

The *pgaABCD* Locus of *Escherichia coli* Promotes the Synthesis of a Polysaccharide Adhesin Required for Biofilm Formation

Xin Wang,¹ James F. Preston III,² and Tony Romeo^{1*}

Department of Microbiology and Immunology, Emory University School of Medicine, Atlanta, Georgia 30322,¹ and
Department of Microbiology and Cell Science, University of Florida, Gainesville, Florida 32611²

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Production of a polysaccharide matrix is a hallmark of bacterial biofilms, but the composition of matrix polysaccharides and their functions are not widely understood. Previous studies of the regulation of *Escherichia coli* biofilm formation suggested the involvement of an unknown adhesin. We now establish that the *pgaABCD* (formerly *ycdSRQP*) locus affects biofilm development by promoting abiotic surface binding and intercellular adhesion. All of the *pga* genes are required for optimal biofilm formation under a variety of growth conditions. A *pga*-dependent cell-bound polysaccharide was isolated and determined by nuclear magnetic resonance analyses to consist of unbranched β -1,6-*N*-acetyl-D-glucosamine, a polymer previously unknown from the gram-negative bacteria but involved in adhesion by staphylococci. The *pga* genes are predicted to encode envelope proteins involved in synthesis, translocation, and possibly surface docking of this polysaccharide. As predicted, if poly- β -1,6-GlcNAc (PGA) mediates cohesion, metaperiodate caused biofilm dispersal and the release of intact cells, whereas treatment with protease or other lytic enzymes had no effect. The *pgaABCD* operon exhibits features of a horizontally transferred locus and is present in a variety of eubacteria. Therefore, we propose that PGA serves as an adhesin that stabilizes biofilms of *E. coli* and other bacteria.

In many natural and artificial habitats, bacteria form sessile communities known as biofilms (9). Biofilms represent a distinct physiological state, designed in part to provide a protected environment for survival under hostile conditions. They are composed of cells embedded within a glycocalyx-like matrix, and their complex structures have been likened to primitive multicellular organisms (9, 10). Biofilms play important roles in interactions of both nonpathogenic and pathogenic bacteria with eucaryotic hosts. Nonpathogenic biofilms in the mammalian gut and on the roots of plants provide barriers to invading pathogens (47, 50). Biofilms protect pathogens from attack by the immune system, complicate chronic infections that are difficult to eliminate with antibiotic therapy, and are involved in prostatitis, biliary tract infection, and urinary catheter cystitis caused by *Escherichia coli* (10, 18).

Biofilm development is a complex process (10, 17, 37). In general, it is initiated by cell attachment to a surface and formation of “microcolonies” on that surface. A variety of surface factors facilitate attachment and microcolony formation by *E. coli* (13, 48, 61). Differentiating microcolonies produce a matrix that encloses the biofilm and typically contains polysaccharides as its major components (59). Ultimately, planktonic cells are released that can complete the development cycle and colonize elsewhere.

Despite intensive interest in biofilm development, the functions of the extracellular polysaccharides (EPS) that form the matrix remain largely undefined. Colanic acid of *E. coli* affects biofilm architecture but not the adhesion of bacteria to surfaces or to themselves (14, 32). Cellulose is needed for biofilm

formation by certain morphotypes of *Enterobacteriaceae* (58, 69). Although some strains of *E. coli* synthesize cellulose and the biosynthetic genes are present in *E. coli* K-12, this strain does not synthesize cellulose under known conditions (70). The length of the O-antigenic side chain of lipopolysaccharide is inversely correlated with biofilm formation by *Salmonella enterica* (45) and *E. coli* (X. Wang and T. Romeo, unpublished data). Perhaps the most widely studied role of EPS in biofilm formation by a gram-negative bacterium is that of alginate, a uronic acid polymer responsible for the mucoid phenotype of *Pseudomonas aeruginosa*. Alginate is a virulence factor and affects biofilm architecture (see, for example, reference 29). Although it is often cited as a component important for biofilm formation, recent investigations have cast doubt on that idea (46, 67).

Staphylococcus epidermidis and *S. aureus* produce β -1,6-*N*-acetyl-D-glucosamine polymers (β -1,6-GlcNAc) that serve as adhesins and are required for biofilm formation. These polymers have been referred to as polysaccharide intercellular adhesin (PIA), PNAG (earlier PS/A), or SAE (28, 34, 39, 40, 42, 43). Although various staphylococcal β -1,6-GlcNAc fractions differ in size, degree of *N* acetylation, and substitution by phosphate, succinate, or other moieties, their synthesis depends upon the *icaABCD* locus, and the basis of the observed chemical differences in the isolated polysaccharides remains to be resolved (reviewed in reference 26).

Biofilm development is guided by several regulatory systems in *E. coli* (1, 8, 19, 32, 33, 49, 61). Perhaps the most dramatic effects are exhibited by the Csr (carbon storage regulatory) system. Csr is a global regulatory system that represses stationary phase processes (reviewed in reference 51), including glycogen synthesis and catabolism (52, 68), gluconeogenesis (54), and biofilm formation (32, 60, 64). Conversely, it activates glycolysis (54), motility (63), acetate metabolism (62), and bio-

* Corresponding author. Mailing address: Department of Microbiology and Immunology, Emory University School of Medicine, 3105 Rollins Research Center, 1510 Clifton Rd. N.E., Atlanta, GA 30322. Phone: (404) 727-3734. Fax: (404) 727-3659. E-mail: romeo@microbio.emory.edu.

film dispersal (32). Its key component, CsrA, is an RNA-binding protein that binds to the untranslated leader sequences of target mRNAs and alters their translation and stability (5, 38, 63). Repression of biofilm formation by CsrA involves the synthesis and catabolism of intracellular glycogen (32). This finding highlights the importance of central carbon metabolism and its regulation in biofilm development. The effect of CsrA on biofilm formation did not require several known surface factors, suggesting that unknown adhesins or other factors are required (32).

We identify here a genetic locus of *E. coli* that promotes surface binding, intercellular adhesion, and biofilm formation. Biochemical and genetic experiments show that this involves the production of a β -1,6-GlcNAc polysaccharide. Loci that are homologous to *pga* are present in variety of bacterial pathogens, suggesting that they may synthesize related polysaccharide adhesins that contribute to biofilm-mediated diseases.

MATERIALS AND METHODS

Bacterial strains, phage, plasmids, and growth conditions. All *E. coli* strains, phage, and plasmids used in the present study are listed in Table 1. Unless otherwise indicated, bacteria were routinely grown at 37°C in Luria-Bertani (LB) medium (44) containing 0.2% glucose. Biofilms were grown at 26°C in LB or colonization factor antigen (CFA) medium (1% Casamino Acids, 0.15% yeast extract, 0.005% MgSO₄, and 0.0005% MnCl₂; pH 7.4 [32]). Kornberg medium (1.1% K₂HPO₄, 0.85% KH₂PO₄, 0.6% yeast extract containing 0.5% glucose for liquid or 1% for solid medium) was used in the selection of transposon mutants. Semisolid CFA or tryptone medium (pH 7.4) containing 1% tryptone, 0.5% NaCl, and 0.35% agar was used to test motility (63). Minimal media (M9 and M63) were prepared as described by Miller (44). Media were supplemented with antibiotics, as needed, at the following final concentrations: ampicillin, 100 µg/ml; chloramphenicol, 25 µg/ml; kanamycin, 100 µg/ml; and tetracycline, 10 µg/ml.

