Depolymerization of β-1,6-*N*-Acetyl-D-Glucosamine Disrupts the Integrity of Diverse Bacterial Biofilms†

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Polymeric β-1,6-*N*-acetyl-D-glucosamine (poly-β-1,6-GlcNAc) has been implicated as an *Escherichia coli* and *Staphylococcus epidermidis* biofilm adhesin, the formation of which requires the *pgaABCD* and *icaABCD* loci, respectively. Enzymatic hydrolysis of poly-β-1,6-GlcNAc, demonstrated for the first time by chromatography and mass spectrometry, disrupts biofilm formation by these species and by *Yersinia pestis* and *Pseudomonas fluorescens*, which possess *pgaABCD* homologues.

Sessile bacterial growth on a surface or interface is generally referred to as a biofilm. Intense interest in biofilms over the past several years stems largely from the recognition of their roles in protecting microbes from the immune system, predation, and stresses; their association with failed antimicrobial therapies; and their potential to facilitate genetic exchange (2, 3, 6, 7, 9, 12, 24, 31).

Adherence of cells to surfaces and to each other is critical for biofilm formation. A variety of surface organelles have been reported to assist in adherence (4, 20, 29, 32, 36). While capsular polysaccharides affect biofilm architecture, mounting evidence indicates that many of these, including colanic acid of *Escherichia coli*, Vi antigen of *Salmonella enterica*, and alginate of *Pseudomonas aeruginosa*, do not play necessary roles in biofilm formation (5, 13, 30, 38). In contrast, genetic studies and polysaccharide analyses indicate that a cell-bound polysaccharide of *E. coli* and staphylococci, polymeric β-1,6-*N*-acetyl-D-glucosamine (poly-β-1,6-GlcNAc), is required for biofilm formation (11, 37). *E. coli* biofilm is dispersed by meta-periodate, but not by protease, consistent with a role of polysaccharide(s) in maintaining its structural stability but short of demonstrating that poly-β-1,6-GlcNAc performs this role (37).

The four genes of the *pgaABCD* operon of *E. coli* are required for biofilm formation and are predicted to encode proteins required for the synthesis and translocation of poly-β-1,6-GlcNAc, also referred to as PGA, through the gram-negative cell envelope (37). Production of staphylococcal β-1,6-GlcNAc polysaccharides, alternatively known as PIA, PS/A, SAE, and PNAG (14, 17, 22, 23, 25, 26), depends upon the *icaABCD* locus. Two gene products of the *pga* and *ica* operons are apparently homologous (37). The UDP-GlcNAc-specific glycosyltransferase, IcaA, polymerizes β-1,6-GlcNAc, and its homologue PgaC is predicted to do the same. IcaB and PgaB are related to polysaccharide *N*-deacetylases but have not been shown to possess this enzymatic activity. Evidence that genetic loci related to *pgaABCD* have undergone broad horizontal transfer suggests that poly-β-1,6-GlcNAc may participate in adhesion processes of diverse species (37). These include mammalian and plant pathogens, e.g., *Yersinia pestis, Yersinia pseudotuberculosis, Yersinia enterocolitica, Actinobacillus actinomycetemcomitans, Actinobacillus pleuropneumoniae, Bordetella pertussis, Bordetella parapertussis, Bordetella bronchiseptica, Xanthomonas axonopodis*, the biocontrol agent *Pseudomonas fluorescens*, and others. The *hmsHFRS* operon of *Y. pestis* is homologous to *pgaABCD* and has been previously implicated in biofilm formation (6). Species closely related to these do not invariably contain homologous loci, e.g., *S. enterica* and *P. aeruginosa* (37).

In the present study, a previously identified β-hexosaminidase and biofilm-dispersing enzyme of *A. actinomycetemcomitans*, DspB or dispersin B (18, 19), is shown for the first time to specifically hydrolyze the glycosidic linkages of poly-β-1,6-GlcNAc. Growth of cultures in the presence of DspB or treatment of preformed biofilms with this enzyme implicate poly-β-1,6-GlcNAc in the formation and stabilization of diverse bacterial biofilms.

