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Characterization of *irvR*, a Novel Regulator of the *irvA*-Dependent Pathway Required for Genetic Competence and Dextran-Dependent Aggregation in *Streptococcus mutans*▽

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Previous studies identified *irvA* as a normally repressed but highly inducible transcription regulator capable of repressing mutacin I gene expression in *Streptococcus mutans*. In this study, we aimed to identify and characterize the regulator(s) responsible for repressing the expression of *irvA*. An uncharacterized open reading frame (SMU.1398) located immediately adjacent to *irvA* and annotated as a putative transcription repressor was identified as a likely candidate. The results of mutation studies confirmed that the expression of *irvA* was greatly increased in the SMU.1398 background. