Identification of Poly-\(N\)-acetylglucosamine as a Major Polysaccharide Component of the \textit{Bacillus subtilis} Biofilm Matrix*§

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**Background:** \textit{Bacillus subtilis} is a model organism for analyzing bacterial biofilms, but the carbohydrate components are undescribed.

**Results:** Genes in the \textit{epsHIJK} locus needed for biofilm formation encode proteins synthesizing the conserved bacterial polysaccharide poly-\(N\)-acetylglucosamine (PNAG).

**Conclusion:** PNAG is a major carbohydrate component of \textit{B. subtilis} biofilms.

**Significance:** PNAG production is essential for formation of \textit{B. subtilis} biofilms.

\textit{Bacillus subtilis} is intensively studied as a model organism for the development of bacterial biofilms or pellicles. A key component is currently undefined exopolysaccharides produced from proteins encoded by genes within the \textit{eps} locus. Within this locus are four genes, \textit{epsHIJK}, known to be essential for pellicle formation. We show they encode proteins synthesizing the broadly expressed microbial carbohydrate poly-\(N\)-acetylglucosamine (PNAG). PNAG was present in both pellicle and planktonic wild-type \textit{B. subtilis} cells and in strains with deletions in the \textit{epsA}–\textit{G} and \textit{L}–\textit{O} genes but not in strains deleted for \textit{epsH}–\textit{K}. Cloning of the \textit{B. subtilis} \textit{epsH}–\textit{K} genes into \textit{Escherichia coli} with in-frame deletions in the PNAG biosynthetic genes \textit{pgaA}–\textit{D}, respectively, restored PNAG production in \textit{E. coli}. Cloning the entire \textit{B. subtilis} \textit{epsHIJK} locus into \textit{pga}-deleted \textit{E. coli}, \textit{Klebsiella pneumoniae}, or alginate-negative \textit{Pseudomonas aeruginosa} restored or conferred PNAG production. Bioinformatic and structural predictions of the \textit{EpsHJK} proteins suggest \textit{EpsH} and \textit{EpsJ} are glycosyltransferases (GT) with a GT-A fold; \textit{EpsI} is a GT with a GT-B fold, and \textit{EpsK} is an \(\alpha\)-helical membrane transporter. \textit{B. subtilis}, \textit{E. coli}, and \textit{pga}-deleted \textit{E. coli} carrying the \textit{epsHIJK} genes on a plasmid were all susceptible to opsonic killing by antibodies to PNAG. The immunochemical and genetic data identify the genes and proteins used by \textit{B. subtilis} to produce PNAG as a significant carbohydrate factor essential for pellicle formation.

Many microbial organisms produce biofilms, structurally complex multicellular communities inside an extracellular matrix of variable factors that can include exopolysaccharides (EPS),\(^7\) proteins, and nucleic acids (1, 2). Biofilms are probably the most common structures for microbial communities, as this state protects against many environmental stresses such as antimicrobial factors (3). Biofilms are also involved in the pathogenesis of many infectious diseases (1, 4, 5). Polysaccharides are often prominent components of biofilms but, like many factors in this structure, make a variable contribution.

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§ This article contains supplemental Table S1.

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⁷ The abbreviations used are: EPS, exopolysaccharides; PNAG, poly-\(N\)-acetylglucosamine; GT, glycosyltransferase; MMG, minimal medium glucose; LB, lysogeny broth; OPK, opsonophagocytic killing; MATE, multiple antimicrobial extrusion protein; PDB, Protein Data Bank; TT, tetanus toxoid.
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depend on the microbial species, strain, growth conditions, and overall environment. Interestingly, numerous species have developed diverse metabolic pathways for production of EPS, alluding to the possibility that these systems have evolved independently. A common EPS associated with microbial biofilm formation is a polymeric β-1,6-linked N-acetylgalcosamine (PNAG) structure that is highly conserved and expressed by a range of bacterial, fungal, and protozoan microorganisms (6, 7). PNAG was first isolated and characterized from Staphylococcus epidermidis (8), where it was referred to as the polysaccharide intercellular adhesin, and then later shown to be produced by Staphylococcus aureus (9, 10). In these two species, PNAG is synthesized by proteins encoded by four genes in the ica operon (9–11), and ica-deleted PNAG-deficient strains were unable to produce biofilms (9, 11). However, PNAG-independent biofilm formation in some staphylococcal strains has also been described (12). In general, depending on the strain and culture conditions, PNAG is often necessary, but not sufficient or essential, for biofilm formation.

PNAG synthesis also occurs in various Gram-negative organisms, including Escherichia coli (13), Acinetobacter baumannii (14) and Burkholderia spp. (15). In these organisms PNAG is synthesized by four proteins encoded by genes in the pga operon. More recently, Cywes-Bentley et al. (7) showed that a much wider spectrum of microbes can synthesize PNAG, including many human bacterial pathogens such as Streptococcus pneumoniae, Streptococcus pyogenes, Streptococcus agalactiae, Neisseria gonorrhoeae, and Neisseria meningitidis, the nontypable Haemophilus influenzae and Mycobacterium tuberculosis, and eukaryotic organisms such as fungal pathogens, and protozoan parasites such as Trichomonas vaginalis and murine and human Plasmodia spp. that cause malaria. Among many of these pathogens there are no readily identifiable genes homologous to those in the ica or pga loci. Nonetheless, the broad conservation of PNAG synthesis implies an important role in microbial biology, perhaps related to growth phases involving aggregation of microbes living in different environments or protection from anti-microbial factors.

Bacillus subtilis is a spore-forming Gram-positive bacillus wherein certain strains grown under specific conditions develop complex biofilms (16–18). A large amount of knowledge has been accumulated that explains how the production and composition of the B. subtilis biofilm are regulated (17), although many aspects of this process are not fully elucidated. Full biofilm formation by some strains of B. subtilis such as NCIB 3610 (3610) is dependent on 15 proteins encoded by genes in the eps locus (epsA–epsO) that are associated with the carbohydrate content and complexity of a surface pellicle (16) involved in the overall biofilm structure. The composition and structure of polysaccharides synthesized by the proteins within this complex are not well described, but mutations in most of the genes within the B. subtilis eps cluster lead to loss of biofilm formation (19). Some genes, such as epsE, have dual functions, affecting both polysaccharide synthesis and flagella-based motility (20, 21).

Given the ubiquity of PNAG synthesis among a range of microbial organisms, we evaluated B. subtilis biofilms for PNAG production and further examined the B. subtilis eps locus for genes potentially encoding PNAG biosynthetic proteins. Both biofilm and planktonic cells produced PNAG, and within the available annotated genome, we identified four genes, epsH–K, as potentially being responsible for PNAG synthesis. These genes are predicted to encode two glycosyltransferases (GT) (epsH and epsI), separated by another GT with potential EPS modifying enzymatic activity (epsL), and a transporter/facilitator of synthesis (epsK). Cloning of the epsH–K genes into PNAG-deficient E. coli or Klebsiella pneumoniae (Δpga), or alginate-negative Pseudomonas aeruginosa, leads to synthesis of a polymer immunologically equivalent to PNAG. Each of the four B. subtilis epsH–K genes could individually trans-complement E. coli strains deleted for the pgaA–D genes, respectively. Also the epsH and epsI genes could partially complement E. coli strains deleted for the pgaC or pgaA genes, respectively. Extracts of both WT B. subtilis and E. coli (Δpga) complemented with B. subtilis epsHJK complemented PNAG-imunoreactive, hexosamine-containing material that was destroyed by treatment with both the PNAG-degrading enzyme dispersin B (22) and by sodium periodate, which can only destroy hydrolyze polymeric hexosamines in a 1–6-linkage. Synthesis of PNAG in E. coli from the epsH–K genes resulted in susceptibility of cells to killing in an opsonophagocytic assay using antibodies specific to PNAG, indicative of functional conservation of PNAG properties when B. subtilis gene products directly synthesize PNAG in E. coli. Overall, we identify the presence of PNAG in B. subtilis biofilms and the genes within the eps locus that encoded proteins that synthesize PNAG. B. subtilis lacking epsH–K genes are unable to form biofilms, indicating PNAG is essential for biofilm/pellicle formation by this organism.

Experimental Procedures

Bacterial Strains and Plasmids—Bacterial strains (B. subtilis, E. coli, K. pneumoniae, and P. aeruginosa) and plasmids used in this study are listed in Table 1. B. subtilis was grown on minimal medium glucose (MMG) agar (23) or lysogenic broth (LB) agar (24) for 3–5 days at room temperature, whereas the other bacterial strains were grown overnight at 37 °C in LB or on LB agar.

BLAST (Basic Local Alignment Search Tool) and Structural Analysis—Sequences of eps genes in B. subtilis strain 168 were obtained from the NCBI website (reference sequence, NZ_CM000487.1). Amino acid sequences from four eps genes (epsH, epsL, epsP, and epsK) were mapped on E. coli and S. aureus sequences using BLAST from the NCBI website. Amino acid sequences from the four pga genes of E. coli IHE3034 were similarly mapped on S. aureus sequences. The amino acid sequences of B. subtilis epsH–K were analyzed by the Protein Homology/analY Recognition Engine Version 2 (PHYRE2) (25) for structural predictions as to their function.

Strain Construction in Gram-negative Bacteria—Deletions of individual pga genes in a clinical isolate of an E. coli K1 strain from a child with meningitis, designated E11 and kindly provided by Kwan Sik-Kim of Johns Hopkins University School of Medicine, and deletion of the entire 4-gene pga locus in both E. coli E11 and K. pneumoniae NTUH-K2044 (26) were constructed as described previously (27).

Briefly, a kanamycin resistance cassette flanked by FLP recombinase recognition target
sites and homology arms to replace the DNA segments of interest in-frame were generated by PCR with deletion primers (supplemental Table 1). Recombination within the targeted chromosomal sequences was mediated by the red recombinase encoded on pRdET (28), resulting in the replacement of the targeted sequence with a kanamycin-resistant cassette; all allelic replacements were confirmed by PCR. Subsequently, the kanamycin marker was removed, using the FLP expression plasmid pCP20 (29).

**Strain Construction in B. subtilis—**All constructs were either directly integrated and resolved in the competent strain DK1042 (comF::Tn501) or integrated in the competent strain DS2569 (ΔpBS32), transferred to the 3610 background using SPP1-mediated generalized phage transduction, and resolved (30). All strains and plasmids used in this study are listed in Table 1. Primer sequences are delineated in supplemental Table 1.

**In-frame Deletions—**To generate the in-frame marker-less deletion constructs, each plasmid was introduced by single crossover integration at the restrictive temperature for plasmid replication (37 °C) using mls resistance as a selection. To evict the plasmid, the strain was incubated in 3 ml of LB broth at a permissive temperature for plasmid replication (22 °C) for 14 h. Cells were then serially diluted and plated on LB agar at 37 °C. Individual colonies were patched onto LB agar plates and LB agar plates containing mls to identify mls-sensitive colonies that had evicted the plasmid. Chromosomal DNA from colonies that had excised the plasmid was purified and screened by PCR to determine which isolate had retained the deletion allele.