Recombinant DspB containing an N-terminal His tag was produced using a strain containing a *dspB* plasmid clone [pHis-DspB1]. This clone was prepared by first amplifying the coding region of the *dspB* gene of *A. actinomycetemcomitans* HK1651 by PCR using the primers 5′-GGGAATTCCATGCATCATC ATCATCATCATAATTGTTGCGTAAAAGGCAATTCCAT (the EcoRI site is underlined) and 5′-TTCTGCAGCTCACTCA TCCCCATTCGTCTTATG (the PstI site is underlined). Chromosomal DNA for the PCR was a gift from David Dyer and Allison Gillaspy (University of Oklahoma Health Sciences Center) and was isolated using a PUREGENE DNA isolation kit (Gentra, Minneapolis, Minn.) under the conditions recommended for gram-negative organisms by the manufacturer, with the addition of a phenol-chloroform extraction step prior to eth-

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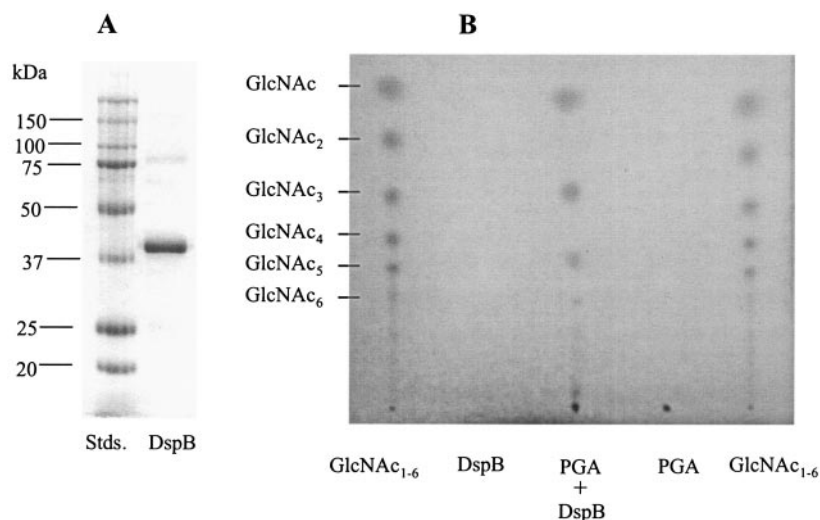
FIG. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of DspB and TLC separation of products generated by treatment of PGA with DspB. (A) Protein molecular weight standards (Stds.) and 10 μg of recombinant DspB were separated on a 12% polyacrylamide gel and stained with Coomassie blue R-250. (B) Reaction products generated by digestion of PGA for 16 h at 37°C are shown, along with untreated PGA and DspB controls. The GlcNAc standards are β-1,4-linked oligosaccharides derived from chitin.

anol precipitation. The resulting PCR product was cloned into the HincII site of pUC19, sequenced (AnaGen Technologies, Inc., Atlanta, Ga.), and found to be free of PCR-generated mutations. The *dspB*-containing EcoRI-PstI fragment was excised and subcloned into the corresponding restriction sites of pKK223-3 (1) to produce pHis-DspB1. Strain JM101[pHis-DspB1]) was inoculated (20 ml/liter) and grown with aeration at 37°C in Luria-Bertani (LB) medium (27) containing ampicillin (100 μg/ml). After 4 h of growth, isopropyl-β-D-thiogalactopyranoside (2 mM final concentration) was added to induce *dspB* expression, and growth was continued for 3 h. The cells were harvested by centrifugation and disrupted by sonication, and DspB protein was purified by affinity chromatography using a His-Trap column (QIAexpressionist), as recommended by the manufacturer (QIAGEN, Valencia, Calif.), and dialyzed against 10 mM sodium phosphate (pH 5.9). This single chromatographic step provided an almost homogeneous preparation of DspB (≥95% purity), as assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis with Coomassie blue staining (Fig. 1A) and densitometry. This enzyme was active against 4-nitrophenyl-N-acetyl-β-D-glucosaminide (18). The yield of DspB was ~6 mg/liter of culture, as determined with a BCA protein assay kit (Pierce, Rockford, Ill.).

