APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Nov. 2009, p. 7037–7043 Vol. 75, No. 22
0099-2240/09/$12.00 doi:10.1128/AEM.01015-09

Role of the *Streptococcus mutans irvA* Gene in GbpC-Independent, Dextran-Dependent Aggregation and Biofilm Formation▽

Min Zhu,[1] Dragana Ajdić,[2] Yuan Liu,[1] David Lynch,[1] Justin Merritt,[2] and Jeffrey A. Banas[1]*

Dows Institute for Dental Research, Department of Pediatric Dentistry, College of Dentistry, University of Iowa, Iowa City, Iowa 52242,[1] and University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma 73104[2]

Received 4 May 2009/Accepted 21 September 2009

Dextran-dependent aggregation (DDAG) of *Streptococcus mutans* is an in vitro phenomenon that is believed to represent a property of the organism that is beneficial for sucrose-dependent biofilm development. GbpC, a cell surface glucan-binding protein, is responsible for DDAG in *S. mutans* when cultured under defined stressful conditions. Recent reports have described a putative transcriptional regulator gene, *irvA*, located just upstream of *gbpC*, that is normally repressed by the product of an adjacent gene, *irvR*. When repression of *irvA* is relieved, there is a resulting increase in the expression of GbpC and decreases in competence and synthesis of the antibiotic mutacin I. This study examined the role of *irvA* in DDAG and biofilm formation by engineering strains that overexpressed *irvA* (IrvA+) on an extrachromosomal plasmid. The IrvA+ strain displayed large aggregation particles that did not require stressful growth conditions. A novel finding was that overexpression of *irvA* in a *gbpC* mutant background retained a measure of DDAG, albeit very small aggregation particles. Biofilms formed by the IrvA+ strain in the parental background possessed larger-than-normal microcolonies. In a *gbpC* mutant background, the overexpression of *irvA* reversed the fragile biofilm phenotype normally associated with loss of GbpC. Real-time PCR and Northern blot analyses found that expression of *gbpC* did not change significantly in the IrvA+ strain but expression of *spaP*, encoding the major surface adhesin P1, increased significantly. Inactivation of *spaP* eliminated the small-particle DDAG. The results suggest that IrvA promotes DDAG not only by GbpC, but also via an increase in P1.

Streptococcus mutans is considered the main etiological agent of dental caries (10, 20). A prominent virulence property of this species is its ability to adhere to tooth surfaces in the presence of dietary sucrose. Dextran-dependent aggregation (DDAG) is an in vitro phenomenon that is believed to represent a property of the organism that is beneficial for sucrose-dependent biofilm development. It can be described as the formation of bacterial aggregates or flocculation upon the addition of dextran to an overnight culture of bacteria grown in the absence of sucrose. In this instance, the dextran simulates the glucan that would be synthesized if sucrose were present. DDAG was originally reported for *Streptococcus sobrinus* and *Streptococcus criceti* (7). It was regarded as specific for these two mutans streptococcal species until Sato et al. demonstrated DDAG of *S. mutans* following growth under a variety of stress conditions, including subinhibitory concentrations of tetracycline, ethanol, or xylitol (27, 28). *S. mutans* DDAG was attributed to GbpC, a wall-anchored surface glucan-binding protein encoded by the *gbpC* gene (27). However, GbpC alone does not appear to account for DDAG, as transcription of *gbpC* does not increase under some growth conditions that promote DDAG (4).

Located just upstream of *gbpC* is a putative transcriptional regulator gene, *irvA* (Fig. 1), that has been found to contribute to repression of mutacin I gene expression and is thought to be part of a stress regulon (24, 31). The predicted IrvA amino acid sequence is 79 residues and bears a helix-turn-helix domain characteristic of the Cro/cI family of transcriptional regulators (2). Under routine growth conditions in the laboratory, *irvA* appears to be repressed by the product of an adjacent gene, *irvR* (25). Similarly, *gbpC* is transcribed under routine laboratory conditions despite the potential to be repressed by CovR (GcrR) (4, 29) and perhaps by another, as yet unidentified, regulator (29). Derepression of *irvA* following mutation of *irvR* leads to prolific DDAG and enhanced GbpC expression (25).

Taken together, these observations suggest that the DDAG phenotype in *S. mutans* is most advantageous under conditions of stress. The ability of bacteria to autoaggregate has been proposed to be an important step in biofilm formation (17), though several other theories have been proposed as well. These include protection against predation or environmental stresses, or responses to nutrient conditions (8). It is curious that the mutans streptococci, which share similar virulence traits responsible for caries in animals or humans, are divided between species for which DDAG is a constitutive property and those for which DDAG is induced only under growth conditions that simulate stress. This study was undertaken to further examine the conditions under which DDAG is induced in *S. mutans* and to investigate its effects on biofilm formation.

MATERIALS AND METHODS

Strains and culture conditions. *Escherichia coli* JM109 was used for the intermediate cloning steps. *Streptococcus mutans* UA159 was used as the parental strain for all genetically engineered *S. mutans* strains, including those harboring *irvA* on an extrachromosomal plasmid, as well as strains wherein *gbpC*, *irvA*, or *spaP* is inactivated. Table 1 lists the strains used in this study. *E. coli* was cultured in Luria broth (Fisher Scientific, Pittsburgh, PA) at 37°C with shaking. Streptococci were cultured on Todd-Hewitt plates (Becton, Dickinson and Co., Franklin Lakes, NJ) and in chemically defined medium (CDM) (SAFC Biosciences, Inc.,

* Corresponding author. Mailing address: Dows Institute for Dental Research, University of Iowa, N436 Dental Science Building, Iowa City, IA 52242. Phone: (319) 335-9911. Fax: (319) 335-8895. E-mail: Jeffrey-Banas@uiowa.edu.

▽ Published ahead of print on 25 September 2009.

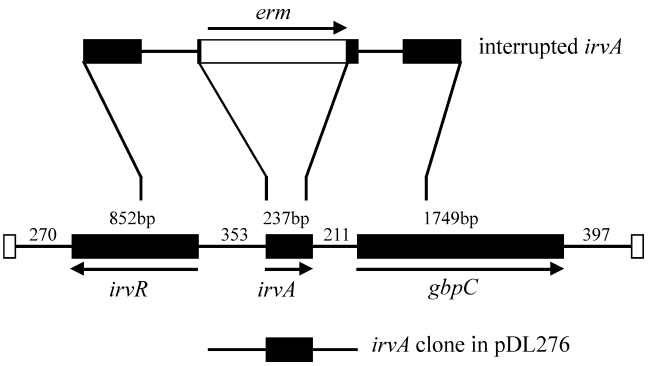

FIG. 1. The chromosomal locus harboring genes *irvR*, *irvA*, and *gbpC* is depicted (middle), along with representations depicting the construction of the *irvA* mutant (top) and the cloning of *irvA* and the flanking DNA (bottom). The arrows indicate gene orientation. The numbers indicate the lengths of the genes or intergenic regions in base pairs.

Lenexa, KS) and were grown at 37°C in an anaerobic chamber (5% CO_2, 10% H_2, 85% N_2). Biofilms were cultured in CDM with 1% sucrose and grown at 37°C in 5% CO_2.

Cloning and overexpression of *irvA*. The *irvA* gene was cloned into the shuttle vector pDL276 (19). The cloned portion of *irvA* extended from 317 bp upstream of the *irvA* start codon (primer 5′-XbaI-CATGCTTCACCTTC-3′) to 206 bp downstream of the *irvA* termination codon (primer 5′-BamHI-AAACCATCCT TTATATT-3′) (Fig. 1). The shuttle vector pDL276 with *irvA* was transformed into the parental strain, *S. mutans* UA159, and *gbpC* mutant strains to generate overexpressing strains (IrvA+). Vector control strains contained pDL276 without the *irvA* insert. For transformation, overnight cultures were diluted 1:100 in medium containing 5% horse serum (23). The bacteria were incubated 2 h following the initial dilution, 0.8 μg/ml of a synthetic competence-stimulating peptide (SGSLSTFFRLFNRSFTQALGK) was added, and the bacteria were incubated another 0.5 h prior to addition of DNA. The culture was then incubated 3 h at 37°C and plated onto medium containing 800 μg/ml kanamycin.

Inactivation of *irvA* and *spaP*. A mutant strain in which *irvA* is inactivated was engineered by allelic replacement. DNA upstream and downstream of *irvA* was amplified by PCR and cloned on either side of an erythromycin resistance cassette (*erm*) (Fig. 1). The upstream amplification began 707 bp upstream of the *irvA* start codon (primer 5′-PstI-CTCAGTCGTTTCATCACTGG-3′) and ended 5 bp into the *irvA* reading frame (primer 5′-XbaI-CTGCATAGGAATCATTA CCT-3′). The downstream amplification began 37 bp prior to the *irvA* termination codon (primer 5′-BamHI-TTACTATGATATCAGTTTAG-3′) and ended 700 bp downstream of the *irvA* termination codon (primer 5′-KpnI-TGAGCTT CATACTCCTTGAC-3′). A linear fragment containing these regions was then used to transform *S. mutans*. Homologous recombination on either side of *erm* resulted in a replacement of *irvA*, which was confirmed using conventional PCR, and real-time PCR was used to confirm the lack of *irvA* transcription. A *spaP* mutant was engineered by insertional inactivation. A 703-bp internal portion of *spaP* from base 466 (primer 5′-ACAGCTGAAGAAGCAGTCCAAAAAGAA A-3′) to base 1142 (primer 5′-TCACAGCTGAAAATACTGCAATTAAGAA-3′) was amplified by PCR and cloned into the suicide vector pVA8912 (30). This construct was transformed into *S. mutans* wherein a single homologous recombination generated two incomplete copies of *spaP*. Conventional PCR was used to confirm the genotype, and real-time PCR was used to confirm the lack of *spaP* transcription.

DDAG. Overnight cultures, grown in CDM with or without 4% ethanol, were washed and resuspended in phosphate-buffered saline (PBS; pH 7.4). Dextran T2000 (Sigma, St. Louis, MO) was added to a final concentration of 100 μg/ml. The tubes were then incubated at 37°C for 1 h, with optical density (OD) measurements at 660 nm taken at 0, 2, 15, 30, and 60 min. Small-particle aggregation was visualized following a 1:2 dilution in PBS.

Biofilm growth and analysis. Biofilms of parental and mutant strains were formed in 24-well polystyrene plates (Costar 3526; Corning Inc., Corning, NY) or in glass-bottom culture dishes (MatTek Corporation, Ashland, MA) when biofilms were to be analyzed by confocal laser scanning microscopy. The culture dishes were first incubated with sterile, diluted saliva for 3 h at 37°C, which was aspirated prior to the addition of medium (CDM with 1% sucrose), and then inoculated with growth from overnight cultures of test strains. The biofilms were grown at 37°C in 5% CO_2 on a fixed-angle rotator at a rotation speed of 10 rpm and an angle of 60° overnight or for 2 days with a change in medium after 24 h (3). Biofilms in polystyrene dishes were washed, stained with safranin, and photographed against a light box. Biofilms for confocal microscopy were rinsed twice in PBS and stained for 35 min with SYTO9 (Molecular Probes, Inc., Eugene, OR) and then rinsed twice more with PBS. One milliliter of PBS was added to the wells to prevent drying of the biofilm during image collection. Biofilm images of the substratum were collected using a Bio-Rad Radiance 2100MP multiphoton/confocal microscope. Particle counts and particle sizes of microcolonies at the substratum were calculated using ImageJ 1.40 and averaged from three random fields.

