplification was performed for 25 cycles with the GeneAMP 9700 PCR system (P.E. Applied Biosystems, Foster City, CA) using Platinum *Taq* High Fidelity DNA polymerase (Invitrogen, Carlsbad, CA) and primer set-dependent annealing temperatures and extension times. DNA nucleotide sequences of all constructs were obtained by automated DNA sequencing (RPCI Biopolymer Facility, Roswell Park Cancer Institute, Buffalo, NY) and were analyzed with MacVector 10.5. Total RNA was isolated using an RNAProtect and RNeasy minikit (Qiagen) and was subjected to RQ1 RNase-free DNase treatment (Promega). Reverse transcription-PCR (RT-PCR) analyses were performed using a OneStep RT-PCR kit (Qiagen) to ensure that transcription of the genes flanking the insertionally inactivated coding region in the mutant strain remained unaffected.

MAb development. *A. baumannii* LPS core-specific monoclonal antibody (MAb) 13C11 was developed by injecting BALB/c mice intraperitoneally with viable *A. baumannii* 307-0294, using previously described methods (2, 18). Hybridoma supernatants were screened by immunodotting, Western blot assays, and flow cytometry using standard procedures to determine the presence of surface carbohydrate-reactive antibodies (18).

Construction of *A. baumannii* mutants and complemented derivatives. *A. baumannii* 307-0294 transposon (TN) mutant derivatives were generated using the Epicentre EZ::TN<KAN-2> Transposome system as described previously (18, 36). A mutant derivative of *A. baumannii* 307-0294 that lost reactivity to MAb 13C11 was identified by a colony lift assay and designated 307::TN17. Chromosomal DNA from 307::TN17 that was purified was restriction enzyme digested with Sau3AI and rescue cloned into BamHI-digested pUC18. Recombinant plasmids containing the TN and flanking *A. baumannii* DNA were isolated by selecting for resistance to both ampicillin and kanamycin after electroporation into *E. coli* XLI-Blue and were subjected to automated DNA sequencing for determination of the TN insertion site. A derivative that was complemented in *trans*, 307::TN17/pSS11, was generated using the recently described *A. baumannii* shuttle vector pNLAC1 developed by our group (36). pNLAC1 was generated by subcloning the *ori-repM* region of pMAC (a recently described 9.5-kb mobilizable extrachromosomal element isolated from *A. baumannii* ATCC 19606 [6]) into pBR322, which allowed replication in both *E. coli* (for subcloning) and *A. baumannii* (for complementation). pNLAC1 constructs (with and without the *lpsB* coding region plus 200 bp of flanking DNA) were transformed into the wild-type and mutant strains and assessed to determine restoration of the phenotype, as described previously.

Phenotypic analyses of *A. baumannii* LPS. LPS samples used for SDS-PAGE and Western blot analyses were prepared using proteinase K (PK)-treated whole-cell lysates or enriched sheared surface preparations, prepared as described previously with additional incubation for 2 h at 60°C with PK (0.4 mg/ml) when necessary (20). LPS was resolved by SDS-16% polyacrylamide gel electrophoresis and visualized by silver staining (19, 37). Mild periodate oxidation at an acidic pH to destroy carbohydrate determinants was performed in the presence (final concentration, 20 mM) and absence (control reactions) of sodium *meta*-periodate as described previously (3). *In vitro* susceptibility assays were performed as described previously (19).

Structural carbohydrate analyses. Compositional and mass spectrometric (MS) analyses were performed at the National Research Council of Canada (Ottawa, Canada). Bacterial cells were killed with 2% (wt/vol) (final concentration) phenol and pelleted by centrifugation. LPS was isolated by standard methods (45). In brief, killed cells were freeze-dried and washed once with ethanol and then twice each with acetone and light petroleum ether to remove lipids and