To investigate the catalytic activity of DspB, a substrate of homopolymeric β-1,6-GlcNAc or PGA was prepared from *E. coli* K-12 DJ4[pPGA372], as described previously (37), except that the strain was grown in LB medium (27). The genotype of this strain (MG1655 *csrA*::*kan cpsE*::Tn*10*) and plasmid pPGA372 (pUC19 containing *pgaABCD*) allow PGA to be overproduced (37). Enzymatic treatment of PGA with DspB was carried out for 16 h at 37°C. Thin-layer chromatography (TLC) was used to detect low-molecular-weight oligosaccharides (GlcNAc$_{1-6}$), as described previously (35). The reaction mixture (75 μl) contained 0.6 mg of PGA in 50 mM ammonium acetate buffer (pH 5.9) along with 7.5 μg of DspB. Apparent reaction products included GlcNAc and GlcNAc oligomers that remain unassigned (Fig. 1B). In contrast, identical enzymatic treatments of two β-1,4-GlcNAc polymers, water-soluble chitin or glycol chitin, produced no degradation products (data not shown). Analysis of the primary amine content of PGA using the ninhydrin reaction (21), with D-glucosamine as a standard, indicated that it contains 3.2% ± 0.2% unacetylated glucosamine, in agreement with the results of previous nuclear magnetic resonance analyses (37). Enzymatic treatment did not significantly increase this value, indicating that DspB does not possess N-deacetylase activity.

Matrix-assisted laser desorption-ionization time of flight (MALDI-TOF) mass spectrometry allowed detection of individual high-molecular-weight reaction products. Spectra were acquired in a linear mode by using a Voyager STR (Applied Biosystems) employing a nitrogen laser (wavelength, 337 nm). One hundred to 600 laser pulses were used to obtain the spectra. Detection employed delayed extraction and a low-mass gate, which deflected ions lower in mass than the scan range detected. The matrix was a saturated solution of 1,4-hydroxyphenylazobenzoic acid in 50% acetonitrile and 1% trifluoroacetic acid and was mixed in a 10:1 ratio with a sample. One microliter of the mixture was transferred to the sample plate and dried. A reaction identical to the one described above for TLC analysis yielded a series of oligosaccharides, each major product of which differed from the previous one by a mass of 204 Da, equivalent to a single anhydro GlcNAc residue (Fig. 2A). These products were confirmed to be reaction products by their absence in the control spectra of DspB and untreated PGA (Fig. 2B). The experimental masses of the reaction products were indicative of oligomers ranging from GlcNAc$_9$ to >GlcNAc$_{28}$. Minor products were also observed, many of which differed from the major ions by the mass of a single acetyl moiety. These minor products apparently represent the release of oligosaccharides with preexisting modifications. The low-molecular-weight products that were detected by TLC (Fig. 1) were masked by matrix ions of the mass
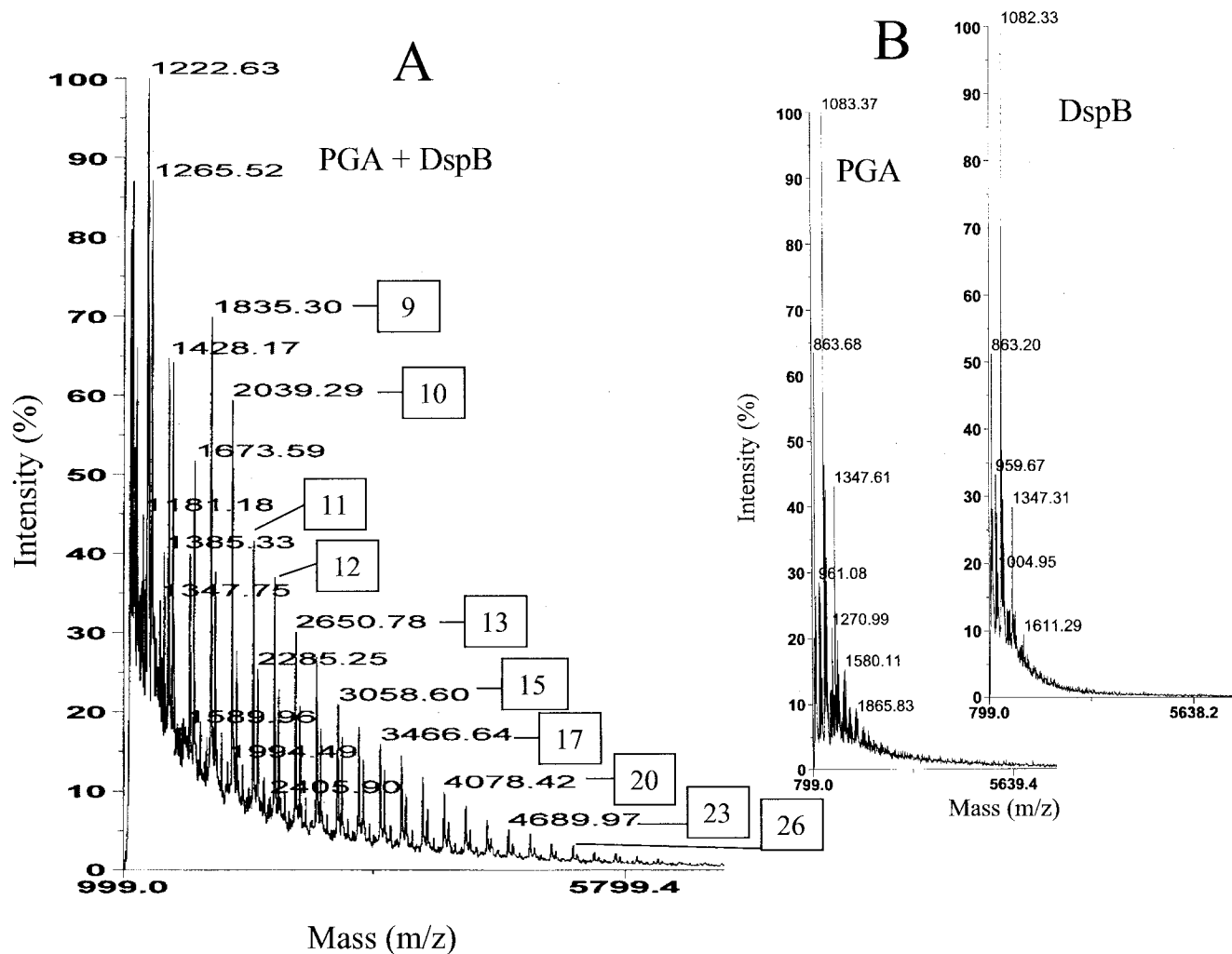
FIG. 2. MALDI-TOF mass spectra of PGA after digestion with DspB for 16 h at 37°C (A) and untreated PGA and DspB (B). The degree of polymerization of a major reaction product is indicated by a boxed number that refers to either the centroid mass (m/z) or ion peak of the spectrum.