Real-time PCR. Total RNA from each bacterial strain was isolated using the RNeasy mini kit (Qiagen Inc., Valencia, CA). Relative expression levels of *gbpC* and *spaP* were measured by quantitative real-time PCR, using equivalent amounts of RNA. Results were normalized to *gyrA* transcription. The *gbpC* or *spaP* expression was set at 1 for the parental strain. Reactions were set up using the iScript one-step reverse transcription-PCR kit with SYBR green (Bio-Rad, Hercules, CA) and tested in an iQ5 real-time PCR detection system. Each sample was tested in duplicate during a trial, and four independent trials were performed.

Northern blotting. mRNA from each bacterial strain was isolated by the MicrobExpress bacterial mRNA purification kit (Ambion, Austin, TX). Northern blot analyses employed equivalent amounts of mRNA (100 ng) for each sample and were performed using the NorthernMax formaldehyde-based system (Ambion, Austin, TX) according to manufacturer's instructions.

RESULTS

Our laboratory has had a longstanding focus on *S. mutans* glucan-binding proteins, so the proximity of *irvA*, a putative transcriptional regulator gene, to *gbpC* was of interest. Initially, it was determined that overexpression of *irvA* in the parental or *gbp* mutant backgrounds enhanced the DDAG of *S. mutans*. In particular, strains carrying a copy of *irvA* on an extrachromosomal plasmid (IrvA+) were capable of DDAG even when grown in the absence of stress (Fig. 2A). Stress was simulated by adding 4% ethanol to the growth medium. The expression of *irvA* was enhanced approximately 2.5-fold in the IrvA+ strains (Fig. 3), whereas ethanol had no effect on *irvA* expression. An *S. mutans irvA* mutant strain behaved similarly to the wild-type parental strain (Fig. 2A).

Since GbpC was known to be linked to DDAG in *S. mutans*, an *irvA*-overexpressing strain was engineered in a *gbpC* mutant background. As expected, large aggregates of bacteria no longer appeared upon the addition of dextran. However, DDAG did not disappear altogether. The plasmid-based copy

TABLE 1. Bacterial strains used in this study

S. mutans strain	Description	Antibiotic resistance[a]	Source
UA159	Wild-type *S. mutans*	None	2
MZ159VC	UA159 with pDL276; vector control	Km	This study
MZ159A	UA159 *irvA* overexpresser (IrvA+)	Km	This study
MZ159i	UA159 *irvA* mutant	Em	This study
MZ159c	UA159 *gbpC* mutant	Sp	This study
MZ159Ac	UA159 *gbpC* mutant, *irvA* overexpresser	Sp, Km	This study
MZ159Acp	UA159 *gbpC spaP* mutant, *irvA* overexpresser	Sp, Km, Em	This study

[a] Km, kanamycin (800 μg/ml); Em, erythromycin (25 μg/ml); Sp, spectinomycin (500 μg/ml).