Isolation of biofilm mutants. DJ25 strain (MG1655 Δ *fimB-H* Δ *motB* *csrA*) was infected with λ NK1324 containing mini-Tn10*cam* at a multiplicity of infection of 0.2, as described previously (36). Biofilm mutants were isolated as described previously (48), with minor modifications. Briefly, chloramphenicol-resistant colonies were selected on Kornberg agar plates containing 2.5 mM sodium pyrophosphate. Individual colonies (11,000) were inoculated into 96-well microtiter plates containing CFA medium and incubated at 26°C for 24 h. The cultures were then diluted 1:100 and subcultured into fresh microtiter plates. After 24 h of incubation at 26°C, cell growth and biofilms were measured as described below. Mutants with altered biofilm production and normal growth were isolated. To assess the genetic linkage between altered biofilm production and the transposon insertion, the mutations were transduced by P1vir into the wild-type *E. coli* K-12 strain MG1655 and/or its *csrA* mutant (TRMG1655). Transposon mutations that were genetically linked to the altered biofilm phenotypes (153 in all) were further analyzed by arbitrarily primed PCR and DNA sequencing (see below).

Quantitative biofilm assay. Biofilms were assayed by crystal violet staining, as described previously (32). Overnight cultures were diluted 1:100 into fresh medium and grown in 96-well microtiter plates. Bacterial growth was determined by measuring the absorbance at 630 nm. At least six replicates were conducted for each sample, and each experiment was performed at least twice. The results were calculated as averages and standard errors of two or more experiments. Tukey multigroup analysis (StatView; SAS Institute, Inc., Cary, N.C.) was used for statistical analysis of data.

PCR amplification of insertion sites and DNA sequence analyses. Chromosomal DNA flanking the transposon insertions was amplified by using arbitrarily primed PCR (25). Sequences of primers used in the first round (ARB1 and OUT1-L) and the second round (ARB2 and PRIMER1-L) of PCRs, as well as those of all other oligonucleotide primers, will be provided upon request. Arbitrarily primed PCR conditions were as described by Gibson and Silhavy (25). PCR products were purified by QIAquick gel extraction kit and sequenced by using PRIMER1-L. All DNA sequence analyses were conducted at the DNA Sequencing Facility at the University of Arizona. Disrupted genes were identified by basic local alignment search tool (BLAST) analysis (2) at the National Center for Biotechnology Information (NCBI) website.

TABLE 1. Strains, plasmids, and bacteriophage used in this study

Strain, plasmid, or phage ^a	Description or genotype	Source or reference
<i>E. coli</i> K-12 strains		
MG1655	F [−] λ [−]	Michael Cashel
TRMG1655	MG1655 <i>csrA::kan</i>	52
CF7789	MG1655 Δ <i>lacI-Z</i> (MluI)	Michael Cashel
TRCF7789	CF7789 <i>csrA::kan</i>	60
DJ4	TRMG1655 <i>cpsE::Tn10</i>	32
DJ6	TRMG1655 Δ <i>fimB-H</i>	32
DJ2	TRMG1655 <i>csgA2::Tn105</i>	32
DJ24	TRMG1655 Δ <i>motB</i> <i>uvrC-279::Tn10</i>	32
DJ25	DJ24 Δ <i>fimB-H</i>	32
TRFMXWA*	DJ25 <i>pgaA672::cam</i>	This study
TRFMXWB*	DJ25 <i>pgaB510::cam</i>	This study
TRFMXWC*	DJ25 <i>pgaC880::cam</i>	This study
TRFMXWD*	DJ25 <i>pgaD146::cam</i>	This study
XWA672	MG1655 <i>pgaA672::cam</i>	This study
XWB510	MG1655 <i>pgaB510::cam</i>	This study
XWC880	MG1655 <i>pgaC880::cam</i>	This study
XWD146	MG1655 <i>pgaD146::cam</i>	This study
TRXWA672	TRMG1655 <i>pgaA672::cam</i>	This study
TRXWB510	TRMG1655 <i>pgaB510::cam</i>	This study
TRXWC880	TRMG1655 <i>pgaC880::cam</i>	This study
TRXWD146	TRMG1655 <i>pgaD146::cam</i>	This study
TRXWEC	DJ4 <i>pgaC880::cam</i>	This study
XWMGΔA	MG1655 Δ <i>pgaA</i>	This study
XWMGΔB	MG1655 Δ <i>pgaB</i>	This study
XWMGΔC	MG1655 Δ <i>pgaC</i>	This study
XWMGΔABCD	MG1655 Δ <i>pgaABCD</i>	This study
TRXWMGΔA	TRMG1655 Δ <i>pgaA</i>	This study
TRXWMGΔB	TRMG1655 Δ <i>pgaB</i>	This study
TRXWMGΔC	TRMG1655 Δ <i>pgaC</i>	This study
TRXWMGΔABCD	TRMG1655 Δ <i>pgaABCD</i>	This study
Plasmids		
pCR-XL-TOPO	Cloning vector	Invitrogen
pCRPGA37	<i>E. coli</i> <i>pga</i> locus in pCR-XL-TOPO	This study
pCR2.1-TOPO	Cloning vector	Invitrogen
pUC19	Cloning vector	55
pPGA372	<i>pgaABCD</i> in pUC19	This study
pCRS5	<i>pgaA</i> gene in pCR2.1-TOPO	This study
pCRR12	<i>pgaB</i> gene in pCR2.1-TOPO	This study
pCRP93	<i>pgaD</i> gene in pCR2.1-TOPO	This study
pETQwt	<i>pgaC</i> gene in pET-Blue	This study
pET-Blue	Expression vector	Novagen
pKD46	For arabinose induction of λ Red system	16
pKD3	Contains the <i>cat</i> gene	16
pCP20	For FLP-recombinase production	16
Bacteriophage		
P1vir	Strictly lytic P1	Carol Gross
λ NK1324	Contains mini-Tn10 <i>cam</i> transposon	36

^a Original transposon insertion mutants, as displayed in Fig. 1A, used in the present study are indicated by an asterisk.

Cloning of the *pgaABCD* locus and individual *pga* genes. Molecular cloning of the *pga* genes involved PCR amplification of chromosomal or plasmid DNA by using Elongase enzyme (Invitrogen) under the reaction conditions described by the manufacturer, with annealing temperatures and extension times that were based on primer melting temperature (*T*_m) and final product size, respectively. PCR fragments of 3,070, 2,167, 1,325, and 596 bp, corresponding to *pgaA*, *pgaB*, *pgaC*, and *pgaD*, respectively, were prepared. The *pgaA*, *pgaB*, and *pgaD* genes were cloned into the vector pCR2.1-TOPO, resulting in pCRS5, pCRR12, and pCRP93, respectively. The *pgaC* gene was cloned into pET-Blue (Novagen) to produce pETQwt. Clones of individual *pga* genes were sequenced and found to be free of mutations. To clone the intact *pga* locus with all noncoding flanking DNA, a 6,933-bp fragment was amplified by PCR with Elongase enzyme (Invitrogen) from chromosomal DNA of MG1655. The 6,933-bp fragment was purified by using the QIAquick gel extraction kit (Qiagen) and cloned into vector pCR-XL-TOPO by using DH5 α as the host for transformation. The plasmid clone pCRPGA37 increased biofilm ~6-fold when expressed in DH5 α . This

clone was subsequently treated with HindIII and XbaI, and the insert DNA was subcloned into pUC19 to yield pPGA372. The genomic DNA of this clone was completely sequenced and found to have a silent mutation in *pgaB* and two missense mutations in *pgaA* (Q130R and N195D). However, it fully complemented biofilm defects caused by mutations in each of the four individual *pga* genes, as well as a deletion of the entire *pgaABCD* operon.