spectrum. Spectra were acquired up to a mass of 25,000 Da, but no additional products were detected beyond the scale shown in Fig. 2. The PGA substrate for the reaction was not observed because its molecular weight is greater than the range of detection, $\geq$400,000 Da (37). Together, these findings imply that DspB endolytically hydrolyzes the glycosidic linkages of PGA, ultimately processing the products to GlcNAc and low-molecular-weight oligosaccharides.

Having established that DspB specifically cleaves poly-β-1,6-GlcNAc, we examined the ability of this enzyme to inhibit bacterial biofilm formation. We tested several species that have *pgaABCD* or *icaABCD* loci, namely, *E. coli* K-12 MG1655; its biofilm-forming *csrA* mutant, TRMG1655; a clinical strain of *E. coli* that was isolated from a colonized urinary catheter, P18 (16, 33, 37); *S. epidermidis* 1457 (23); a nonpathogenic *Y. pestis* strain lacking the 72-kb virulence plasmid, KIM6+, and KIM6, which additionally lacks the 102-kb Pgm locus containing *hmsHFRS* and the high pathogenicity island (8, 15); and *P. fluorescens* WCS365 (28). *S. enterica* serovar Typhimurium ATCC 14028 and *P. aeruginosa* PAO1 (provided by M. E. Hart) were also tested as exam-

ples of related species that lack *pga* loci (37). As indicated in Fig. 3, these bacteria were grown in microtiter wells containing LB medium (27), tryptic soy broth (TSB) medium (23), colony-forming antigen medium (16), M63 medium (27) supplemented with citric acid (0.4%) and MgSO$_4$ (1 mM), or YPG medium (10). The antibiotics ampicillin (100 μg/ml) and kanamycin (100 μg/ml) were included in growth media as needed.

Biofilm formation was measured by crystal violet staining (16). *Y. pestis* biofilm was somewhat unstable and was examined using a modified procedure. Planktonic cells and spent media were discarded, and adherent cells were gently rinsed twice with deionized water, treated with methanol (100%) for 1 min to fix the cells, and allowed to air dry prior to being stained (http://www.engscientific.com/micro.html). All biofilm experiments were conducted at least twice, with at least six replicas per sample.