To generate the ∆epsA in-frame marker-less deletion construct, the region upstream of epsA was amplified by PCR using the primer pair 3971/3972 and digested with EcoRI and XhoI, and the region downstream of epsA was amplified by PCR using the primer pair 3973/3974 and digested with XhoI and BamHI. The two fragments were then simultaneously ligated into the EcoRI and BamHI sites of pMiniMAD that carries a temperature-sensitive origin of replication and an erythromycin resistance cassette to generate pMPP201.

The same method was used to generate the other eps gene in-frame marker-less deletion constructs. Plasmids are detailed in Table 1 and primers in supplemental Table 1.

**Genetic Complementation—**Wild-type alginate overproducing mucoid *P. aeruginosa* FRD1 and an alginate-deficient strain (*P. aeruginosa* (Tn501::algF)) due to a polar effect of the Tn501 insert in the algF gene on alginate synthesis (31) were used for extraction and immunologic detection of PNAG above on MMG agar or LB agar plates, respectively, and cells grown on LB agar plates with tetracycline (10 mg/liter) under the same conditions with protection from light. Microbial samples were suspended in PBS, then spotted onto microscope slides, air-dried, and fixed for 1 min at room temperature with ice-cold methanol. After washing, slides were reacted with control or PNAG-specific mAbs directly conjugated to Alexa Fluor 488 at 5.2 μg/ml along with nucleic acid stain, Syto 83 (Molecular Probes) (7). After 2 h at room temperature or overnight at 4 °C, slides were washed and evaluated by confocal microscopy. For enzymatic and periodate treatments, samples fixed to slides were incubated in Tris-buffered saline (pH 6.4) containing either 50 μg of dispersin B/ml (digests PNAG) or 50 μg of chitinase/ml (no effect on PNAG) overnight at 37 °C or in 0.4 M periodate (destroys PNAG) for 2 h at 37 °C in a humidified environment. After washing, cells were treated with the Alexa Fluor 488 directly conjugated mAbs.

**Extraction and Detection of PNAG—**B. subtilis DS991 and *E. coli* ∆pga (pUCP18Tc-epsHJK) were grown as described above on MMG agar or LB agar plates, respectively, and cells were used for extraction and immunologic detection of PNAG by a slot blot, as described previously (14), and chemical detection of hexosamines, as described previously (33).

**B. subtilis** DS991, *B. subtilis* DK2055 ∆epsK, *E. coli* WT, and *E. coli* ∆pga as a negative control were tested for intracellular PNAG by a direct-binding ELISA using lysates of these cells prepared by sonication. Briefly, cells from blood agar plates grown overnight at 37 °C were suspended in normal saline, washed, and then suspended in Tris-buffered saline containing 1.25 μg/ml dispersin B to remove surface PNAG during a 24-h 37 °C incubation step. After washing, bacterial cells were suspended in 0.04 M phosphate buffer (pH 7.2) at an absorbance at 650 nm (*A*_{650 nm}) of 0.8, sonicated with 10-s bursts, 10 times, and insoluble debris was removed by centrifugation, and dilutions of the lysate were used to directly sensitize Immunol 4 ELISA plates. A standard curve was generated using purified PNAG (0.015–0.1 μg/ml) to sensitize ELISA wells in duplicate, and samples were probed with 10 μg/ml mAb F598 followed by an alkaline phosphatase-conjugated goat antibody to human IgG. After assay development with the p-nitrophenyl phosphate substrate, *A*_{405 nm} readings were obtained; a standard curve of the
Purified PNAG concentration versus A405 nm was calculated by linear regression, and the amount of PNAG in bacterial cell lysates was calculated after subtracting out the background from the negative control. Samples of cells taken before and after dispersin B treatment were probed as described above for immunochemical detection of PNAG in microbial cells.

Opsonophagocytic Assay—Opsonophagocytic killing (OPK) of B. subtilis DS991, E. coli WT, E. coli (Δpga), and E. coli (Δpga) (pUCP18Tc-epsHIJK) was carried out, as described previously (34), except differentiated human promyelocytic HL60 cells were used as the phagocyte source (35). The percent killing mediated by antibodies in immune sera raised to a conjugate of nine residues of β-1–6-linked glucosamine and tetanus toxoid (TT; 9GlcNH₂-TT vaccine) (36) was calculated by dividing the colony-forming units (cfu) in the test sera by those in the corresponding dilution of the nonimmune control serum.

Results

Detection of PNAG in B. subtilis Biofilms/Pellicles—B. subtilis pellets formed over 10 days of growth at the air-liquid interface in MMG medium were reacted with control mAb F429 or mAb F598 to PNAG, both directly conjugated to Alexa Fluor 488 and visualized for immunofluorescence by confocal microscopy. Bacilli embedded in a strongly immunoreactive matrix of PNAG were readily observed, and binding to mAb F598 was lost after treating the B. subtilis biofilms with the PNAG-degrading enzyme dispersin B or with PNAG-hydrolyzing sodium periodate (Fig. 1).