Nonpolar deletion of *pgaA*, *pgaB*, and *pgaC* and deletion of the *pgaABCD* locus. The chromosomal *pgaABCD* locus and *pgaA*, *pgaB*, and *pgaC* genes were deleted by targeted gene substitutions (16). The *cat* gene flanked by FLP recognition target (FRT) was amplified from pKD3 by PCR and introduced by electroporation into arabinose-treated MG1655(pKD46). Transformants were selected on chloramphenicol, and their insertion sites were confirmed by PCR. The *camR* marker of each strain was subsequently moved into TRMG1655 by P1vir transduction. The *cat* genes of these strains were eliminated as described previously (16). The resulting deletions were confirmed by PCR analyses. The single gene deletions were determined to be nonpolar by complementation of their biofilm phenotypes with plasmids containing the corresponding genes, pCRS5 (*pgaA*), pCRR12 (*pgaB*), and pETQwt (*pgaC*). pPGA372 complemented the *pgaABCD* deletion. The *pgaD146* transposon insertion was complemented by pCRP93 (*pgaD*).

Detection of *pga*-dependent polysaccharide. The *pgaC880* mutant (TRXWEC) containing pPGA372 or pUC19 was grown for 24 h at 37°C with shaking at 250 rpm in CFA medium. Cells were harvested from 2 liters of each culture and resuspended in 20 ml of 50 mM Tris-Cl buffer (pH 8.0). Lysozyme (100 mg) and 4 ml of 0.1 M EDTA were added, and each suspension was incubated at room temperature for 30 min. Then, α -amylase (100 mg), DNase I (5 mg), and RNase A (20 mg) were added, and the suspension was incubated at room temperature for 1 h and then at 37°C for 2 h. Polysaccharide was separated from proteins, and cell debris by phenol extraction (65). The aqueous phase was extracted with an equal volume of chloroform, concentrated by ultrafiltration (Amicon YM-10 membrane; 10,000 molecular weight cutoff) and fractionated by fast protein liquid chromatography (FPLC) on Sephacryl S-200 (HiPrep 16/60; Amersham Pharmacia Biotech). The column was equilibrated with 0.1 M phosphate-buffered saline (PBS; pH 7.4) and eluted with the same buffer. Fractions (1.6 ml) were collected and assayed for hexosamine after acid hydrolysis with 3-methyl-2-benzothiazolone hydrazone hydrochloride (MBTH) (57). Neutral-sugar content was determined by using the phenol-sulfuric acid assay (20). *N*-Acetyl-D-glucosamine and D-glucose, respectively, were used as standards in these two assays. Proteins and nucleic acids were detected by UV absorbance and ethidium bromide staining after agarose gel electrophoresis, respectively. Spent medium was also collected from these two strains and treated with ethanol to precipitate polysaccharides, and the resulting precipitates were dissolved in 0.1 M PBS. Hexosamine content was determined by using the MBTH assay.

Nuclear magnetic resonance (NMR) analyses of *pga*-dependent polysaccharide. A cell lysate from 24 liters of cell culture (135 g of cells) was prepared by using a *pga* wild-type strain (DJ4) carrying pPGA372. The polysaccharide was purified as described above, except that fractionation was performed on a semi-preparative Sephacryl S-300 column (HiPrep 26/60; Amersham Pharmacia Biotech), and 5-ml fractions were collected. The hexosamine-containing high-molecular-weight fractions from three columns were combined, precipitated with 67% ethanol at 4°C, and collected by centrifugation. The resulting white precipitate (0.7 g containing ~35-mg GlcNAc equivalents) was suspended in 10 ml of D₂O (99.9%; Aldrich Chemical) and subjected to filtration and dialysis by using a Centriprep YM-10 (Amicon/Millipore). This process was repeated several times until the conductivity reached a level less than that of 0.02 M NaCl. The retentate was then lyophilized to provide 52 mg of a white residue. A portion (28 mg) was suspended in 2.0 ml of 5.0 M DCl, prepared from 35% DCl in D₂O (99% D; Aldrich Chemical), resulting in a faintly turbid suspension after dissolution of most of the residue. The suspension was clarified by brief centrifugation (microfuge) and neutralized to pH 6 to 7 with 5.0 M NaOH dissolved in D₂O. The faintly opalescent solution was further triturated with D₂O by using the YM-10 Centriprep. The retentate solution, estimated to contain 4 mg of polymer/ml and 99.6% D₂O, was analyzed by ¹H- and ¹³C-NMR. NMR spectra were obtained by using a Bruker Avance 500 Console, a Magnex 11.75 T/54-mm magnet, and a 5-mm BBO probe. Acquisitions were obtained at 300°K without spinning. ¹H spectra were obtained at 500 MHz; ¹³C spectra were obtained at 125 MHz. ¹H shift assignments were established by COZY-45 and corroborated with ¹³C assignments by 500/125-MHz ¹H/¹³C-HMQC spectral analyses.

Treatment of biofilms with H₂O₂ and hydrolytic enzymes. Biofilms were grown for 24 or 48 h in 96-well microtiter plates containing CFA or LB media. The planktonic cells were aspirated, and the biofilms were incubated at 4°C for 23 h after adding 200 μ l of the following agents, respectively: H₂O₂, 40 mM NaO₄ (metaperiodate [pH 5.0]), 40 mM NaO₄ plus 40 mM glucose or lactose

(quenched metaperiodate), 40 mM glucose or 40 mM lactose, 0.1 M PBS, 40 mM EDTA, and 40 mM NaCl. Dispersed cells were examined by microscopy, and biofilms were quantified with crystal violet staining. The quenched periodate was prepared by incubation with 40 mM glucose or lactose at 4°C for 23 h before it was added to preformed biofilms. For enzymatic treatment of biofilms, DNase I and RNase A were dissolved in 10 mM Tris-Cl (pH 8.0) containing 2 mM MgCl₂ at final concentrations of 200 and 50 μ g/ml, respectively. Proteinase K was dissolved in 100 mM Tris-Cl to a final concentration of 1 mg/ml (41). These enzyme solutions were added to biofilms (200 μ l per well), and the reaction mixtures were incubated at 37°C for 3 or 6 h. For treatment with *Trichoderma reesei* cellulase, 200 μ l of a mixture containing 70 U of enzyme/ml in 0.05 M sodium citrate buffer (pH 5.0) was added to the biofilm, and the reaction was incubated at 45°C for 72 h as previously described (58). Remaining biofilm was assayed by crystal violet staining.

Microscopy. Sterile borosilicate coverslips were aseptically placed into 15-cm petri dishes containing 50 ml of a freshly inoculated (1:100) culture. The petri dishes were incubated at 26°C, and coverslips were removed at various times and rinsed gently with water. Adherent cells were viewed by transmitted light with an Olympus 1X71 microscope (\times 40 objective lens with a \times 1.6 selector). The images were captured by using a charge-coupled device camera (COHU-4915) and Image Pro-Plus 4.1 software (MediaCybernetics) and then stored as separate digital files for subsequent analysis.

Molecular biology, genetics, and bioinformatics. Standard procedures were used for plasmid isolation, restriction digests, ligations, transformation, and transduction of antibiotic markers (44, 55). Predictions of protein domains and envelope localization are available from the Entrez Protein database at the NCBI website.

RESULTS

Isolation of transposon insertions in *pgaABCD*. Random transposon mutagenesis to identify novel genes required for biofilm development was performed with a Δ *motB* Δ *fimB-H* *csrA* mutant of MG1655 (DJ25) as the parent strain. This strain was designed to avoid the isolation of predominant mutants that affect type I pili and motility (48). Because the combined Δ *motB* Δ *fimB-H* deletions severely disrupt biofilm development, a *csrA* mutation was introduced to increase biofilm formation and permit us to isolate biofilm-deficient mutants (Materials and Methods). Altogether, 17 such mutations were isolated in a predicted operon, *ycdSRQP* (6), renamed here as *pgaABCD* (Fig. 1A). These insertions were genetically linked to the biofilm-deficient phenotype and decreased biofilm ~10-fold when transduced back into the parent strain (data not shown).

The intact *pgaABCD* locus is required for optimal biofilm formation. Four insertions—*pgaA672*, *pgaB510*, *pgaC880*, and *pgaD146*—were tested and found to significantly decrease biofilm formation in both the MG1655 strain background (~3-fold) and its isogenic *csrA* mutant (>10-fold) (Fig. 1B and C; compare bar 1 with bars 2 and 3; other data not shown). A plasmid containing the complete *pga* locus, pPGA372, complemented the biofilm defects caused by these mutations (Fig. 1B and C, compare bars 4 and 6 to bars 5 and 7, respectively; data not shown). In *csrA* wild-type strains, this plasmid complemented the *pga* defects and increased biofilm formation to a level ~3-fold greater than that of the wild-type parent strain, MG1655 (Fig. 1B; compare bar 1 with bars 5 and 7). Similar effects of *pga* mutations were observed in all growth media that have been tested, including LB medium containing 0.2% glucose, CFA with or without 0.2% glucose, and minimal medium (M9 and M63) containing 0.2% glucose (data not shown). We also noticed that the parent strain (DJ25) and other *csrA* mutants formed distinct pellicles at the air-liquid interface in shaking flasks or borosilicate test tubes, whereas *pga* mutants

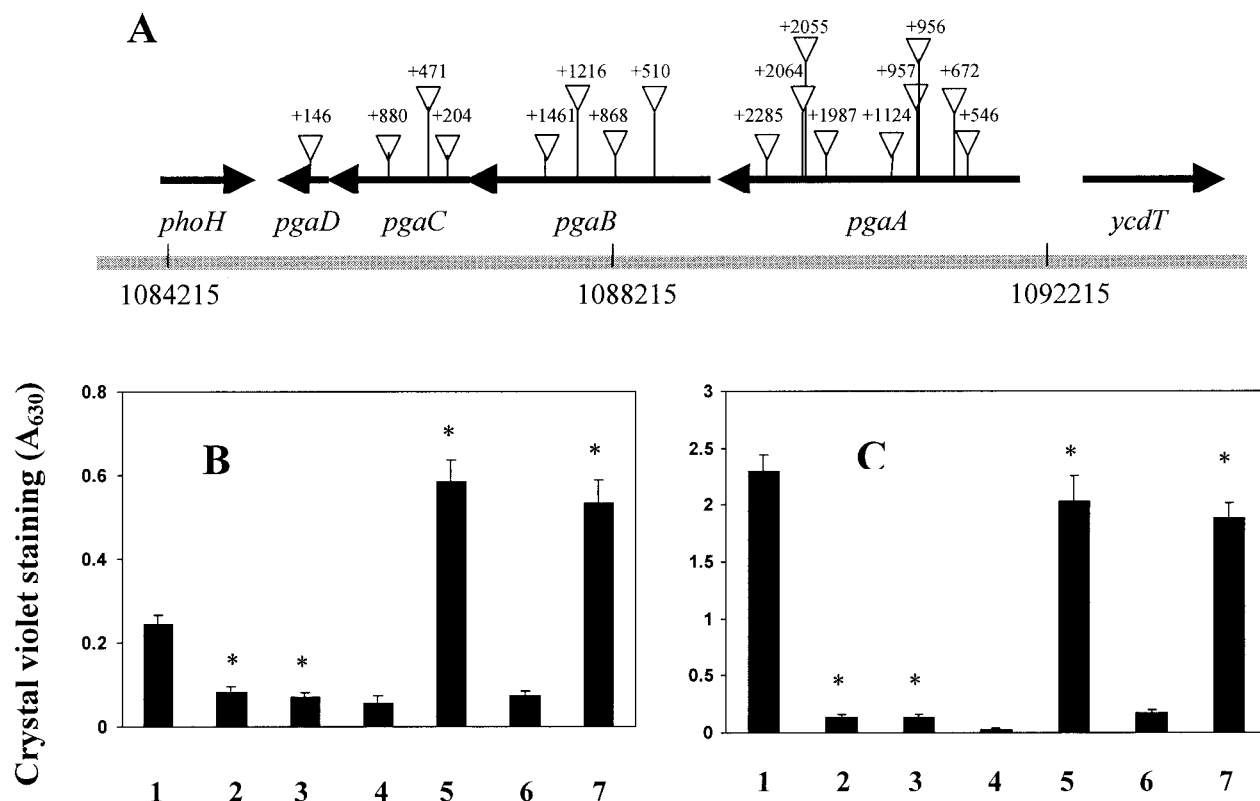


FIG. 1. Transposon insertions in the *pga* locus of *E. coli* K-12 and their effects on biofilm formation. (A) Insertions are numbered relative to the first nucleotide (+1) of the coding region of the corresponding gene. The coordinates of this locus on the *E. coli* K-12 genome (6) are shown. (B) Biofilm formation in polystyrene microtiter plates. Isogenic strains are represented by bars numbered as follows: 1, MG1655; 2, XWC880 (*pgaC880::cam*); 3, XWA672 (*pgaA672::cam*); 4, XWC880(pUC19); 5, XWC880(pPGA372)(*pgaABCD*); 6, XWA672(pUC19); and 7, XWA672 (pPGA372). (C) Strains were as in panel B, except that they were *csrA* mutants. All biofilms were grown in LB medium at 26°C for 24 h. The asterisks denote significant differences relative to the corresponding parent strain ($P < 0.001$ [Tukey multigroup analysis]).

of these strains did not (data not shown). The *pga* mutations did not affect the growth curves of the strains (data not shown), suggesting that they block one or more steps specifically needed for biofilm development.

To investigate the roles of individual *pga* genes in biofilm formation, we constructed nonpolar *pgaA*, *pgaB*, and *pgaC* chromosomal deletions, as well as a deletion of the entire *pgaABCD* locus, in both MG1655 and its *csrA* mutant. These deletions and the *pgaD146* insertion decreased biofilm formation in both strain backgrounds (Fig. 2A and B). Furthermore, they caused almost complete loss of the ability to adhere to borosilicate glass coverslips (Fig. 2C and data not shown). In similar experiments, the single-gene mutants were inoculated in all pairwise combinations, and biofilm formation was tested. No strain combination was able to restore biofilm development (data not shown), revealing that these mutations are incapable of intercellular complementation. Thus, all four *pga* genes must be functional within the cell to promote surface attachment and biofilm formation.

Predicted functions of *pga* genes. A search for conserved protein domains (3) predicted that PgaC is a 441-amino-acid *N*-glycosyltransferase belonging to family 2 (GT-2; *afmb.cnrs-mrs.fr/CAZY*) (7). It is also predicted to be an inner membrane protein with two N-terminal and three C-terminal transmembrane domains. IcaA from *S. epidermidis* and NodC from *Rhizobium loti* are processive glycosyltransferases, which contain

an N-terminal catalytic domain and a C-terminal domain that is needed both for catalysis and interaction with nascent polysaccharide chains (24, 56). Overall, IcaA and PgaC share 35% amino acid identity and 57% similarity. Five amino acids that appear to be essential for the catalytic activities of processive glycosyltransferases are found in PgaC (Asp¹⁶³, Asp²⁵⁶, Gln²⁹², Arg²⁹⁵, and Trp²⁹⁶) (24). These analyses suggested that PgaC is a polysaccharide polymerase that uses UDP-GlcNAc as a substrate.

PgaB is a predicted 672-amino-acid lipoprotein with a 20-amino-acid signal sequence and is homologous to the second gene product of the staphylococcal *ica* locus, IcaB. Both PgaB and IcaB contain putative polysaccharide *N*-deacetylase domains and belong to carbohydrate esterase family 4 (CE4) (11), suggesting that they may modify polysaccharides during synthesis.