Growth in the presence of DspB (50 μg/ml) caused essentially complete inhibition of biofilm formation in species with *pgaABCD* or *icaABCD* loci: *E. coli* MG1655, its *csrA* mutant, the catheter isolate *E. coli* P18, *S. epidermidis, P. fluorescens,*
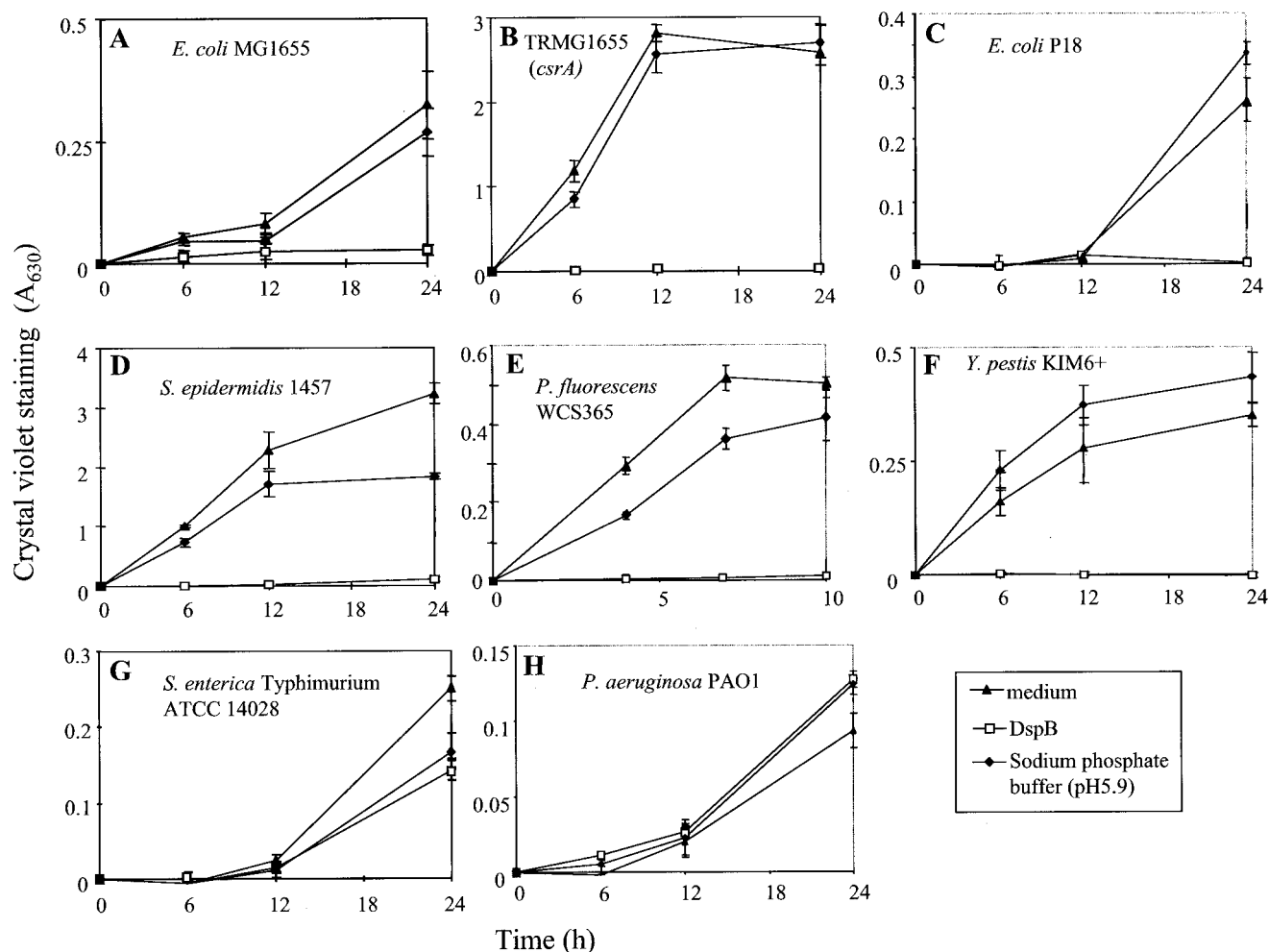
FIG. 3. Effect of DspB on biofilm formation by the following strains with the listed growth conditions: *E. coli* MG1655, LB medium, 26°C (A); TRMG1655 (*csrA::kan*), LB medium, 26°C (B); *E. coli* P18, TSB, 26°C (C); *S. epidermidis* 1457, TSB, 26°C (D); *P. fluorescens* WCS365, M63 supplement, 26°C (E); *Y. pestis* KIM6+, TSB, 26°C (F); *S. enterica* serovar Typhimurium, colony-forming antigen medium, 26°C (G); and *P. aeruginosa* PAO1, YPG, 26°C (H). Biofilm formation in the indicated media, in presence or absence of recombinant DspB or an enzyme buffer control, was determined at the indicated times by crystal violet staining (A_{630}). Error bars represent standard deviations (six replicates) of a representative experiment.