other lipophilic components. Washed cells were extracted by the hot phenol-water method, and the retentate was dialyzed and freeze-dried. A 2% solution of this crude LPS preparation was treated with DNase and RNase at 37°C for 4 h and then with proteinase K at 37°C for 4 h. Small peptides were removed by dialysis. After freeze-drying, the retentate was again rehydrated to obtain a 2% solution and centrifuged at 8,000 × *g* for 15 min, and then the supernatant was centrifuged at 100,000 × *g* for 5 h. The pellet from the high-speed centrifugation, which contained purified LPS, was redissolved in water and freeze-dried. The LPS was treated with anhydrous hydrazine to prepare O-deacylated LPS as described previously (12, 37). KOH-treated LPS was isolated by treating LPS with 4 N KOH (10 mg/ml, 125°C, 30 h) and neutralizing the preparation with HCl after cooling; salts were removed by centrifugation (5,000 × *g*) and by elution from a Sephadex G-25 gel permeation chromatography column. Purified carbohydrate-containing material was assessed to determine its sugar content as derived alditol acetates and by performing compositional analyses using capillary electrophoresis-electrospray mass spectrometry (CE-ES-MS) as previously described (39).

Serum bactericidal assay. Complement-mediated bactericidal assays were performed by measuring the change in bacterial titer over time in the presence of 90% active or inactive (heated at 56°C for 30 min) human serum. An input bacterial titer of approximately 1×10^5 CFU was used, and titers were measured at 0, 1, 2, and 3 h as described previously (19, 35, 36). Numbers of surviving CFU were determined by plating duplicate serial 10-fold dilutions in duplicate or triplicate. A minimum of three independent assays were performed for each strain.

Rat soft tissue infection model. The rat soft tissue infection model has recently been established by our group as a clinically relevant animal model for assessing *A. baumannii* infection (35, 36). In brief, 1% croton oil (1 ml) was injected into a space created by subcutaneous injection of 30 to 50 ml of air on the back of anesthetized Long-Evans rats. This space matured into an encapsulated, fluid-filled "pouch" within 7 days, mimicking a subcutaneous abscess replete with exudative fluid. On day 8, approximately 10^6 CFU of *A. baumannii* was injected into the pouches of anesthetized animals. Competition assays were performed by introducing equal numbers of CFU of the mutant and wild type. Plating the organisms on the appropriate selective media was used to distinguish between the two strains. Serial dilutions of pouch fluid aliquots (0.5 ml) obtained from anesthetized animals at 0, 6, 24, and 48 h after bacterial challenge were plated to determine bacterial titers. Assays were performed using cohorts of three rats per strain on at least two separate occasions.

Statistical analyses. Data are expressed below as means ± standard errors of the means. To normalize *in vitro* and *in vivo* data, $\log_{10}$-transformed values were used. The area under each curve was calculated from $\log_{10}$-transformed values, and the areas were compared using the two-tailed unpaired *t* test for statistical significance, using GraphPad Prism 5 software.

RESULTS

Development of an LPS-specific MAb. Mice were immunized with *A. baumannii* 307-0294, and fusion was performed using standard methods. The resulting hybridomas were screened by an immunodot assay versus whole-organism and proteinase K (PK)-treated whole-cell lysates. Clone 13C11 reacted to a PK-resistant, periodate-sensitive, low-molecular-mass bacterial cell surface component (Fig. 1) and was selected for further analysis. Based on the data, we hypothesized that MAb 13C11 was specific for a carbohydrate epitope in the *A. baumannii* 307-0294 LPS core. To further characterize the putative LPS epitope recognized by MAb 13C11, a panel of *A. baumannii* clinical isolates from infected military personnel were evaluated by colony lift analysis to determine their reactivity to MAb 13C11. MAb 13C11 reacted to a conserved surface-exposed epitope expressed by 34.2% (26/78) of the *A. baumannii* isolates in our collection. The surface reactivity of MAb 13C11 was confirmed by flow cytometry, and additional Western blot analyses indicated that MAb 13C11 reacted to a single, conserved band at approximately 7-kDa for all positive strains (data not shown). Furthermore, we performed immunodot assays with a variety of Gram-negative organisms, and

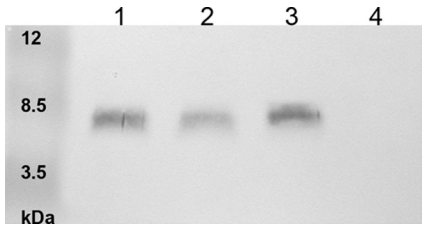

FIG. 1. Western blot analysis of *A. baumannii* 307-0294 whole-cell lysates (lane 1) treated with PK (lane 2), demonstrating that MAb 13C11 reacts to an approximately 7-kDa PK-resistant epitope. The epitope recognized by MAb 13C11 is sensitive to periodate treatment (lane 4) but not to the reaction buffer alone (lane 3), which is consistent with the presence of a carbohydrate molecule in the LPS core. The positions of molecular size standards are indicated on the left.