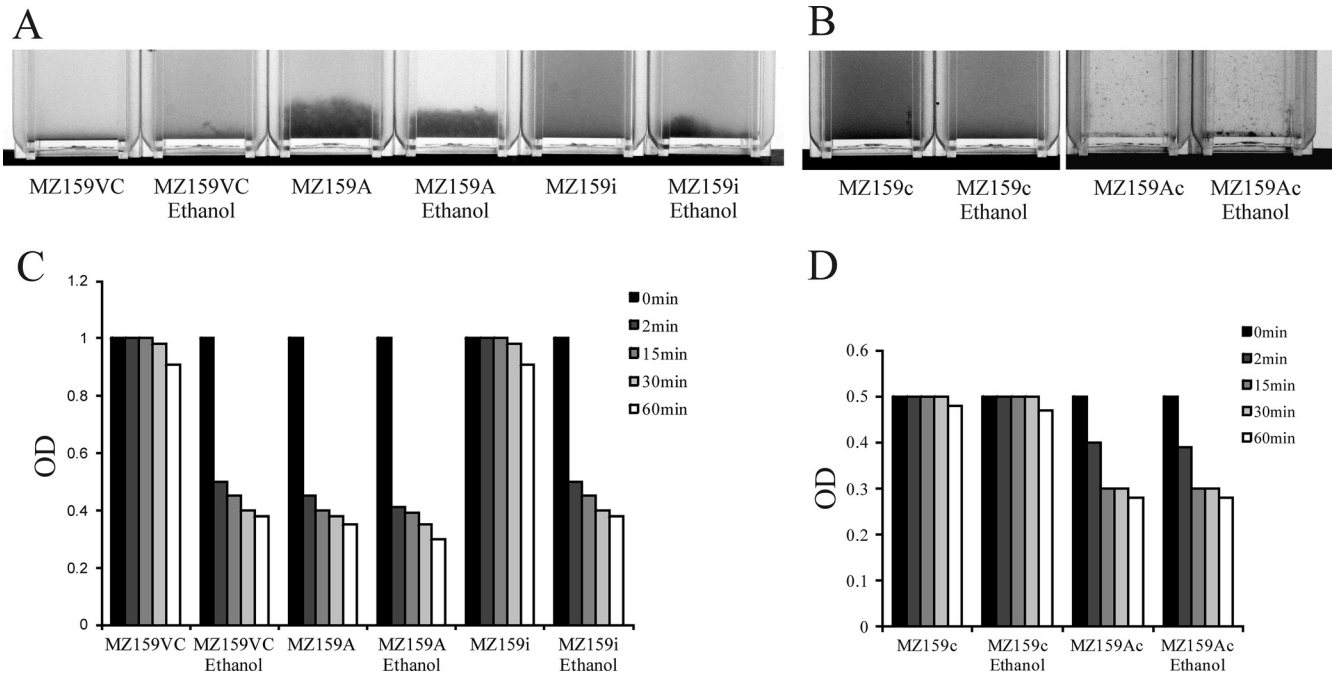

FIG. 2. Strains carrying a copy of *irvA* on an extrachromosomal plasmid (IrvA+), or parental vector control strains carrying the plasmid without *irvA*, were tested for DDAG when grown with or without 4% ethanol added to the growth medium. (A) In *S. mutans*, the IrvA+ strain (MZ159A) was capable of DDAG under both growth conditions, whereas the parental vector control (MZ159VC) and *irvA* mutant (MZ159i) strains displayed DDAG only when grown with ethanol. (B) In a *gbpC* mutant background (MZ159c), *S. mutans* lost the ability to form large aggregates as a result of DDAG, but the plasmid-based copy of *irvA* (MZ159Ac) resulted in tiny, dense aggregates under either growth condition. In order to visualize these aggregates more easily, the suspension of bacteria was diluted and the photographic contrast exaggerated. (C) The magnitudes of DDAG in the IrvA+ and parental vector control strains were quantified by measuring the decreases in OD over a period of 60 min. The histogram shows that DDAG by *S. mutans* resulted in an almost immediate decrease in OD when the parental vector control (MZ159VC) or *irvA* mutant (MZ159i) strains were grown in the presence of ethanol. An identical reduction in OD occurred in the IrvA+ (MZ159A) strain but was independent of the growth condition. (D) In the *S. mutans gbpC* mutant background (MZ159c), reductions in OD were evident in the strain carrying a plasmid copy of *irvA* (MZ159Ac), albeit more modestly than when large aggregates were formed. These reductions were independent of the growth condition.

of *irvA* resulted in tiny, dense aggregates that quickly fell to the bottom of the tube (Fig. 2B). The two forms of DDAG could be distinguished spectrophotometrically as well. Figure 2C shows that DDAG resulted in an almost immediate decrease in OD when the parental or *irvA* mutant strains were grown in the presence of stress. An identical reduction in OD occurred in the IrvA+ strain but was independent of stress. When *irvA* was overexpressed in a *gbpC* mutant background (Fig. 2D), the reductions in OD due to small-particle aggregation were more modest than when large aggregates were formed, and were independent of stress.

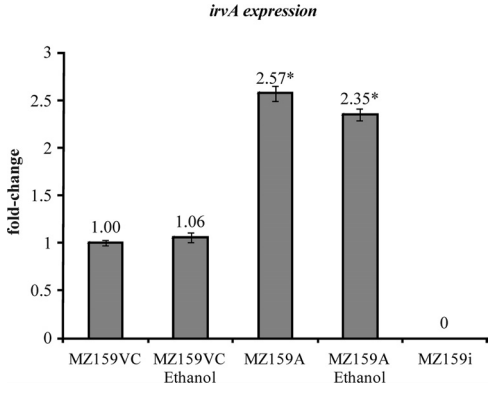

FIG. 3. Real-time PCR was used to measure the relative transcriptions of *irvA* in parental vector control (MZ159VC) and IrvA+ (MZ159A) strains. The data were normalized to *gyrA* expression, and then between strains by setting expression in the parental strain to 1. Addition of ethanol to the growth medium had no effect on *irvA* expression. The expression of *irvA* was increased approximately 2.5-fold in the IrvA+ strain, a difference that was statistically significant (marked by an asterisk) based upon a Student *t* test ($P < 0.05$). As expected, no transcription could be detected in an *irvA* mutant strain (MZ159i).

Not only did the IrvA+ strain display stress-independent DDAG, the DDAG that occurred following growth under stress appeared to be more robust than that formed by the parental vector control strain. To determine whether this observation could be explained by increased expression of GbpC, transcription of *gbpC* was measured by quantitative real-time PCR. As shown in Fig. 4A, the IrvA+ strain displayed only a slight increase in expression that appeared to be within the margin of experimental error. A Northern blot (Fig. 4B) also supported a conclusion that *gbpC* expression was not appreciably changed in the IrvA+ strain.

Since the prominent surface antigen P1 was once described as a possible glucan-binding protein (5), the expression of the gene encoding P1, *spaP*, was examined in the IrvA+ strain and found to be significantly enhanced (Fig. 4C). The induction of *spaP* was further confirmed by Northern blot analysis (Fig. 4D). To determine whether an increase in P1 could account for the small aggregates formed in the absence of GbpC, DDAG was tested in an IrvA+ strain that had mutations of both *gbpC*

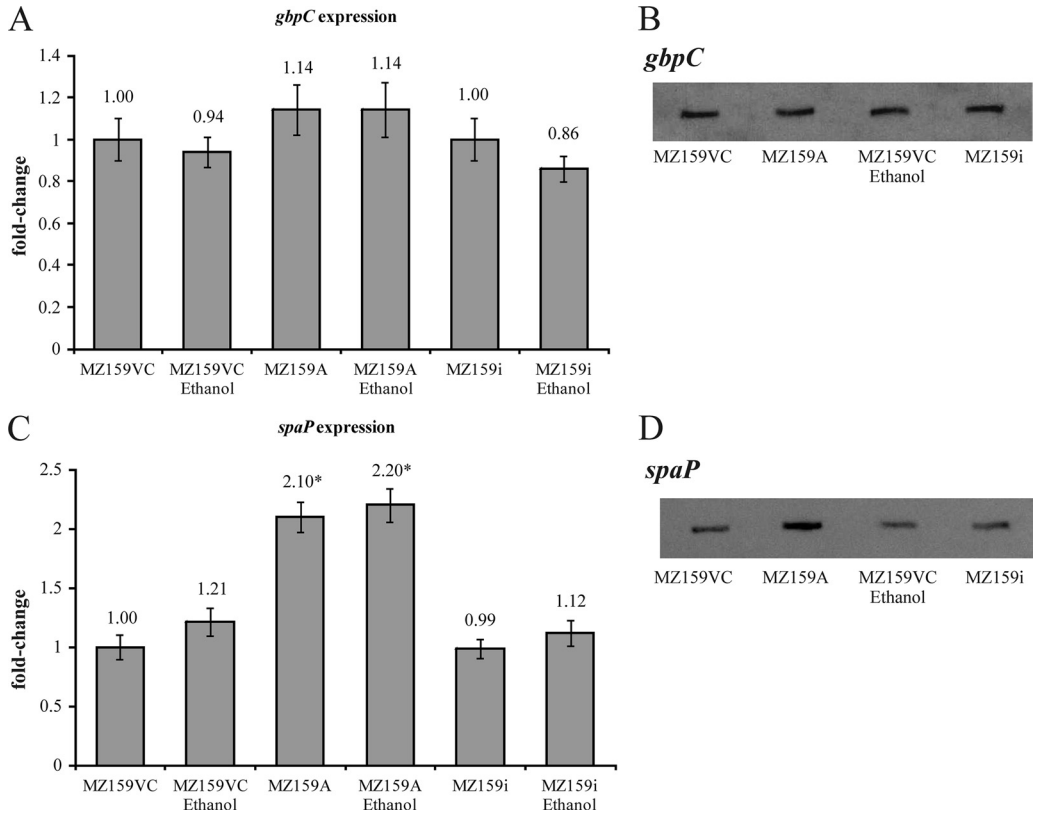

FIG. 4. Transcription of *gbpC* and *spaP* was measured by real-time PCR (A and C) and by Northern blotting (B and D). (A) The IrvA+ strain (MZ159A) displayed only a slight increase in expression of *gbpC* relative to that for the parental vector control (MZ159VC). The difference was within the margin of experimental error. Growth in the presence of ethanol did not increase expression of *gbpC*. (B) A Northern blot also supported a conclusion that *gbpC* expression was not appreciably changed in the IrvA+ strain (MZ159A). (C) The expression of the gene encoding P1, *spaP*, was examined in the IrvA+ strain (MZ159A) and found to be significantly enhanced (marked by an asterisk) compared to expression in the parental vector control (MZ159VC) as determined by a Student *t* test comparing the mean values of four independent trials ($P < 0.05$). (D) The induction of *spaP* was further confirmed by Northern blot analysis. In all instances, the *irvA* mutant (MZ159i) was similar to the parental vector control (MZ159VC).

and *spaP*. The loss of P1 resulted in the ablation of DDAG (Fig. 5).

Next, the impact of DDAG on biofilm formation was analyzed. Biofilms were generated in saliva-coated polystyrene wells and stained with safranin to view the overall appearance of the mature biofilm (Fig. 6). The overexpression of *irvA* (MZ159A) in the parental background did not alter biofilm appearance, nor did a mutation in *irvA* (MZ159i) (Fig. 6A). The parental vector control strain (MZ159VC), the *irvA* mutant strain (MZ159i), and the IrvA+ strain (MZ159A) all formed similar biofilms whether or not ethanol, which stimulates DDAG, was present (Fig. 6A). However, GbpC, the main mediator of large-particle DDAG, was necessary to maintain a biofilm morphology similar to that of the parental strain (Fig. 6B; compare the parental vector control, MZ159VC, with the *gbpC* mutant, MZ159c), even when grown in the absence of ethanol. The overexpression of *irvA* in the *gbpC* mutant background (MZ159Ac) compensated for the loss of GbpC, resulting in a biofilm similar to that generated by the parental strain (MZ159VC). When the *spaP* gene was inactivated (MZ159Acp), the overexpression of *irvA* was no longer able to restore the biofilm appearance that resulted from loss of GbpC (Fig. 6B).

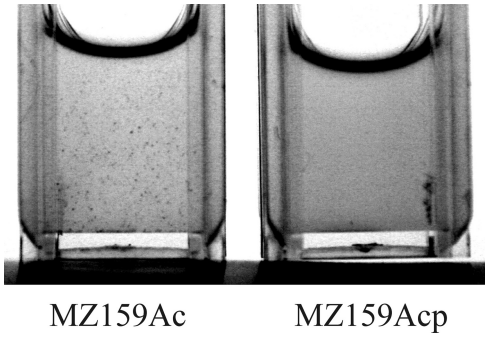

FIG. 5. DDAG was tested in an IrvA+ strain (MZ159Acp) that had mutations in both *gbpC* and *spaP* and compared to DDAG in an IrvA+ strain that had a mutation only in *gbpC* (MZ159Ac). The loss of P1 resulted in the ablation of the small-particle DDAG.

In an effort to determine the basis for the changes in biofilm appearance, mature biofilms were examined by confocal microscopy. Differences at the substratum were immediately apparent. To quantify these differences, substratum images from three random fields were analyzed by the program ImageJ to calculate the average numbers and sizes of microcolonies. These are designated particle count and particle size in Table