and *Y. pestis* KIM6+ (Fig. 3). In contrast, DspB did not exhibit a discernible effect on biofilm formation by *S. enterica* or *P. aeruginosa*, which lack *pga/ica* loci. Growth curves in the presence or absence of DspB showed that this enzyme does not inhibit the planktonic growth of these six species (data not shown). *Y. pestis* KIM6, which lacks *hmsHFRS*, failed to form biofilm (data not shown).

The ability of recombinant DspB to disperse biofilms of these species was examined after 24 h of growth or 10 h for *P. fluorescens* (28). The media and planktonic cells were removed, M63 minimal salts solution (16) was added with and without DspB (50 μg/ml), and biofilm was measured at various times thereafter. The addition of DspB to *E. coli* K-12 strains and *S. epidermidis* led to complete dispersal of the biofilm within 30 min (Fig. 4). DspB exhibited weaker dispersal effects on *E. coli* P18 and *P. fluorescens* biofilms, which nevertheless were reproducible. Biofilm of *Y. pestis* was not dispersed by DspB (data not shown), suggesting that, while growth in the presence of

DspB prevents biofilm formation, poly-β-1,6-GlcNAc is inaccessible to the enzyme or other factors are involved in stabilizing the biofilm of *Y. pestis*. As expected from the results of the biofilm-blocking experiments, biofilms of *S. enterica* and *P. aeruginosa* were not dispersed by DspB treatment. The apparent modest stimulation of *P. aeruginosa* biofilm growth in the presence of DspB has not been further examined but may involve the proteolysis of DspB by secreted proteases (reference 34 and references therein) and uptake of the resulting amino acids and peptides, which might also have affected DspB activity in biofilm inhibition experiments (Fig. 3).