MAb 13C11 did not react with any of the isolates, suggesting that this antibody may be specific to *A. baumannii* LPS (data not shown). As anti-core LPS antibodies have been shown to protect against lethal Gram-negative sepsis in animal models, we hypothesized that further characterization of this LPS epitope may result in a better understanding of the mechanism of pathogenesis used by *A. baumannii* to establish infections.

Identification of an *A. baumannii* LPS mutant using TN mutagenesis. *A. baumannii* 307-0294 was subjected to random TN mutagenesis, and approximately 500 kanamycin-resistant transformants were immunoscreened for loss of reactivity to MAb 13C11. One of the mutants that did not react to MAb 13C11, designated 307::TN17, was selected for further study, and the TN insertion point was determined by DNA sequencing. Analysis of the subcloned fragment comprised of DNA flanking the site of TN insertion indicated that the TN had inserted into the 1,101-bp *lpsB* open reading frame (at bp 686) that was predicted to encode a 366-amino-acid protein (GenBank accession number ACJ59103, locus tag ABBFA_003104 [1]).

***In silico* analysis of *lpsB*.** BlastP queries using the NCBI database detected conserved domains that placed the predicted coding region in the GTB-type superfamily of nucleotide-sugar-dependent glycosyltransferases (GTs). The deduced protein sequence is most closely related to sequences of the WavL-like group 1 GT (GT1) family. WavL has been shown to be involved in the biosynthesis of the LPS core oligosaccharide in *Vibrio cholerae* (25). Members of the GT1 family transfer activated UDP-, ADP-, GDP-, or CMP-linked sugars to the LPS core. LpsB exhibited the highest levels of homology (40% to 48% identity [ID]) to other putative GT1 homologues identified by genome sequencing of predominately environmental bacteria, including *Thiobacillus denitrificans* ATCC 25259, *Nitrosococcus oceani* ATCC 19707, and *Psychrobacter arcticus* 273-4. Structural searches revealed that the highest levels of homology were the levels of homology to the crystal structure of the *Bacillus anthracis* strain Ames family GT4 enzyme designated ORF Ba1558 (24% ID) and the UDP-glucose:(heptosyl)-LPS-α-1,3-glucosyltransferase WaaG (formerly RfaG) of *E. coil* (22% ID). WaaG, a well-described GT involved in LPS biosynthesis in many bacteria, is responsible for addition of the first glucose moiety to the LPS core via an α-1,3-glycosidic linkage to heptose II, the distal heptose residue of the inner core Hep–Hep–3-deoxy-D-manno-octulosonic acid (Kdo) trisaccharide. The homology to

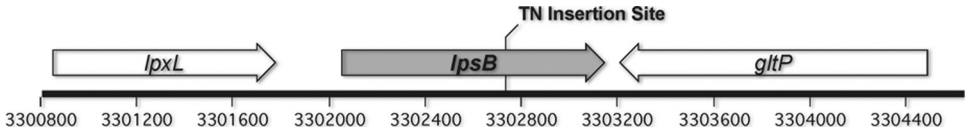

FIG. 2. Genetic organization of the *A. baumannii* 307-0294 *lpsB* gene cluster comprised of *lpxL* (locus tag ABBFA_003103), *lpsB* (locus tag ABBFA_003104), and *gltP* (locus tag ABBFA_003105); the numbers indicate the genomic coordinates (NCBI accession number CP001172) (1). The arrows indicate the sizes and directions of the transcription coding regions. The location of the TN insertion site identified in the *lpsB* of 307::TN17 is indicated.