Expression of PNAG by WT and eps-mutant B. subtilis—Planktonic cells of B. subtilis that overproduced EPS (due to mutation of the master repressor SinR) and also defective for the biofilm-organizing protein TasA (DS991) were also positive for PNAG expression by immunofluorescence microscopy (Fig. 2A), and immunoreactivity was lost following treatment with...
### TABLE 1

**Strains and plasmids used in this study**

| Strain or plasmid | Relevant characteristics | Source or ref. |
|-------------------|--------------------------|----------------|
| **E. coli sm10** | thi-1 relA1 thi-1 thy leu tonA lacy supE recA RP4-2::Mu Km' | 57 |
| **B. subtilis 3610** | Undomesticated wild strain | 58 |
| **B. subtilis DS991** | sinR:kan tasA::Tn10 spec | 59 |
| **B. subtilis DK1042** | comF | 60 |
| **B. subtilis DS2569** | ΔpSB32 | 60 |
| **B. subtilis DK1943** | ΔpSB32 comF | This study |
| **B. subtilis DK1806** | ΔpSB comF | This study |
| **B. subtilis DK1807** | ΔpSB comF | This study |
| **B. subtilis DS4248** | ΔpSB | This study |
| **B. subtilis DS2152** | ΔpSB | This study |
| **B. subtilis DS1646** | ΔpSB | This study |
| **B. subtilis DS7499** | ΔpSB | This study |
| **B. subtilis DS6776** | ΔpSB | This study |
| **B. subtilis DK1758** | ΔpSB comF | This study |
| **B. subtilis DS4166** | ΔpSB | This study |
| **B. subtilis DK2055** | ΔpSB K | This study |
| **B. subtilis DS7432** | ΔpSB | This study |
| **B. subtilis DS4901** | ΔpSB | This study |
| **B. subtilis DS4900** | ΔpSB | This study |
| **B. subtilis DK1759** | ΔpSBO comF | This study |
| **E. coli E11** | K1 capsule type; clinical isolate | K. Silk-Kim |
| **E. coli E11 Δpga** | E. coli E11 with in-frame deletion of entire pga locus | Baltimore, MD |
| **E. coli E11 ΔpgaA** | E. coli E11 in-frame deletion of pgaA | This study |
| **E. coli E11 ΔpgaB** | E. coli E11 in-frame deletion of pgaB | This study |
| **E. coli E11 ΔpgaC** | E. coli E11 in-frame deletion of pgaC | This study |
| **E. coli E11 ΔpgaD** | E. coli E11 in-frame deletion of pgaD | This study |
| **K. pneumoniae** | K2 capsule type; clinical isolate | This study |
| **K. pneumoniae Δpga** | K. pneumoniae K2 with in-frame deletion of entire pga locus | This study |
| **P. aeruginosa** | WT strain FRD1 | 31 |
| **P. aeruginosa Tn::algF** | FRD1 Tn560::algF, alginate-deficient | 31 |

**Plasmid**

| Plasmid | Relevant characteristics | Source or ref. |
|---------|--------------------------|----------------|
| **pRED/ET** | Red/ET expression plasmid | 61 |
| **pCP20** | Helper plasmid; FLP*, temperature-sensitive, AmpR, CmR | 62 |
| **pUCP18Tc** | Broad host range vector pUC18 derivative; TetR | 63 |
| **pUCP18Tc-pga** | TetR, pgaABCD (pga) locus from E. coli E11 cloned into pUCP18Tc | This study |
| **pUCP18Tc-pgaA** | TetR, pgaA from E. coli E11 cloned into pUCP18Tc at XbaI-SbfI sites | This study |
| **pUCP18Tc-pgaB** | TetR, pgaB from E. coli E11 cloned into pUCP18Tc at XbaI-SbfI sites | This study |
| **pUCP18Tc-pgaC** | TetR, pgaC from E. coli E11 cloned into pUCP18Tc at XbaI-SbfI sites | This study |
| **pUCP18Tc-pgaD** | TetR, pgaD from E. coli E11 cloned into pUCP18Tc at XbaI-SbfI sites | This study |
| **pUCP18Tc-epshHIJK** | TetR, epshHIJK from B. subtilis DS991 cloned into pUCP18Tc at XbaI-SbfI sites | This study |
| **pUCP18Tc-epsh** | TetR, epsh locus from B. subtilis DS991 cloned into pUCP18Tc at XbaI-SbfI sites | This study |
| **pUCP18Tc-epsI** | TetR, epsI locus from B. subtilis DS991 cloned into pUCP18Tc at XbaI-SbfI sites | This study |
| **pUCP18Tc-epsK** | TetR, epsK locus from B. subtilis DS991 cloned into pUCP18Tc at XbaI-SbfI sites | This study |
| **pMiniMAD** | oriBsTs amp mls | 64 |
| **pMP201** | ΔepsA mls amp | This study |
| **pMP202** | ΔepsB mls amp | This study |
| **pMP203** | ΔepsC mls amp | This study |
| **pKB90** | ΔepsD mls amp | This study |
| **pKB91** | ΔepsF mls amp | This study |
| **pSG52** | ΔepsG mls amp | This study |
| **pSG57** | ΔepsH mls amp | This study |
| **pMP204** | ΔepsI mls amp | This study |
| **pKB92** | ΔepsI mls amp | This study |
| **pMP215** | ΔepsK mls amp | This study |
| **pMP5** | ΔepsL mls amp | This study |
| **pMP206** | ΔepsO mls amp | This study |