PgaA is predicted to be a large (807-amino-acid) outer membrane protein, suggesting that it might mediate translocation and/or docking of PGA to the cell surface. PgaA has no functional homologues, including in the staphylococci, which lack outer membranes.

PgaD is predicted to be a small (137-amino-acid) inner membrane protein with two N-terminal membrane-spanning domains. IcaD of staphylococci is also a small cytoplasmic membrane protein, which enhances PIA synthesis by IcaA

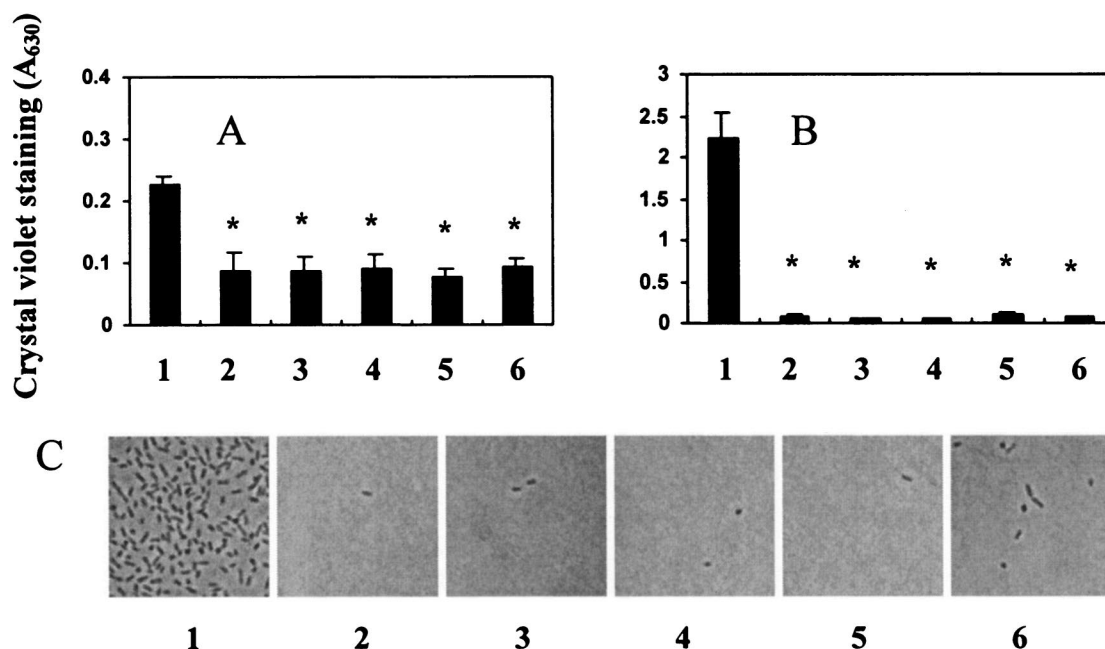


FIG. 2. Effects of nonpolar *pga* gene disruptions on crystal violet binding of biofilms grown in polystyrene microtiter wells (A and B) and adherence of cells to borosilicate coverslips (C). Panels A and C depict results in the MG1655 strain background. Strains are represented by bars in panels A and B as follows: 1, MG1655; 2, XWMGΔABCD (Δ*pgaABCD*); 3, XWMGΔA (Δ*pgaA*); 4, XWMGΔB (Δ*pgaB*); 5, XWMGΔC (Δ*pgaC*); and 6, XWD146 (*pgaD146::cam*). (B) Strain identities were the same as in panel A, except that the strains were *csrA* mutants. Cultures were grown in LB medium at 26°C for 24 h. Asterisks denote significant differences relative to the corresponding parental strain ($P < 0.001$ [Tukey multigroup analysis]).

(24). Although PgaD and IcaD are not related in sequence, perhaps they may nevertheless function similarly.

Detection of a *pga*-dependent hexosamine-rich polysaccharide. To test the hypothesis that the *pgaABCD* locus is required for the synthesis of a novel *E. coli* polysaccharide, two isogenic strains were constructed. One strain, TRXWEC (MG1655 *csrA cpsE pgaC880*) was defective for the *pga* locus in the chromosome and contained the plasmid vector pUC19, whereas the second strain contained a multicopy plasmid clone of the *pgaABCD* locus, pPGA372. The *csrA* mutation was introduced with the intention of enhancing central carbon flux into synthesis of the polysaccharide. Colanic acid production (*cpsE*) was eliminated to avoid contamination of the extracts with this polymer. The *cpsE* mutation does not affect the quantity of biofilm formed by these strains (32; data not shown). Cell extracts were prepared, fractionated by FPLC on Sephacryl S-200, and fractions were assayed for hexosamine, neutral sugars, proteins, and nucleic acids (Materials and Methods). The extract from the *pga*-overexpressing strain contained material rich in hexosamine, which eluted in the void volume of the column, and which was absent from extracts of the *pga*-defective strain (Fig. 3A and B, respectively). The separation range of Sephacryl S-200 for dextrans (1,000 to 80,000 Da) suggested that the hexosamine component is at least 80,000 Da. Chromatography on Sephacryl S-300 suggested a size of $\geq 400,000$ Da (data not shown). The void fraction contained $\leq 10\%$ neutral sugar with respect to hexosamine (Fig. 3A), and proteins and nucleic acids were not detectable (data not shown). The hexosamine-containing polymer was not detected in the spent medium from either strain (data not shown). These analyses revealed that *E. coli* synthesizes a cell-bound, hexosamine-rich

polysaccharide whose production depends upon the *pgaABCD* locus.

NMR analysis of the purified polysaccharide. To determine the chemistry of the hexosamine-rich polysaccharide, ~ 35 -mg GlcNAc equivalents were prepared and then analyzed by NMR spectroscopy (see Materials and Methods). The ^1H -NMR spectrum (Fig. 4, x axis) has three defined signals— $d = 4.56$ ppm, $d = 3.45$ ppm, and $d = 2.08$ ppm—that correspond to H-1, H-4, and *N*-acetyl protons, respectively, of β -1,6-linked GlcNAc residues, as originally reported for PIA of *S. epidermidis* (39). COZY spectra established that each of these signals represents ^1H attached to a single carbon atom. The integration of these signals results in a 1.01:1.05:3.00 ratio that is consistent with these assignments and indicates that unacetylated glucosamine residues account for $\leq 3\%$ of the residues of the polymer. A barely detectable broad signal at 2.875 ppm that could not be further defined by COZY analysis may be that of ^1H of free amino groups of the unacetylated residues. Other signals have shift assignments that are essentially identical to those reported (39) and which are supported by COZY analysis. The spectrum is nearly identical to that of the polysaccharide adhesin (PS/A) from *Staphylococcus aureus* (42). The small signal at 2.17 ppm might represent ^1H of the methylene carbons of succinyl groups, suggestive of a small extent of succinylation.