WaaG is particularly interesting in light of the recent determination of the structure of the LPS carbohydrate backbone of *A. baumannii* strain ATCC 19606 (a MAb 13C11-positive strain), which showed that the *A. baumannii* LPS core is heptose deficient (42). Additional sequence analysis of the entire 4.4-kb subcloned insert revealed the presence of a lipid A biosynthetic lauroyl acyltransferase (LpxL) coding region upstream of *lpsB* (GenBank accession number ACJ56415.1) and a coding region for a glutamate-aspartate symport protein GltP homologue downstream in the opposite orientation (GenBank accession number ACJ57010.1) (Fig. 2). BlastN analyses of the entire coding region for all three genes revealed 97% to 100% ID in the six publically available annotated *A. baumannii* genomes currently accessible in the GenBank database (strains *A. baumannii* 307-0294 [accession number CP001172], AB0057 [accession number CP001182], AYE [accession number CU459141], SDF [accession number CU468230], ACICU [accession number CP000863], and ATCC17978 [accession number CP000521]).

Analysis of the LPS produced by 307::TN17. SDS-PAGE analysis demonstrated that the more slowly migrating approximately 7- kDa LPS glycoform of *A. baumannii* wild-type strain 307-0294 is not present in mutant 307::TN17 (Fig. 3A). A corresponding Western blot was probed with MAb 13C11, which reacted only to the wild-type LPS and specifically to the 7-kDa band (Fig. 3B). These data suggest that the loss of functional LpsB activity specifically affected the *A. baumannii* 307-0294 LPS glycoform and that the mutant did not express the LPS epitope recognized by MAb 13C11.

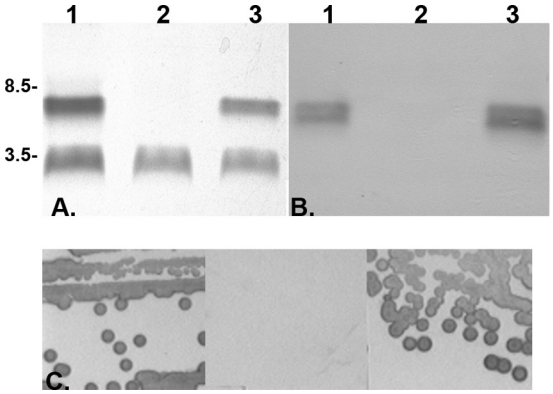

FIG. 3. SDS-PAGE analysis (A) and corresponding MAb 13C11-probed Western blot (B) of LPS from *A. baumannii* wild-type strain 307-0294 (lane 1), the 307::TN17 mutant (lane 2), and the 307::TN17/pSS11 derivative complemented in *trans* (lane 3). (C) Composite results of colony lift assays demonstrating that MAb 13C11 reacts with a cell surface epitope expressed by *A. baumannii* wild-type strain 307-0294 (left panel) and 307::TN17/pSS11 (right panel) but not with the LpsB-defective mutant 307::TN17 (middle panel).

LPS was isolated from flask-grown cells by using well-documented, standard protocols that included an initial organic solvent wash to delipidate the cells in order to enhance the efficiency of the subsequent phenol extraction step. Phenol extraction is the universally accepted method for isolating LPS from Gram-negative bacterial cells (45). The resulting crude LPS extracts are then treated with DNase, RNase, and proteinase K in order to remove nucleic acids and any residual proteins, which results in a pure LPS preparation.

Sugar analyses of both LPS and KOH-treated LPS from *A. baumannii* 307-0294 identified glucose, *N*-acetylglucosamine, and Kdo, whereas glucose was not present in LPS and KOH-treated LPS from the 307::TN17 *lpsB* mutant in the same analyses. CE-ES-MS analyses were performed to compare the compositions of the LPS elaborated by 307::TN17 and *A. baumannii* 307-0294. CE-ES-MS is a well-established technique for ascertaining the compositions of isolated LPS-derived samples, such as the O-deacylated and completely deacylated samples described here (39). Analyses of the O-deacylated LPS of *A. baumannii* 307-0294 suggested a composition of lipid A-OH, 3 Kdo residues, 2 HexN residues, HexNAcA, and 4 Hex residues, which is consistent with the LPS composition observed previously for *A. baumannii* strain ATCC 19606 (43). In contrast, analysis of both LPS-OH and fully deacylated (KOH-treated) LPS preparations obtained from the 307::TN17 mutant revealed a highly truncated molecule containing just 2 Kdo residues and the lipid A region (Table 1).