**Cassette**

| Cassette | Relevant characteristics | Source or ref. |
|----------|--------------------------|----------------|
| **FRT-PGK-gh2-neo-FRT** | PGK-gh2-neo cassette flanked by FRT sites | Gene Bridges GmbH, Heidelberg Germany |

dispersin B or periodate (Fig. 2A). When PNAG production was evaluated in *B. subtilis* strains with in-frame deletions in all 15 identified *eps* genes (Table 1), most of which are unable to form biofilms, but disruption of the *epsH–K* genes led to loss of PNAG production (Fig. 2B). The *epsH* and *epsF* genes are annotated as putative GTs and show similarity to proteins encoded by *S. aureus icaA* and *E. coli pgaC* (Table 2). The *epsF* gene is currently annotated as a putative polysaccharide pyruvyltransferase (Table 2), but as shown below, its structural predictions suggest other functions. The *epsK* gene is currently annotated as a putative extracellular matrix component exporter similar to the *wzx* proteins in *E. coli* (Table 2). Two other *eps* genes (*epsE* and *epsF*) are annotated as putative GTs, but deletions in these genes did not lead to loss of PNAG production (Fig. 2B). Similarly, an *epsG* deletion mutant, known to be deficient in biofilm production like the *epsH* mutant (17), was still able to produce PNAG (Fig. 2B). As with other microbial species, PNAG production appears to be necessary, but not sufficient, for full biofilm formation in *B. subtilis*.
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### TABLE 2

| Organism strain | Gene    | Location          | No of amino acids | Known or putative annotated protein function | Similar to       | Coverage | Amino acid identities | Positive amino acids |
|-----------------|---------|-------------------|-------------------|---------------------------------------------|------------------|----------|----------------------|----------------------|
| *E. coli* E11 IHE3034 | pgmA    | 1,119,576–1,119,999 | 808               | Putative outer membrane–β-PNAG translocation/docking protein | Unclear           | None     | %                    | %                    |
| *E. coli* E11 IHE3034 | pgpB    | 1,117,549–1,119,567 | 673               | β-PNAG N-deacetylase, belonging to CAZY family CE4 | *S. aureus* IcaB  | 37       | 22                   | 42                   |
| *E. coli* E11 H1002 | pgaC    | 1,116,231–1,117,556 | 442               | PNAG N-glycosyltransferase belonging to CAZY family GT2, and PNAG co-transporter | *S. aureus* IcaA  | 92       | 40                   | 63                   |
| *E. coli* E11 H1002 | pgaD    | 1,115,816–1,116,229 | 138               | Inner membrane c-di-GMP receptor and PNAG co-transporter | Unclear           | None     | %                    | %                    |
| *B. subtilis* DS991 | epsK    |                    | 350               | Putative extracellular matrix component exporter | *E. coli* Wzx    | 60–97    | 28                   | 48                   |

### TABLE 3

Summary for detection of PNAG synthesis by immunofluorescence in indicated strain

The following symbols are used: −, no immunofluorescence detected; +, 25–50% of WT levels of PNAG observed; ++, 51–80% of WT levels of PNAG observed; ++++, >80% of WT levels of PNAG observed.

| Strain | Complemented with | Control mAb | mAb to PNAG |
|--------|-------------------|-------------|-------------|
| *E. coli* K1 WT |                    | –           | ++          |
| *E. coli* K1 ΔpgmA | (pUCP18Tc) | –           | +           |
| *E. coli* K1 ΔpgaB | (pUCP18Tc) | –           | +           |
| *E. coli* K1 ΔpgaC | (pUCP18Tc) | –           | ++          |
| *E. coli* K1 ΔpgaD | (pUCP18Tc) | –           | +           |
| *E. coli* K1 ΔpgaA | (pUCP18Tc) | –           | ++          |
| *E. coli* K1 ΔpgaA | (pUCP18Tc) | –           | +           |
| *E. coli* K1 ΔpgaB | (pUCP18Tc) | –           | ++          |
| *E. coli* K1 ΔpgaC | (pUCP18Tc) | –           | ++          |
| *E. coli* K1 ΔpgaD | (pUCP18Tc) | –           | +           |
| *E. coli* K1 Δpga | (pUCP18Tc) | –           | ++          |
| *E. coli* K1 ΔpgmA | (pUCP18Tc) | –           | +           |
| *E. coli* K1 ΔpgpB | (pUCP18Tc) | –           | ++          |
| *E. coli* K1 ΔpgpC | (pUCP18Tc) | –           | ++          |
| *E. coli* K1 ΔpgpD | (pUCP18Tc) | –           | +           |
| *E. coli* K1 Δpga | (pUCP18Tc) | –           | ++          |
| *E. coli* K1 ΔpgmA | (pUCP18Tc) | –           | +           |
| *E. coli* K1 ΔpgpB | (pUCP18Tc) | –           | ++          |
| *E. coli* K1 ΔpgpC | (pUCP18Tc) | –           | ++          |
| *E. coli* K1 ΔpgpD | (pUCP18Tc) | –           | +           |
| *E. coli* K1 ΔpgmA | (pUCP18Tc) | –           | ++          |
| *E. coli* K1 ΔpgpB | (pUCP18Tc) | –           | ++          |
| *E. coli* K1 ΔpgpC | (pUCP18Tc) | –           | ++          |
| *E. coli* K1 ΔpgpD | (pUCP18Tc) | –           | +           |
| *E. coli* K1 ΔpgmA | (pUCP18Tc) | –           | ++          |
| *E. coli* K1 ΔpgpB | (pUCP18Tc) | –           | ++          |
| *E. coli* K1 ΔpgpC | (pUCP18Tc) | –           | ++          |
| *E. coli* K1 ΔpgpD | (pUCP18Tc) | –           | +           |