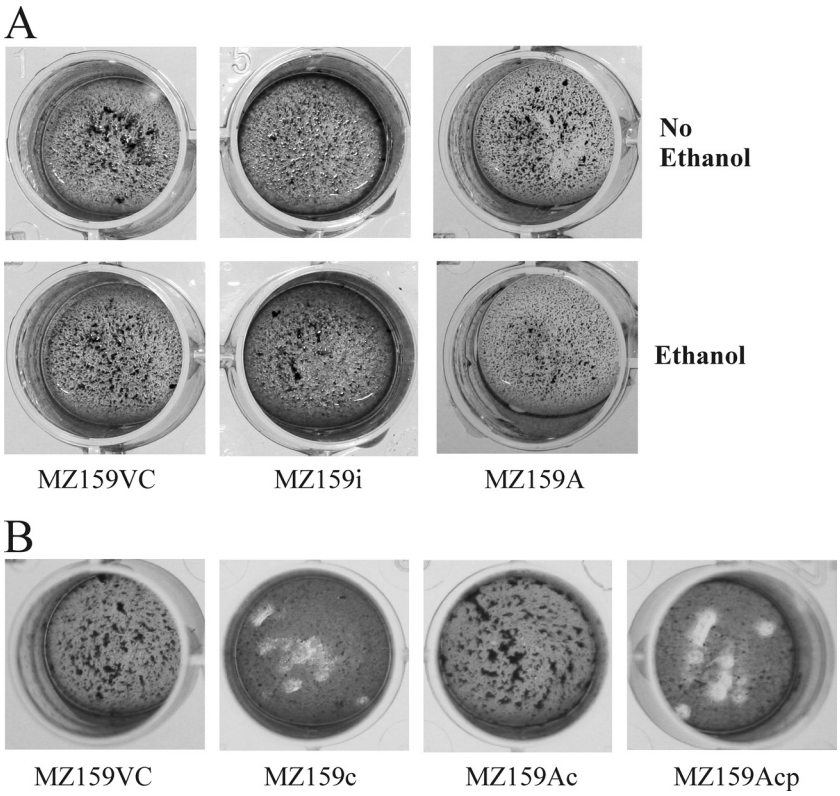

FIG. 6. Photographic images of mature biofilms formed by parental vector control and IrvA+ strains. (A) IrvA made little, if any, contribution to the macroscopic appearance of biofilms since biofilms produced by an *irvA* mutant (MZ159i) and an IrvA+ strain (MZ159A) appeared to be similar to those of the parental vector control (MZ159VC) strain. Growth in ethanol (second row of images), which stimulates DDAG, did not affect biofilm morphology for any of the strains. (B) The loss of GbpC (MZ159c) changed the biofilm appearance compared to that of the parental vector control strain (MZ159VC), despite growth under a condition that does not promote DDAG (no ethanol). Overexpression of *irvA* (MZ159Ac) overcame the change in biofilm appearance caused by the loss of GbpC, unless the *spaP* gene was inactivated (MZ08Acp).

2. The IrvA+ strain (MZ159A) formed biofilms with significantly fewer microcolonies of larger average size than did the parental strain (MZ159VC). Microcolonies within biofilms formed by an *irvA* mutant (MZ159i) did not differ from those formed by the parental strain. The inactivation of *gbpC* (MZ159c) led to very small microcolonies with significantly reduced particle size and significantly increased particle count relative to those for the parental strain. These GbpC-based changes had previously been reported in a qualitative manner (22). When *irvA* was added to the *gbpC* mutant background (MZ159Ac), the particle count significantly decreased, and the particle size significantly increased relative to those for the *gbpC* mutant strain (MZ159c). These data demonstrate that the overexpression of *irvA* significantly increases the size of individual microcolonies.

TABLE 2. Confocal image analysis of biofilm microcolonies at the substratum

S. mutans strain	Particle count	Particle size (bp)
MZ159VC	4,549 ± 186	70.0 ± 7.6
MZ159A	3,917 ± 67[a]	110.9 ± 12.1[a]
MZ159i	5,521 ± 191	72.9 ± 7.8
MZ159c	11,376 ± 879[a]	38.2 ± 5.4[a]
MZ159Ac	2,149 ± 102[b]	65.7 ± 8.0[b]

[a] Significantly different from the parental strain (MZ159VC) based on a Student *t* test comparing means.
[b] Significantly different from MZ159c based on a Student *t* test comparing means.

DISCUSSION

The precise significance of DDAG as it relates to *S. mutans* sucrose-dependent adhesion and biofilm formation is still a subject of speculation. DDAG is apparent under routine laboratory culture conditions for some closely related species within the mutans streptococci but is induced in *S. mutans* only under stressful growth conditions, such as the inclusion of 4% ethanol or sublethal dosages of antibiotics in the growth medium (27). *S. mutans* DDAG has been attributed to GbpC (27), yet *gbpC* is expressed even in the absence of DDAG, and Biswas et al. (4) report that transcription of *gbpC* does not increase when ethanol is added to the growth medium. Still, it appears that increased *gbpC* expression can promote DDAG. Sato et al. (28) reported that growth in the presence of xylitol increased both *gbpC* expression and DDAG. In addition, Niu et al. (25) demonstrated that inactivation of *irvR*, encoding a transcriptional repressor of *irvA* and reminiscent of the lambda cI/Cro repressor system, resulted in elevated levels of GbpC and DDAG that did not require special growth conditions. The

results of the current study add another layer of complexity to DDAG and its contribution to biofilm development.

The initial observation in this study was that overexpression of *irvA*, accomplished by providing a copy of *irvA* on an extrachromosomal plasmid, induced DDAG that was independent of stressful growth conditions. A similar phenotype was observed when *S. mutans irvA* was introduced into *Streptococcus gordonii*, whereas *Streptococcus mitis* was unaffected (data not shown). These results suggested that DDAG is a property shared by at least one primary plaque-colonizing species and that it may be regulated by a similar mechanism. The induction of DDAG in the IrvA+ strain was not accompanied by a significant increase in transcription of *gbpC*. To reconcile this observation with the report that derepression of *irvA* leads to increased *gbpC* expression, we propose that the latter increase in *gbpC* expression is due to transcriptional read-through, rather than regulatory activity of IrvA at the *gbpC* promoter. Since *irvA* was overexpressed in *trans* in our experiments, transcriptional read-through was not a possibility.