In *trans* complementation of 307::TN17. *lpsB*, along with approximately 200 bases of flanking DNA, was subcloned into the *A. baumannii* complementation plasmid pNLAC1, and the sequence was confirmed to be correct by a sequence analysis (36). The resulting construct, designated pSS11, was introduced into 307::TN17 to generate the in *trans* complemented strain 307::TN17/pSS11. Analyses of the chromosomal *lpsB* coding region and extraction of the plasmid from 307::TN17/pSS11 indicated that the complementing plasmid was maintained as an independent replicon in 307::TN17. SDS-PAGE and Western blot assays, as well as colony lift analyses of the wild type, the LPS-deficient TN mutant 307::TN17, and the complemented derivative 307::TN17/pSS11, demonstrated that the MAb 13C11-reactive LPS moiety was restored (Fig. 3A and B, lane 3, and 3C).

LPS affects *A. baumannii* sensitivity to the bactericidal activity of NHS. *A. baumannii* 307-0294 is resistant to the bactericidal activity of normal human serum (NHS), exhibiting complete resistance even in the presence of 90% NHS after 3 h (35, 36). To evaluate whether the loss of functional LpsB enzymatic activity and corresponding production of a severely

TABLE 1. Negative-ion CE-ES-MS data and compositions of O-deacylated LPS and fully deacylated LPS (KOH treated) for *A. baumannii* 307-0294 and 307::TN17

Strain	Observed ions (m/z)[a]			Observed molecular ion (m/z)	Calculated molecular ion (m/z)	Proposed composition
	$(M-H)^-$	$(M-2H)^{2-}$	$(M-3H)^{3-}$			
O-deacylated LPS						
A. baumannii 307-0294		1400.5	933.5	2803.3	2800.7	Lipid A-OH, 3 Kdo, 2 HexN, HexNAcA, 4 Hex[b]
307::TN17	1391.0			1392.0	1392.36	Lipid A-OH, 2 Kdo
	1172.0			1173.0	1172.18	Lipid A-OH, Kdo
	952.5			953.5	952.0	Lipid A-OH
Fully deacylated LPS						
A. baumannii 307-0294		1153.0	768.1	2307.7	2307.0	3 Kdo, 2 HexN, HexNA, 4 Hex, 2 HexN, 2 P, H_2O
307::TN17	939.3	469.2		940.4	940.6	2 Kdo, 2 HexN, 2 P, H_2O
	719.1			720.1	720.5	Kdo, 2 HexN, 2 P, H_2O
	499.0					2 HexN, 2 P, H_2O

[a] Average mass units (in daltons) were used for calculation of molecular masses based on the proposed composition, as follows: Lipid A-OH, 952.00; Hex, 162.15; Kdo, 220.18; HexN, 161.19; HexNAc, 203.19; HexNAcA, 218.19; and P, 79.95.
[b] Lipid A-OH contains two N-acylated glucosamine residues, two phosphate residues, and a water molecule.

truncated LPS molecule affected serum sensitivity, comparative bactericidal assays were performed. These assays indicated that 307::TN17 exhibited significantly ($P = 0.0001$) increased serum sensitivity compared to the parental strain (Fig. 4). In contrast to the survival of *A. baumannii* 307-0294 in 90% serum after 3 h, only 12.8% of the 307::TN17 inoculum survived under these conditions. Importantly, in the complemented derivative 307::TN17/pSS11 serum resistance was completely restored, and the levels of resistance were comparable to wild-type levels. All strains were also assessed in the presence of 90% heat-inactivated (56°C for 30 min) NHS and in MH broth; all growth and survival rates were comparable (data not shown).