### Bioinformatic Analysis of *B. subtilis* PNAG Biosynthetic eps Genes and Proteins—The above results, along with bioinformatic analysis of the *B. subtilis* eps locus, indicated the genes required for PNAG production, epsH–K, could comprise a four-gene locus somewhat similar to the *S. aureus* icaADBC and *E. coli* pgaABCD loci encoding the PNAG biosynthetic proteins in these two organisms (Table 2) (11, 13). To obtain a more precise idea of the functions of the EpsH–K proteins, as well as other proteins within the eps locus, the predicted protein structures were analyzed using the PHYRE2 server (Fig. 3) (25). Overall, the entire eps locus contains genes encoding proteins known to be involved in polysaccharide synthesis using either the Wzx/Wzy or ABC transporter pathways for lipopolysaccharides, capsules, colonic acid, and S-layer components (37–39). Both EpsH and EpsJ have nearly identical predicted structures (Fig. 3) with homology to the GT domains of IcaA and PgaC proteins used in *S. aureus* and *E. coli* for PNAG synthesis, respectively (13, 40), as well as the BcsA protein used for cellulose (β-1–4-linked glucose) synthesis (41). With 60–70% sequence coverage and 15–22% sequence identity, EpsH and EpsJ are predicted with 100% confidence to be UDP-N-acetyl-glucosamine transferases with a GT-A fold. They are not predicted to have any transmembrane domains and therefore would be unable to translocate the synthesized polymer across the membrane.

BLAST analysis showed that EpsK belongs to the polysaccharide biosynthesis protein family, pfam01943. Members of this family are integral membrane proteins that encode for multiple antimicrobial extrusion protein (MATE)-like transporters, such as the O-antigen flippase Wzx. EpsK was predicted to be an α-helical membrane transporter with 14 transmembrane helices using PHYRE2 and various transmembrane domain prediction servers, respectively (Fig. 3) (42). PHYRE2 predicts EpsK to be structurally similar to DinF, a member of the MATE family, covering 84% of the sequence with 10–12% amino acid identity and 100% confidence of the fold prediction.

The analysis of the EpsI protein (Fig. 3) indicated it was not related structurally to the PNAG deacetylases IcaB or PgaB (43, 44) or any members of the family 4 carbohydrate esterases (deacetylases), but rather it is predicted to be a cytoplasmic GT with homology to genes in lipopolysaccharide biosynthesis. There were no predicted signal sequences or transmembrane helices but almost complete coverage (80–90%) of the EpsI sequence with 95% confidence in fold prediction to known lipopolysaccharide transferases.

### Functional Equivalence of *B. subtilis* EpsH–K Proteins to *E. coli* PNAG Biosynthetic Proteins—To determine whether each of these four *B. subtilis* eps genes encoded proteins that could replace those encoded by the pga operon, we constructed individual in-frame mutations in each of the four *E. coli* pga genes, pgaA–D, and complemented each of these different mutants with an eps-related gene judged to most likely encode a protein of potential similar function. As controls, we also complemented some of the *E. coli* mutants with a mismatched gene from *B. subtilis*. The WT *E. coli* strain produced PNAG, and deletion of any of the four individual pga genes abolishes PNAG production (Table 3). Complementation with an empty vector (pUCP18Tc) did not restore the phenotype in any strain with an in-frame pga mutation. Complementation of the *E. coli* pga mutant strains with individual clones of *B. subtilis* epsH, epsI, epsJ, or epsK resulted in restoration of PNAG production in each *E. coli* mutant strain with the gene from the *B. subtilis* locus judged most likely to be a functional equivalent (Table 3). Interestingly, we found that PNAG production in the
E. coli ΔpgaC strain could be restored by complementation with either the B. subtilis epsJ gene and, to a lesser extent, the epsH gene (Table 3). Similarly, we could achieve phnotypic complementation of PNAG production in the E. coli ΔpgaA mutant strain most strongly with the B. subtilis epsH gene and, to a lesser extent, the epsJ gene. The E. coli pgaB mutant was efficiently complemented with the B. subtilis epsJ gene, and the E. coli pgaD mutant was complemented with the B. subtilis epsK gene (Table 3). Cloning of the B. subtilis epsI and epsK genes into the E. coli ΔpgaA mutant did not result in PNAG production. Finally, when the entire pga locus was deleted from either E. coli or K. pneumoniae, the loss of PNAG production could be restored in both strains by complementation with the entire B. subtilis epsHJK locus in pUCP18Tc-epsHJK (Table 3).

We also analyzed the B. subtilis DS991 WT and ΔepsK strains as well as WT E. coli for the presence of intracellular PNAG. WT E. coli had a low, but detectable, level of intracellular PNAG (Table 4). Both WT and ΔepsK B. subtilis had detectable intracellular PNAG (Table 4), with the B. subtilis ΔepsK strain having about 60% more intracellular PNAG, indicating that in the absence of the predicted EpsK transporter, there was accumulation of the PNAG polysaccharide inside the B. subtilis ΔepsK cells.

Finally, we found that the predicted MATE function of the B. subtilis EpsK protein had similarity to PelG and PslK in P. aeruginosa used for the synthesis of the PEL and PSL polysaccharides, respectively. P. aeruginosa, unlike E. coli and K. pneumoniae, does not normally synthesize PNAG, so we introduced the pUCP18Tc-epsHJK plasmid into WT and algF-interrupted (Tn::algF) P. aeruginosa strain FRD1 to ascertain whether PNAG could be synthesized. We did not detect PNAG synthesis in either the WT P. aeruginosa strain or the strain carrying pUCP18Tc-epsHJK, but we did show alginate production by virtue of binding of mAb F429 to these bacterial cells (Fig. 4). We hypothesized that it might be problematic to produce or detect low level PNAG production in WT FRD1 P. aeruginosa if this organism is expressing both the positively charged PNAG and the negatively charged alginate in the same cell. We thus cloned pUCP18Tc-epsHJK into P. aeruginosa FRD1 (Tn501::algF), wherein the Tn (transposon) insertion has a polar effect on the alginate biosynthetic locus, leading to loss of alginate production, as verified by loss of binding of mAb F429 to cells of this strain (Fig. 4). Addition of the pUCP18Tc-epsHJK plasmid resulted in expression of PNAG on the recombinant P. aeruginosa strain FRD1 as detected by immunofluorescence analysis of binding of mAb F598 to PNAG. This binding was lost after treatment of the recombinant P. aeruginosa strains with dispersin B and periodate, indicative of PNAG synthesis in alginate-negative P. aeruginosa by proteins encoded by the B. subtilis epsHJK genes.