The ^{13}C spectrum (Fig. 4, y axis) provided further definition to the structure, with chemical shifts of 102.5, 75.4, 74.8, 71.5, 68.5, 56.5, and 23 ppm corresponding to C-1, C-5, C-3, C-4, C-6, C-2, and CH_3 , respectively, values close to those reported for PIA (39). The $^1\text{H}/^{13}\text{C}$ -HMQC spectrum defined the exact relationships of ^1H signals to the carbon atoms to which they

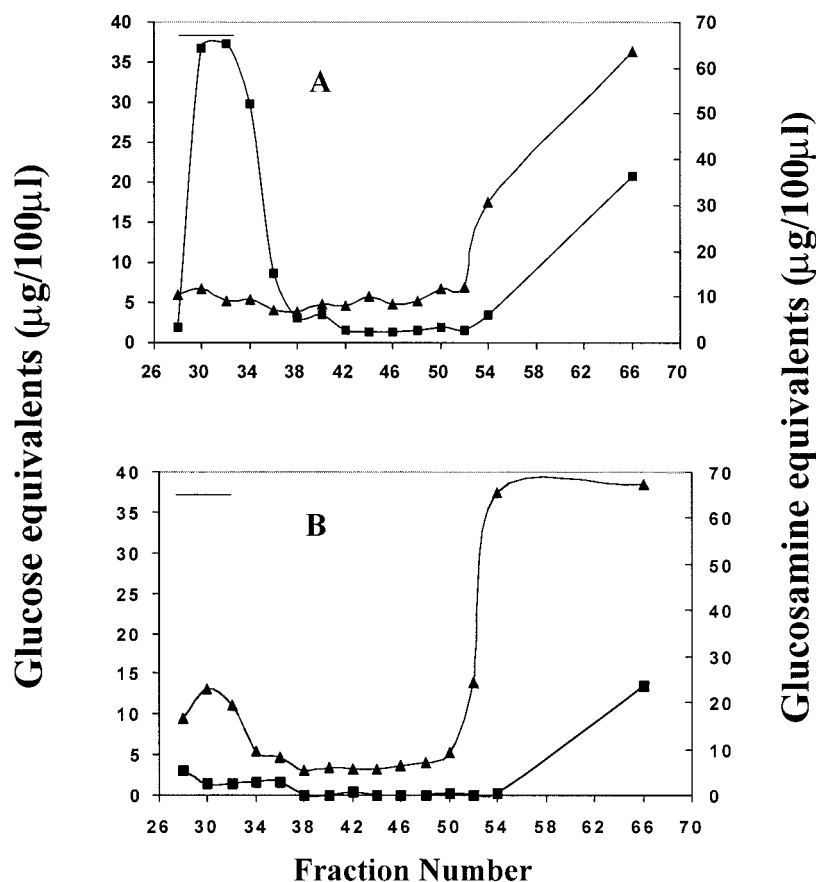


FIG. 3. Fractionation of polysaccharide extracts by gel filtration FPLC. Extracts from strain TRXWEC (MG1655 *csrA cpsE pgaC880*) containing either pPGA372 (*pgaABCD*) (A) or pUC19 (B) were fractionated by using a Sephacryl S-200 (HiPrep 16/60) column. Fractions (1.6 ml) were analyzed for neutral-sugar (\blacktriangle) and, after hydrolysis, for hexosamine (\blacksquare). The column void volume, as determined with 2-MDa blue dextran, is indicated by a horizontal line.

are attached. This analysis further confirms the assignments made for β -1,6-GlcNAc polymers of *S. aureus* (42) and *S. epidermidis* (39). These data establish that the *pga*-dependent polysaccharide is a linear polymer of β -1,6-GlcNAc residues.

Time course of surface attachment by strains defective for PGA versus other components. During biofilm development, initial attachment and subsequent microcolony formation utilize a variety of surface structures. To further investigate the role of PGA in biofilm development, isogenic strains that were defective for factors that promote biofilm formation, type I pili, motility, and curli were compared to a *pgaC880::cam* mutant for binding to microscope coverslips and biofilm formation on a polystyrene surface (Fig. 5). The parent strain (TRMG1655) began to form a dispersed monolayer on the glass coverslips by 4 h. Over the next several hours, a progressively more dense layer of cells was formed. By 8 h, multiple layers of cells were attached, which produce biofilm with a characteristic three-dimensional structure (32). The extent of biofilm formed by the mutant strains was closely correlated with the capacity to attach to coverslips. The *pgaC* mutant exhibited the most severe defect; few cells attached to coverslips during the entire 48 h of incubation. Mutations in motility (Δ *motB*), type I pili, and curli caused defects of decreasing severity, as previously observed (32). Although the biofilm phenotypes of the *pgaC*

and Δ *motB* mutants were somewhat similar, the *pgaC* mutation did not affect the motility of these strains (data not shown). The addition of purified PGA (8 or 38 μ g/ml) to the growth medium of a Δ *pgaC* nonpolar mutant (TRXWMG Δ C) at 0, 16, or 20 h failed to restore biofilm development in this strain (data not shown). This suggested that PGA must be synthesized in situ to promote biofilm formation. These experiments revealed that disruption of the *pga* locus does not simply delay surface attachment and biofilm formation but causes severe, persistent defects in these processes.

HIO₄ treatment releases biofilm. If PGA itself serves as an adhesin in *E. coli*, then its cleavage should disrupt a preformed biofilm. Although no enzyme is known to degrade PIA-like polysaccharides, metaperiodate (HIO₄) is able to do so, by oxidizing the carbons (3 and 4) bearing vicinal hydroxyl groups and cleaving the C-C bonds (39, 42). Treatment of 24-h biofilms of TRMG1655 (*csrA::kanR*) at 4°C with metaperiodate (see Materials and Methods) led to near-complete release (>90%) of the biofilm, whereas similar incubation with 0.1 M PBS (pH 7.4), 40 mM EDTA (pH 8.0), or 40 mM NaCl had minimal or no effect (Table 2). To determine whether this requires metaperiodate to serve as an oxidant, i.e., to cleave C-C bonds, it was quenched by preincubation with glucose. In this case, metaperiodate-mediated biofilm release was inhib-

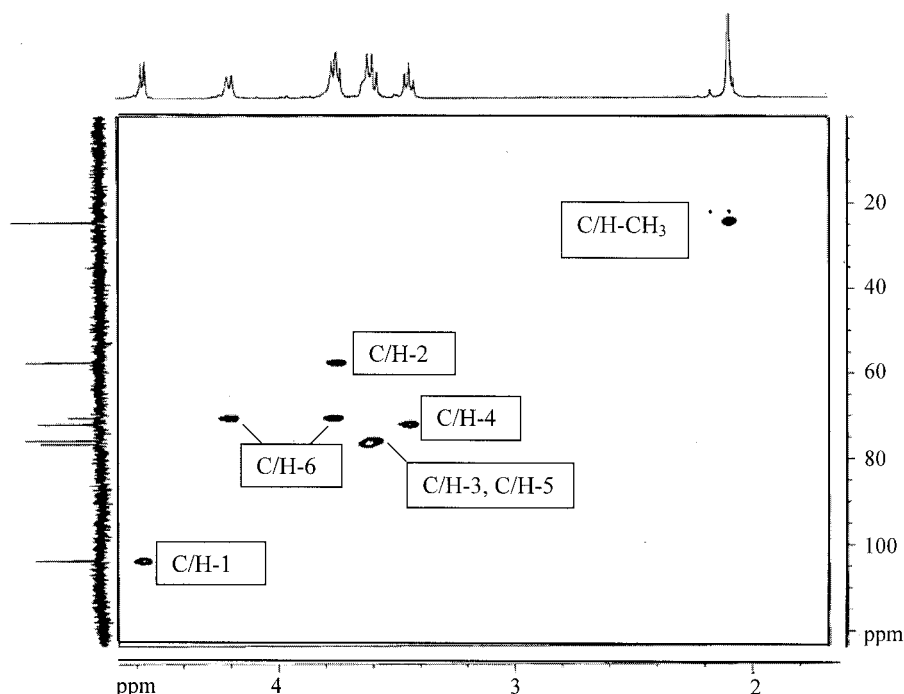


FIG. 4. 500/125 MHz $^1\text{H}/^{13}\text{C}$ -HMQC spectrum of *E. coli* *pga*-dependent polysaccharide with projected ^1H (1.55 to 4.85 ppm) and ^{13}C (0 to 124) spectra.

ited (Table 2). Treatment of biofilm at 4°C with glucose alone had no effect (data not shown). Essentially the same results were observed when 48-h biofilms were examined (data not shown). Biofilm of a strain that was defective for colanic acid synthesis was released similarly by metaperiodate treatment (Table 2), revealing that colanic acid depolymerization does not account for the observed detachment. Similar results were obtained with an isogenic strain that cannot metabolize lactose and by quenching with lactose instead of glucose (Table 2). This control experiment confirmed that inhibition of biofilm release was not due to sugar metabolism. During these studies, we observed that metaperiodate-mediated dispersal of biofilm produced turbid suspensions comprised of intact cells (data not shown). This revealed, as expected, that metaperiodate does not release biofilm by causing cell lysis. Finally, an experiment was performed in which biofilm was grown on borosilicate test tubes and then removed by gentle scraping with a pipette tip. Under microscopic observation, the resulting cell aggregates were dissociated with metaperiodate treatment but not by treatment with the other agents that are shown in Table 2 (data not shown). These experiments suggest that polysaccharide(s) stabilizes the intercellular structure of biofilm.