Evaluation of 307::TN17 in the rat soft tissue infection model. To investigate if LPS expression contributes to *A. baumannii* infection *in vivo*, we examined the growth and survival of the isogenic LPS mutant 307::TN17, both in competition with *A. baumannii* wild-type strain 307-0294 and by itself, in a rat soft tissue infection model. Compared to *A. baumannii* 307-0294, 307::TN17 alone did not exhibit a significant decrease ($P = 0.1185$) in survival in this model (Fig. 5A). In contrast, when the challenge inoculum contained *A. baumannii* 307-0294 and 307::TN17 at a ratio of 1:1 (Fig. 5B), the expression of a truncated LPS molecule by the mutant affected the growth and survival of 307::TN17 compared to the growth and

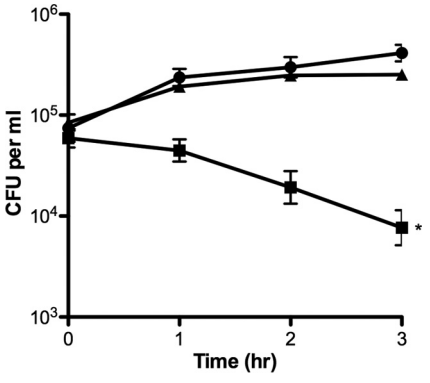

FIG. 4. Bactericidal effects of 90% NHS on *A. baumannii* 307-0294 (▲), 307::TN17 (■), and the complemented derivative 307::TN17/pSS11 (●) over time. The survival of 307::TN17 was significantly decreased compared to that of *A. baumannii* 307-0294 (*, $P = 0.0001$). The data are the means ± standard errors of the means for 6 to 9 replicates for each strain.

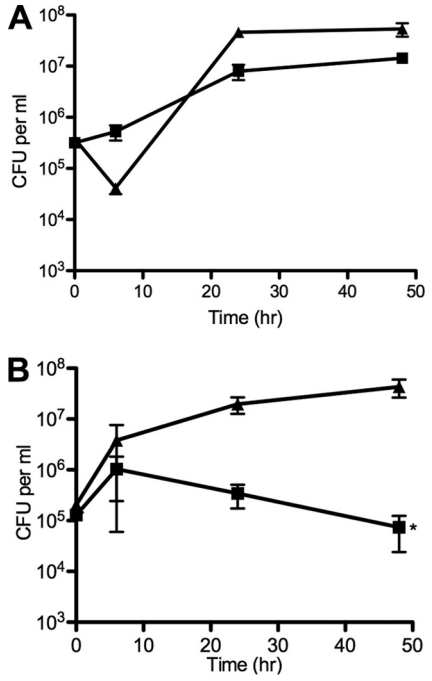

FIG. 5. Survival of *A. baumannii* 307-0294 and the isogenic derivative 307::TN17 in the rat soft tissue infection model. Rat pouches were prepared and challenged with approximately 10^6 CFU of *A. baumannii* 307-0294 (■) and 307::TN17 (●) separately (A) or in combination (1:1 mixture) (B). Bacterial titers were determined at 0, 6, 24, and 48 h and are expressed as means ± standard errors of the means. The survival and growth of 307::TN17 were significantly decreased compared with the survival and growth of *A. baumannii* 307-0294 in the competition experiment (*, $P = 0.0282$).

survival of *A. baumannii* 307-0294 ($P = 0.0282$). Since the growth of *A. baumannii* 307-0294 and the growth of 307::TN17 were similar in laboratory media *in vitro* (data not shown), these data support the conclusion that the expression of a deep rough LPS phenotype is disadvantageous *in vivo*. These results suggest that the full-length LPS molecule is important for the survival of *A. baumannii* 307-0294 and provides a competitive advantage for establishment and persistence of *A. baumannii* infections *in vivo*.