Detection of PNAG by WT and Recombinant B. subtilis and E. coli Strains—To confirm that PNAG was produced by WT B. subtilis but not B. subtilis ΔepsH or ΔepsJ, and by WT E. coli E11
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FIGURE 4. Production of PNAG in alginate-deficient P. aeruginosa FRD1 in the presence of the B. subtilis epsHIJK locus. WT P. aeruginosa FRD1 produces alginate and binds Alexa Fluor 48-labeled mAb F429 but not PNAG-specific mAb F598. A polar Tn-insertion in the afg gene eliminated alginate production and insertion of the B. subtilis epsHIJK locus leads to PNAG production. Immunoreactive PNAG in this latter strain is lost after treatment with dispersin B or periodate. White bars, 10 μm.

FIGURE 5. Detection of PNAG in extracts of E. coli and B. subtilis strains. Extracts of the indicated E. coli or B. subtilis strain were immobilized on membranes and probed with mAb F598 to PNAG followed by anti-human IgG conjugated to HRP. Both WT E. coli and E. coli Δpga carrying the cloned B. subtilis epsHIJK genes expressed PNAG, while the Δpga strain did not contain detectable PNAG. Similarly, extracts of cells of B. subtilis DS991 had detectable PNAG, but none was present in the strains lacking either the epsH or epsJ genes. Extracts with detectable PNAG lost reactivity with mAb F598 following dispersin B or periodate treatment and contained detectable hexosamine. N/A, not applicable; Pos, positive; Neg, negative.

Opsonic Killing Mediated by Antibody to PNAG—The susceptibility of the B. subtilis and E. coli strains to OPK was tested in an assay using antibodies raised in either a rabbit or goat to the 9GlcNH₂-TT vaccine (36). Antibodies in both of these antisera readily mediated OPK of WT B. subtilis and WT E. coli (Fig. 6). Deletion of the pga locus in E. coli resulted in no effect of antibody to PNAG on cell survival in an OPK assay, whereas introduction of the pUCP18Tc-epsHIJK plasmid into pga-deleted E. coli restored the susceptibility to OPK. Thus, B. subtilis EpsH–K proteins produced an antigen in E. coli functionally equivalent to native PNAG from this organism.

Discussion

B. subtilis has served as one of the major model organisms for scrutinizing biofilm formation and multicellular activity in bacterial communities (18, 48). Many of the factors, genes, and conditions needed for producing and modulating formation of biofilms by this organism are well studied (49). Components of the B. subtilis biofilm include EPS and polymeric substances such as poly-DL-glutamic acid and proteins, including TapA, TasA, and BslA (48). However, the chemistry of the biofilm EPS constituents are not defined, and no definitive polysaccharide structures have previously been identified. Here, we found that within the 15-gene eps cluster of B. subtilis there was a 4-gene locus encompassing epsHIJK that encodes proteins that can synthesize either the conserved bacterial surface polysaccharide PNAG, a common component of microbial biofilms, or an antigenically cross-reactive material. In the B. subtilis biofilm, PNAG, or a related structure, likely serves as a scaffold as well as an anchoring substrate for the other components in the biofilm, which requires gene products from the eps locus other than epsHIJK for full matrix formation, as many of the eps gene products are needed to establish the biofilm phenotype (48).

To synthesize PNAG using the B. subtilis EpsH–K proteins, we speculate that EpsH is an undecaprenyl priming transferase that makes undecaprenyl-3-O-acetyl N-acetylgalactosamine. EpsJ could either be modifying this first GlcNAc or possibly adding on another sugar monomer and also providing a deacetylase function. EpsI is potentially the poly-GlcNAc transferase that is needed for long chain extension to the lipid linker of the UDP-N-acetylgalactosamine precursor to synthesize the PNAG-like molecule, and EpsK either