Macromolecules that have been reported to stabilize biofilm structure include cellulose, nucleic acids, and proteins (12, 48, 58, 59, 66). However, treatment of *E. coli* biofilms that were grown as described above with DNase I, RNase A, proteinase K, or cellulase (see Materials and Methods) failed to disrupt biofilm or cause the release of cells (data not shown).

Phylogenetic distribution of *pga* homologues. BLAST analysis (2) at the NCBI website with the *pga* gene products as query sequences revealed homologous loci in eubacteria but not in archaea or eukaryotes. Species with complete *pgaABCD*

loci include *E. coli* O157:H7 and uropathogenic *E. coli* strain CFT073, *Yersinia pestis*, *Y. enterocolitica*, *Xanthomonas axonopodis*, and *Pseudomonas fluorescens*. Species with a locus containing *pgaABC* but lacking an apparent *pgaD* homologue include *Actinobacillus actinomycetemcomitans*, *A. pleuropneumoniae*, *Ralstonia solanacearum* megaplasmid, *Bordetella pertussis*, *B. paraptussis* and *B. bronchiseptica*. A *pga* locus was not apparent in some closely related species, e.g., *Salmonella* and *Shigella* species, *P. aeruginosa*, or *Vibrio cholerae*. The gram-positive species *Lactococcus lactis* contains colocalized homologues of the *E. coli* *pgaC* and *pgaB* genes (staphylococcal *icaA* and *icaB*). This locus of *L. lactis* also contains a homologue of staphylococcal *icaC*, a third gene involved in PIA production. *Streptomyces coelicolor* and *Streptomyces avermitilis* have *pgaC* homologues that are predicted to encode proteins with *N*-deacetylase activity, a characteristic suggestive of bifunctional PgaC-PgaB enzymes.

Many species contain genes with limited sequence similarity to *pgaC*, which may encode glycosyltransferases, but without colocalization of the other *pga* genes. For example, *Streptococcus pyogenes* *hasA* encodes an enzyme that synthesizes hyaluronic acid, another GlcNAc-containing polysaccharide. Thus, the presence of a *pgaC* homologue alone is not a reliable indicator of PGA synthesis.

The observation that diverse species contain loci homologous to *pga* even though these loci are not typical of all members of a given family or genus suggests that these genes are horizontally transferred (21). This view is further supported by the low G+C content of the *pgaABCD* locus of *E. coli* (44 versus 51% for the genome), as recognized previously (23). The homologous locus of *Y. pestis* is present on an unstable

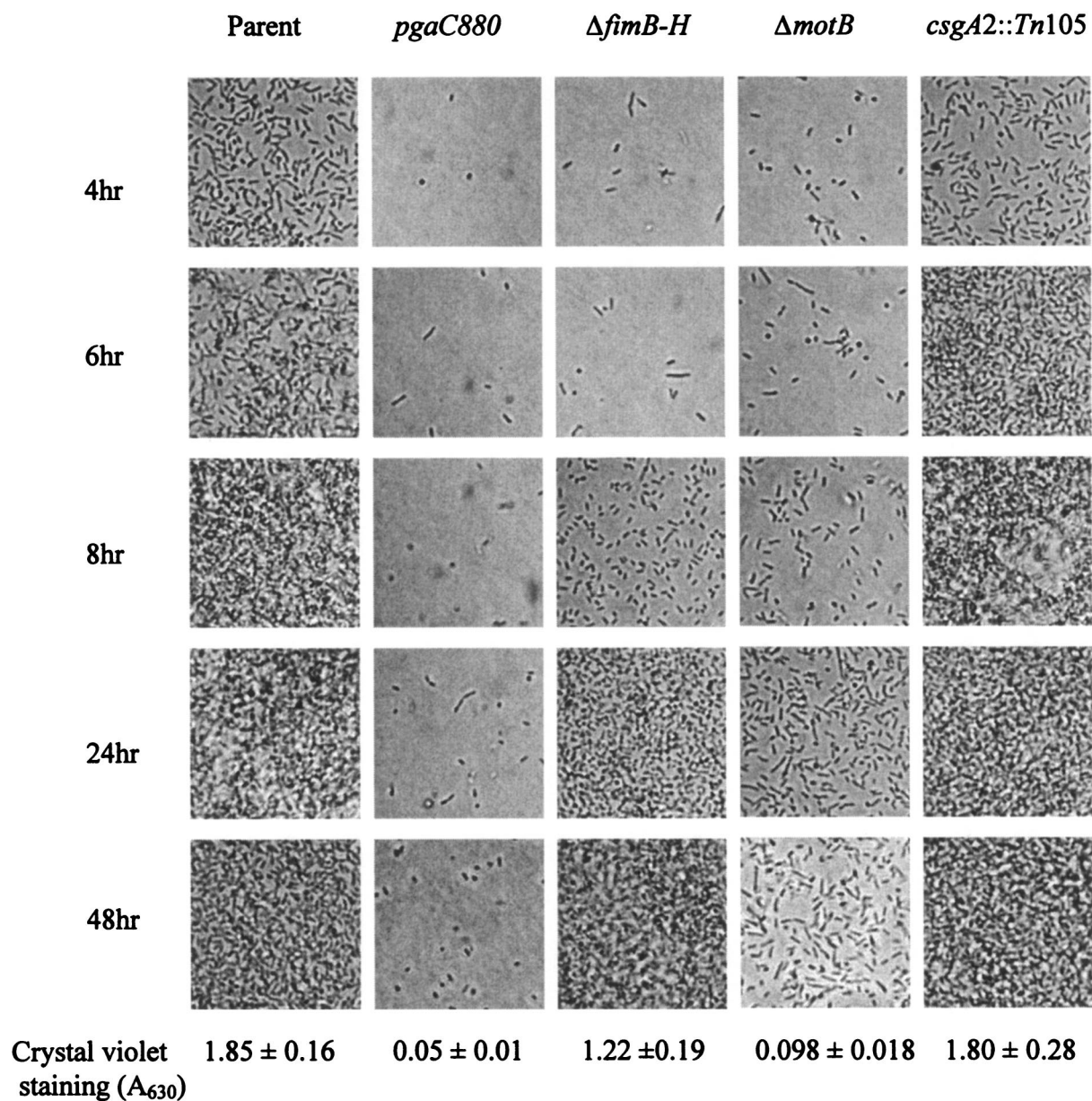


FIG. 5. Time course of adherence to coverslips by strains defective for PGA or other surface factors. The parent strain TRMG1655 and isogenic mutants defective in PGA production (*pgaC880*), type I pili (Δ *fimB-H*), motility (Δ *motB*), or curli (*csgA2::Tn105*) were inoculated in parallel into petri dishes containing CFA medium and sterile borosilicate glass coverslips. Cultures were incubated at 26°C, and attached cells were analyzed at the indicated times. Representative fields are shown. The quantitative results of crystal violet staining of 24-h biofilms, grown in a polystyrene microtiter plate under similar conditions, are shown for comparison.

region of the genome (22), a finding consistent with this possibility.