DISCUSSION

In this paper, we describe the use of random TN mutagenesis for identification of an LPS core GT in *A. baumannii*. This LPS core GT is highly conserved, as the sequence of *A. baumannii* 307-0294 *lpsB* exhibits significant homology to the *lpsB* coding regions in six other *A. baumannii* strains (96 to 100% ID); in addition, this gene was identified by PCR amplification in all *A. baumannii* isolates evaluated regardless of the MAb 13C11 reactivity phenotype (data not shown). The LpsB-deficient mutant 307::TN17 did not produce a 7-kDa carbohydrate moiety detectable by SDS-PAGE analysis and also did not exhibit reactivity to the LPS core-specific MAb 13C11. Furthermore, compositional analyses of this mutant indicated that it expresses a deeply truncated LPS molecule consisting of only 2 Kdo residues and lipid A. In contrast, wild-type *A. baumannii* 307-0294 expresses an LPS core glycoform with the same composition as the previously published LPS core of *A. baumannii* ATCC 19606, which is also an MAb 13C11-reactive strain, comprised of lipid A-OH, 3 Kdo residues, 2 HexN residues, HexNAcA, and 4 Hex residues (43).

The results of this study indicate that an *A. baumannii* LpsB mutant defective in biosynthesis of full-length LPS is sensitive to the bactericidal activity of NHS. When it was compared to *A. baumannii* wild-type strain 307-0294, 307::TN17 exhibited a survival rate that was 29.6% of the wild-type rate at 1 h and was 4.6% of the wild-type rate at 3 h. Not only did a previous study report that clinical isolates of *A. baumannii* obtained from bacteremic patients exhibited intrinsic resistance to 90% NHS, but the authors also postulated that the expression of LPS might be the essential factor for this resistance and suggested that this surface molecule might be important for allowing the strains to survive in blood and contribute to the pathogenesis of this species in human infections (10). Our results obtained using a defined mutant expressing a truncated LPS molecule and a derivative complemented in *trans*, which restored the LPS glycoform to the wild-type phenotype, confirm this hypothesis and conclusively demonstrate that LPS is essential for resistance to NHS. Moreover, detergent sensitivity assays indicated that the deep core LPS mutant 307::TN17 exhibits increased sensitivity ($P < 0.05$) to SDS, Triton X-100, and deoxycholate compared to the wild type, further confirming that the complete core LPS glycoform is essential for the functional integrity of the *A. baumannii* outer membrane (data not shown). Importantly, the growth of 307::TN17 was comparable to the growth of *A. baumannii* wild-type strain 307-0294 *in vitro*, indicating that the deep core LPS truncation exhibited by the mutant did not affect the cell viability or growth rate.

The ability of the mutant to survive *in vivo* was assessed by performing direct challenge assays, as well as competitive survival assays, using the rat soft tissue infection model. We recently established this animal model, which is clinically relevant to *A. baumannii* since this organism is increasingly recognized as a cause of soft tissue infections, as a method for evaluating the importance of *A. baumannii* genes *in vivo* (35, 36). These analyses demonstrated that the 307::TN17 *lpsB* mutant could survive and grow in the rat soft tissue infection model for at least 48 h; however, the expression of a truncated LPS molecule was disadvantageous compared to the expression of the native molecule in this model system. Interestingly, *lpsB* mutants of *Sinorhizobium meliloti* also exhibit deep core LPS biosynthetic defects and are competitively compromised for alfalfa nodulation compared to wild-type strains (13, 16). The data describing defective infections by LpsB mutants support the conclusion that production of full-length LPS plays an important functional role in survival *in vivo*.

Although structural analyses of the wild-type LPS molecules expressed by *A. baumannii* strains have been described previously, this is the first report describing the identification, characterization, and mutagenesis of a gene involved in the biosynthesis of *A. baumannii* LPS. Serum susceptibility assays demonstrated that the expression of full-length LPS on the surface of *A. baumannii* is critical for protection against the bactericidal effects of NHS. Further, *in vivo* studies using a rat soft tissue infection model indicated that LPS contributes to the survival and fitness of *A. baumannii*. Taken together, the data suggest that full-length LPS plays a critical role in the pathogenesis of *A. baumannii* infections and supports the hypothesis that LPS-deficient mutants are likely attenuated for virulence *in vivo*. These findings strongly support the conclusion that additional studies investigating the contribution of the biosynthesis and cell surface expression of LPS to infections caused by *A. baumannii* should be performed.

ACKNOWLEDGMENT

We thank Amy Howlett for skilled technical assistance.

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Editor: S. R. Blanke