Despite the fact that *irvA* overexpression did not result in increased expression of *gbpC*, inactivation of *gbpC* severely diminished DDAG in the IrvA+ strain. However, a novel finding was that small-particle aggregates remained even in the absence of GbpC. These were specific for the IrvA+ strain and were eventually shown to require the presence of P1. At one time, it was speculated that P1 was a glucan-binding protein (5), but no published evidence of that possibility exists. The fact that P1 shares some sequence homology with GbpC (27), along with its apparent role in forming small, dextran-induced aggregates, suggests that a possible glucan-binding role for P1 is not out of the question. Such a role might also explain why biofilms formed in the presence of sucrose by a mutant missing GbpA, -C, and -D remain significantly more robust than biofilms formed in the absence of sucrose (22). However, contributions from other cell components cannot be ruled out. For example, it has been reported that a mutation in *spaP* is associated with a decreased retention of WapA on the cell wall (12) and that WapA has the property of binding dextran (11).

Another unexpected conclusion from this study was that the changes that accompanied the overexpression of *irvA* had a significant impact on certain characteristics of sucrose-dependent biofilms. At the macroscopic level, the appearance of biofilms formed by the parental, *irvA* mutant, or IrvA+ strains did not appear to differ regardless of whether the biofilms were formed in the presence or absence of ethanol. Thus, the conditions that enhanced DDAG did not visibly alter biofilm morphology. Yet the loss of GbpC, the main component responsible for large-particle DDAG, resulted in biofilms that were noticeably different from those formed by the parental strain. Interestingly, the overexpression of *irvA* rescued the *gbpC* mutant biofilm phenotype. Thus, the small-particle DDAG induced by overexpression of *irvA* had an effect that was noticeable macroscopically only when GbpC was absent. A microscopic examination of the biofilms was made in an effort to further understand the basis for these observations. The results showed that small-particle aggregation accompanying *irvA* overexpression affected the size and quantity of microcolonies at the biofilm substratum. The small-particle aggregation was eliminated when the *spaP* gene was mutated, as was the ability of *irvA* overexpression to compensate for the loss of *gbpC* in biofilm development. These data suggest that small-particle DDAG is mediated by P1, though we did not distinguish between P1 being necessary and it being both necessary and sufficient to bring about the observed results. This study also did not address whether the increased expression of *spaP* in the IrvA+ background was due to direct activation by IrvA acting as a transcriptional regulator.

It is relatively common for autoaggregation to be linked to enhanced adhesion and biofilm formation in a number of human pathogens and environmental species (6, 9, 13, 18, 21, 32). Aggregation of *S. mutans* by salivary agglutinins, however, can either promote removal by swallowing or promote adhesion to the tooth surface (1). Since the property of DDAG in *S. mutans* is induced only under specific in vitro laboratory culture conditions, it is unclear whether DDAG promotes efficient adhesion and accumulation of *S. mutans* within the dental plaque biofilm. There appears to be at least one (CovR), if not more, repressor that can regulate *gbpC* expression (4, 29). In addition, induction of *irvA* coincides with greater *gbpC* expression (25). Biswas et al. (4) report that expression of *gbpC* is reduced at low pH, a condition that might be more common within an established biofilm.

An alternative possibility is that DDAG is a response to stress, with the aggregates being collectively more resistant to environmental conditions (8). In this scenario, the repression of *irvR* would induce expression of *irvA* and *gbpC*. In turn, IrvA would induce expression of *spaP* and perhaps several other genes.

Determining the environmental signals that regulate *gbpC* and remove repression of *irvA* may hold the keys to understanding the role of DDAG in *S. mutans* plaque survival. Clearly, however, DDAG encompasses aggregation of various sizes that holds substantial potential to influence how this organism interacts with its environment.

ACKNOWLEDGMENTS

This work was supported by grant DE10058 (J.A.B.) from the National Institute of Dental and Craniofacial Research and by COBRE grant RR018741 (J.M. and D.A.) from the National Center for Research Resources.

REFERENCES

1. **Ahn, S.-J., S.-J. Ahn, Z. T. Wen, L. J. Brady, and R. A. Burne.** 2008. Characteristics of biofilm formation by *Streptococcus mutans* in the presence of saliva. Infect. Immun. **76:**4259–4268.
2. **Ajdić, D., W. M. McShan, R. E. McLaughlin, G. Savic, J. Chang, M. B. Carson, C. Primeaux, R. Tian, S. Kenton, H. Jia, S. Lin, Y. Qian, S. Li, H. Zhu, F. Najar, H. Lai, J. White, B. A. Roe, and J. J. Ferretti.** 2002. Genome sequence of *Streptococcus mutans* UA159, a cariogenic dental pathogen. Proc. Natl. Acad. Sci. USA **99:**14434–14439.
3. **Banas, J. A., K. R. Hazlett, and J. E. Mazurkiewicz.** 2001. An in vitro model for studying the contributions of the *Streptococcus mutans* glucan-binding protein A to biofilm structure. Methods Enzymol. **337:**425–433.
4. **Biswas, I., L. Drake, and S. Biswas.** 2007. Regulation of *gbpC* expression in *Streptococcus mutans*. J. Bacteriol. **189:**6521–6531.
5. **Curtiss, R., III.** 1985. Genetic analysis of *Streptococcus mutans* virulence, p. 253–277. *In* W. Goebel (ed.), Current topics in microbial immunity. Springer-Verlag, New York, NY.
6. **De Windt, W., H. Gao, W. Kromer, P. Van Damme, J. Dick, J. Mast, N. Boon, J. Zhou, and W. Verstraete.** 2006. AggA is required for aggregation and increased biofilm formation of a hyper-aggregating mutant of *Shewanella oneidensis* MR-1. Microbiology **152:**721–729.
7. **Drake, D., K. G. Taylor, A. S. Bleiweis, and R. J. Doyle.** 1988. Specificity of the glucan-binding lectin of *Streptococcus cricetus*. Infect. Immun. **56:**1864–1872.
8. **Farrell, A., and B. Quilty.** 2002. Substrate-dependent autoaggregation of *Pseudomonas putida* CP1 during the degradation of monochlorophenols and phenol. J. Ind. Microbiol. Biotechnol. **28:**316–324.