Conclusions. Studies have recently shown that DspB or dispersin is able to release biofilms of two species, *A. actinomycetemcomitans* and *S. epidermidis* (18, 19). However, the biochemical reaction responsible for this activity had not been previously established. Analyses of *E. coli* PGA depolymerization now reveal that DspB specifically hydrolyzes the glycosidic linkages of poly-β-1,6-GlcNAc. It does not cleave the related
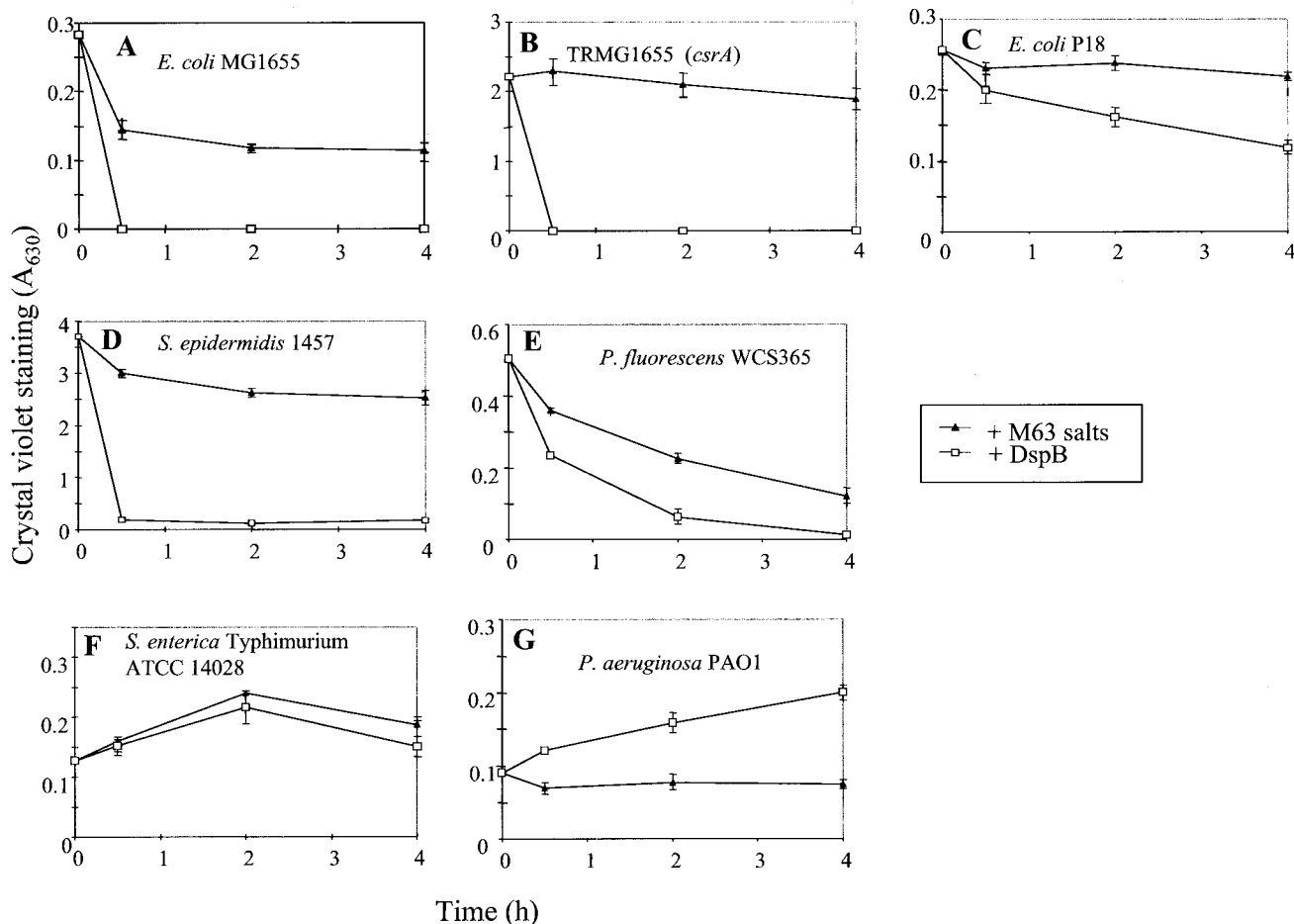
FIG. 4. Treatment of biofilms of *E. coli* MG1655 (A), TRMG1655 (*csrA*::*kan*) (B), *E. coli* P18 (C), *S. epidermidis* 1457 (D), *P. fluorescens* WCS365 (E), *S. enterica* serovar Typhimurium (F), and *P. aeruginosa* PAO1 (G) with DspB or a buffer control (M63 salts). Biofilms were grown under the conditions indicated in the legend to Fig. 3. Error bars represent standard deviations (six replicates) of a representative experiment.

β-1,4 linkages of some chitin derivatives or catalyze N deacetylation of PGA. In conjunction with the results of BLAST analyses for *pgaABCD* loci of other species (37), this information permitted a systematic assessment of the role of this type of polysaccharide in biofilm formation by several eubacteria. The results of this assessment demonstrate that poly-β-1,6-GlcNAc serves as a biofilm adhesin in phylogenetically diverse species, which exploit diverse hosts and environmental niches. Thus, depolymerization of poly-β-1,6-GlcNAc by DspB or perhaps other enzymes that cleave this polymer offers a possibility for inhibiting biofilm formation by several important pathogens which colonize host tissues and/or surfaces of catheters and prosthetic devices. While it is reasonable to expect that species that produce poly-β-1,6-GlcNAc may likewise produce enzymes that degrade this polysaccharide, BLAST analyses of the NCBI microbial-genome database (http://www.ncbi.nlm.nih.gov/BLAST/) revealed the presence of DspB homologues only in *Actinobacillus* species (data not shown). The possibility that distinct depolymerases have convergently evolved to facilitate biofilm dispersal by other species that utilize this polysaccharide adhesin should be investigated.

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