and E. coli E11 (Δpga) (pUCP18Tc-epsHIJK) but not E. coli (Δpga), we extracted surface material from cells as described (14) and used slot blots for immunologic detection of extracted antigen. As shown in Fig. 5, WT B. subtilis as well as WT E. coli E11 produced immunoreactive PNAG, whereas the B. subtilis ΔpsH and ΔepsJ strains did not, nor did E. coli (Δpga). Complementation of the PNAG-deficient E. coli (Δpga) strain with the B. subtilis epsHIJK genes restored detectable PNAG production. Confirmation that the immunoreactive material was likely PNAG was obtained by treating extracts with dispersin B or periodate, both of which destroy PNAG. Such treated materials had no reactivity with the mAb to PNAG in a slot-blot assay (Fig. 5). Finally, chemical analysis of the extracts indicated hexosamine was only detectable in the PNAG-positive extracts. Attempts to further purify PNAG to a degree sufficient for analysis by NMR were unsuccessful. This is consistent with almost all prior publications indicating that methods have not yet been developed to purify PNAG sufficiently for NMR analysis in the absence of hyper-expression of the biosynthetic proteins in organisms like S. aureus, E. coli, or A. baumannii (13, 14, 45, 46). In organisms such as S. epidermidis (8) and Vibrio parahaemolyticus (47), isolation of PNAG-related small molecular weight fragments has been achieved only by use of natural hyper-producers of biofilms as sources of the initial extracts.
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Although PNAG itself, or a structure containing a PNAG component, appears to be an EPS element involved in B. subtilis biofilm formation, it is likely that other EPS molecules are also needed for full pellicle formation. Studies of the eps locus indicate that deletion of numerous other genes also disrupts biofilm formation, and some of these appear to be classic EPS biosynthetic genes (17, 18, 48). The EpsM–O proteins are predicted to be an acetyltransferase (EpsM), an aminotransferase/sugar dehydratase (EpsN), and a GT with a GT-B fold (EpsO). The EpsE protein is involved in both motility and biofilm formation (21) acting as a clutch of the flagellar cellular motility apparatus to inhibit movement, while also having a predicted GT structure.

Recently, Elsholz et al. (52) reported that the B. subtilis EPS serves as a positive regulator of its own synthesis by binding to the extracellular portion of a receptor encoded by the epsA gene that interacts with a tyrosine kinase encoded by epsB and inhibits EpsB autophosphorylation. Dephosphorylated EpsB is associated with enhanced EPS production. In an epsH mutant unable to produce biofilm and, as shown here, PNAG, a His-tagged recombinant EpsB protein was highly phosphorylated due to lack of EPS production. We’ve found neither EpsA nor EpsB was needed for synthesis of immunoreactive PNAG in B. subtilis. This is consistent with the finding from Elsholz et al. (52) that PNAG-related material prepared using a modified method from Mack et al. (8) that yields small molecular weight polysaccharide intercellular adhesin had no effect on EpsB autophosphorylation. Given that the epsH mutant was not producing a factor binding to the EpsA extracellular domain, it appears that other EPS components either depend on EpsH for synthesis or PNAG is needed to facilitate the inhibition of EpsB autophosphorylation. However, the actual factors regulating B. subtilis EPS production via EpsA and EpsB are currently uncharacterized in regard to specific chemical properties.

One final aspect to consider is that the polysaccharide synthesized by the B. subtilis EpsH–K proteins may not be PNAG but a molecular entity with sufficient β-1–6-linked N-acetylglucosamine in it to make it immunoreactive with antibodies to PNAG but also containing other components. mAb F598 binding to PNAG requires a minimum of seven β-1–6-linked N-acetylglucosamine residues (36, 53), indicating a minimal PNAG-related constituent present in the material synthesized by the EpsH–K proteins. We do note that every microbial strain wherein we or others have identified a dispersin B and perio-
date-sensitive, hexosamine-containing antigen that reacts with antibodies to native PNAG, and wherein the reactive material has been isolated, turns out to be chemically verified PNAG (14, 45, 54–56). Furthermore, only 1–6-linked hexosamines, and no other possible amino-sugar linkages, are sensitive to periodate, and only β1–6-linked N-acetylglucosamine can be digested by dispersin B (22). Also, for every microbe for which genes encoding PNAG biosynthetic proteins have been identified and deleted, there is loss of antibody reactivity with material on the cell surface due to gene loss. Thus, we consider it highly unlikely that the material produced by the EpsH–K proteins is not PNAG, but at a minimum, it is at least a PNAG-containing molecular structure.

Overall, our results show PNAG, or a closely related entity, is a component of the *B. subtilis* biofilm matrix synthesized by the EpsH–K proteins. These proteins can also be expressed and are functional in Gram-negative organisms, including *E. coli*, *K. pneumoniae*, and *P. aeruginosa*. However, it is possible that the EpsH–K proteins are also required for producing other polysaccharide components of the biofilm matrix, inasmuch as PNAG does not appear to be the only carbohydrate entity in this organism’s biofilm (48). However, no carbohydrate component other than PNAG identified here has been fully characterized as being synthesized by proteins encoded by genes within the *B. subtilis eps* locus, leaving open the question as to the chemical composition of additional eps-dependent polymeric carbohydrates present in this structure. Analysis of EPS composition would undoubtedly advance the understanding of the *B. subtilis* biofilm formation process, but it should be noted that published methods used to isolate EPS (52) would contain very little native PNAG, as this molecule is poorly soluble at neutral pH, particularly after alcohol precipitation (14, 45). Thus, in the absence of an appropriate method, researchers have not been able to purify PNAG to obtain an appreciable yield for detailed chemical structural determination. We did find, however, that extracts from the WT *B. subtilis* cells and recombinant *E. coli* cells carrying the pUC18Tc-epsHJK plasmid contained immunoreactive dispersin B and the periodate-sensitive hexosamine-containing material present. Thus, the analysis of the products of the *B. subtilis epsHJK* locus described here are fully consistent with the production by WT *B. subtilis* of PNAG, which is likely a necessary component of this organism’s biofilm based on the genetic data showing the essentiality of epsH–K gene products for production of this structure (19).

**Author Contributions**—D. R. performed experiments, analyzed data, contributed to the study concept, and wrote the manuscript. C. C. B., Y. F. Z., S. P., M. K., D. B. K., D. J. L., P. L. H., and D. S. performed experiments, analyzed data, provided reagents, edited the manuscript, and contributed to the study concept. G. B. P. supervised the project, developed the study concept, analyzed data, and wrote the manuscript.

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