DISCUSSION

A crucial biological function that could be provided by EPS, either solely or in complex with ions, proteins such as lectins, or other matrix components, is that of an adhesin. A biofilm adhesin should permit bacterial cells to bind to a surface and/or to each other and thereby stabilize the structure of the biofilm. The present study provides evidence that the *pgaABCD* locus of *E. coli* is needed for the biosynthesis and

function of a polysaccharide that is a biofilm adhesin, i.e., PGA. This polysaccharide affects biofilm in either the presence or absence of other factors, curli fimbriae, type I pili, or motility. This class of polysaccharides was previously unknown from *E. coli* and other gram-negative bacteria but serves an adhesive role in staphylococcal biofilms. Although these are not the only polysaccharides that may serve as biofilm adhesins (59), we expect them to function as adhesins in species that produce them.

What evidence supports the role of PGA as a biofilm adhesin? First, the *pga* locus is needed for attachment to abiotic surfaces, intercellular adherence, biofilm formation, and the

TABLE 2. Dispersal of biofilm by metaperiodate^a

Strain	Mean dispersal \pm SE					
	Control	HIO ₄	HIO ₄ + polyol ^b	PBS	EDTA	NaCl
Parent	2.1 \pm 0.3	0.08 \pm 0.03	1.3 \pm 0.3	2.1 \pm 0.4	1.6 \pm 0.2	1.8 \pm 0.3
<i>cpsE</i>	2.0 \pm 0.3	0.07 \pm 0.03	2.0 \pm 0.3	1.8 \pm 0.4	1.5 \pm 0.2	1.6 \pm 0.3
Δ <i>lacIZ</i>	2.3 \pm 0.2	0.09 \pm 0.02	1.5 \pm 0.1	2.7 \pm 0.2	2.7 \pm 0.1	2.7 \pm 0.2

^a Biofilms formed after 24 h by TRMG1655 and isogenic mutants defective for colanic acid production (*cpsE*) or lactose metabolism (Δ *lacIZ*) were treated at 4°C with the indicated reagents, and the remaining biofilm was quantified by crystal violet staining as described in Materials and Methods. Values represent the mean \pm the standard error of two experiments with six replicates per sample.

^b The polyol used for quenching metaperiodate was glucose for the parental strain and *cpsE* mutant or lactose for the Δ *lacIZ* strain.

accumulation of PGA (Fig. 1, 2, and 5). Second, PGA is structurally related to the staphylococcal polysaccharide adhesins. Third, PGA is cell bound and was not detected in the spent medium. The four *pga* gene products are predicted to be cell envelope proteins (discussed below), further suggesting that PGA is surface associated. Fourth, the dissociation of biofilm by metaperiodate suggests that polysaccharide(s) maintains its structure (Table 2). Neither colanic acid (Table 2) nor cellulose (data not shown) performs this function. Furthermore, enzymatic digestion of RNA, DNA, or protein failed to cause release (data not shown), suggesting that under our experimental conditions these macromolecules are not responsible for maintenance of biofilm structure. Viewed together, these findings offer a compelling case that PGA serves as a biofilm adhesin in *E. coli*.

The predicted localization and biological functions of the Pga gene products suggest a model in which PGA is synthesized at the cytoplasmic side of the inner membrane, where UDP-GlcNAc is present, and is transported through the cell surface by an envelope complex of the Pga proteins. Biofilm formation by nonpolar *pga* mutants was not restored by intercellular complementation or by providing external PGA to growing cultures (data not shown). These observations are fully consistent with this model.

Although the picture is far from complete, some information is available concerning the metabolic processes involved in PGA production. A large number of transposon insertions that disrupt biofilm formation were isolated in the *pgaABCD* locus (Fig. 1 and 2). Because UDP-GlcNAc is an essential sugar nucleotide, no mutations that disrupt the synthesis of this apparent precursor of PGA were isolated. CsrA represses biofilm formation in *E. coli* and its relatives, as well as the synthesis and catabolism of intracellular glycogen, which are required in this process (32). Thus, we previously proposed that redirection of central carbon flux through a reserve polymer might be a general principle of biofilm formation, designed to provide precursor(s) for adhesins or other factors (32). The main product of glycogen catabolism, glucose-1-phosphate, is a precursor via fructose-6-phosphate of the apparent substrate for PGA synthesis, UDP-GlcNAc. In view of the present findings on the role of PGA, we hypothesize that the main influence of glycogen synthesis and catabolism in biofilm formation is to overcome a limitation for UDP-GlcNAc in PGA synthesis, which must compete with peptidoglycan and lipopolysaccharide for this precursor. The finding that *pga* mutations cause more

severe defects in biofilm formation in *csrA* mutant strains (Fig. 1), which exhibit elevated glycogen synthesis and subsequent catabolism (52, 68), supports this notion. It is notable that although *Salmonella enterica* lacks a *pga* locus, glycogen synthesis nevertheless is positively correlated with biofilm formation and CsrA represses biofilm development in this bacterium (discussed in reference 32). This example suggests that relatives of *E. coli* that do not synthesize PGA may use similar strategies for regulating central carbon flux into other polysaccharide adhesins. Unlike bacterial cellulose (58), synthesis of staphylococcal PIA does not require a lipid carrier (24), and we failed to isolate any mutants that were consistent with this requirement for PGA biosynthesis.

Chromatographic and NMR analyses revealed that PGA consists of high-molecular-mass β -1,6-GlcNAc ($\geq 400,000$ Da) containing <3% deacetylated residues and no other major substituents (Fig. 3 and 4). The PGA was prepared from a strain containing multiple copies of the *pga* locus, and even in this strain there was only ~ 1 mg of PGA per liter of culture. Although we recognize that the recombinant genotype of the strain may have affected the structure of the polysaccharide, overexpression of the *pga* operon greatly enhanced biofilm formation, indicating that the resulting polysaccharide is functional. Furthermore, the staphylococcal β -1,6-GlcNAc polymers appear to vary in molecular weight, N acetylation, phosphorylation, and succinylation, and yet all apparently function as adhesins. It has been suggested that structural variations in the staphylococcal polysaccharides may reflect different growth conditions or purification conditions (34). Whether such structural variations have subtle effects on adhesion or other biological functions remains to be determined.

The production of β -1,6-GlcNAc may affect a variety of disease processes that involve biofilm development. In the most definitive example, PIA has been shown to be a virulence factor in model catheter and foreign body infections caused by *S. epidermidis* (see reference 53 and references therein). The *hmsHFRS* locus of *Y. pestis*, the plague bacillus, is homologous to *pgaABCD* and is needed for biofilm formation in vitro (27) and in invertebrates (15). Part of the life cycle of this bacterium involves blockage of the flea gut by the accumulation of an extracellular material that depends upon the *hmsHFRS* locus and appears to protect the bacteria from expulsion from the gut (30). In conjunction with this information, the present study raises the possibility that β -1,6-GlcNAc may be important in the transmission of this deadly disease. Although definitive evidence is lacking, other diseases might also be affected by this polysaccharide, based on the presence of *pga* loci in the etiological agents. These include bladder infections by uropathogenic strains of *E. coli*, which produce substantial amounts of EPS (4), and food-borne illness by enterohemorrhagic *E. coli*, which may be transmitted through biofilm formation on food or food processing equipment (31). β -1,6-GlcNAc might also participate in microbial interactions with higher plants, e.g., formation of a protective biofilm on plant roots by *P. fluorescens* (discussed in reference 47) or diseases caused by *Xanthomonas* or *Ralstonia* species (35).

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