9. **Felek, S., M. B. Lawrenz, and E. S. Krukonis.** 2008. The *Yersinia pestis* autotransporter YapC mediates host cell binding, autoaggregation and biofilm formation. Microbiology **154:**1802–1812.
10. **Hamada, S., and H. D. Slade.** 1980. Biology, immunology, and cariogenicity of *Streptococcus mutans*. Microbiol. Rev. **44:**331–384.
11. **Han, T. K., and M. L. Dao.** 2005. Differential immunogenicity of a DNA vaccine containing the *Streptococcus mutans* wall-associated protein A gene versus that containing a truncated derivative antigen A lacking in the hydrophobic carboxyterminal region. DNA Cell Biol. **24:**574–581.
12. **Harrington, D. J., and R. R. B. Russell.** 1993. Multiple changes in cell wall antigens of isogenic mutants of *Streptococcus mutans*. J. Bacteriol. **175:**5925–5933.
13. **Hart, E., J. Yang, M. Tauschek, M. Kelly, M. J. Wakefield, G. Frankel, E. L. Hartland, and R. M. Robins-Browne.** 2008. RegA, an AraC-like protein, is a global transcriptional regulator that controls virulence gene expression in *Citrobacter rodentium*. Infect. Immun. **76:**5247–5256.
14. Reference deleted.
15. Reference deleted.
16. Reference deleted.
17. **Johnson, L. R.** 2008. Microcolony and biofilm formation as a survival strategy for bacteria. J. Theor. Biol. **251:**24–34.
18. **Kirisits, M. J., L. Prost, M. Starkey, and M. R. Parsek.** 2005. Characterization of colony morphology variants isolated from *Pseudomonas aeruginosa* biofilms. Appl. Environ. Microbiol. **71:**4809–4821.
19. **LeBlanc, D. J., L. N. Lee, and G. M. Dunny.** 1991. A shuttle vector containing a polylinker region flanked by transcription terminators which replicates in *Escherichia coli* and in many gram-positive cocci, p. 306. *In* G. M. Dunny, P. P. Cleary, and L. L. McKay (ed.), Genetics and molecular biology of streptococci, lactococci, and enterococci. American Society for Microbiology, Washington, DC.
20. **Loesche, W. J.** 1986. Role of *Streptococcus mutans* in human dental decay. Microbiol. Rev. **50:**353–380.
21. **Luo, F., S. Lizano, S. Banik, H. Zhang, and D. E. Bessen.** 2008. Role of Mga in group A streptococcal infection at the skin epithelium. Microb. Pathog. **45:**217–224.
22. **Lynch, D. J., T. L. Fountain, J. E. Mazurkiewicz, and J. A. Banas.** 2007. Glucan-binding proteins are essential for shaping *Streptococcus mutans* biofilm architecture. FEMS Microbiol. Lett. **268:**158–165.
23. **Macrina, F. L., C. L. Keeler, Jr., K. R. Jones, and P. H. Wood.** 1980. Molecular characterization of unique deletion mutants of the streptococcal plasmid, pAMβ1. Plasmid **4:**8–16.
24. **Merritt, J., J. Kreth, W. Shi, and F. Qi.** 2005. LuxS controls bacteriocin production in *Streptococcus mutans* through a novel regulatory component. Mol. Microbiol. **57:**960–969.
25. **Niu, G., T. Okinaga, L. Zhu, J. Banas, F. Qi, and J. Merritt.** 2008. Characterization of *irvR*, a novel regulator of the *irvA*-dependent pathway required for genetic competence and dextran-dependent aggregation in *Streptococcus mutans*. J. Bacteriol. **190:**7268–7274.
26. Reference deleted.
27. **Sato, Y., Y. Yamamoto, and H. Kizaki.** 1997. Cloning and sequence analysis of the *gbpC* gene encoding a novel glucan-binding protein of *Streptococcus mutans*. Infect. Immun. **65:**668–675.
28. **Sato, Y., Y. Yamamoto, and H. Kizaki.** 2000. Xylitol-induced elevated expression of the *gbpC* gene in a population of *Streptococcus mutans* cells. Eur. J. Oral Sci. **108:**538–545.
29. **Sato, Y., Y. Yamamoto, and H. Kizaki.** 2000. Construction of region-specific partial duplication mutants (merodiploid mutants) to identify the regulatory gene for the glucan-binding protein C gene in vivo in *Streptococcus mutans*. FEMS Microbiol. Lett. **186:**187–191.
30. **Steiner, K., and H. Malke.** 1995. Transcription termination of the streptokinase gene of *Streptococcus equisimilis* H46A: bidirectionality and efficiency in homologous and heterologous hosts. Mol. Gen. Genet. **246:**374–380.
31. **Tsang, P., J. Merritt, W. Shi, and F. Qi.** 2006. IrvA-dependent and IrvA-independent pathways for mutacin gene regulation in *Streptococcus mutans*. FEMS Microbiol. Lett. **261:**231–234.
32. **Walter, J., C. Schwab, D. M. Loach, M. G. Ganzle, and G. W. Tannock.** 2008. Glucosyltransferase A (GtfA) and inulosucrase (Inu) of *Lactobacillus reuteri* TMW1.106 contribute to cell aggregation, in vitro biofilm formation, and colonization of the mouse gastrointestinal tract. Microbiology **154:** 72–80.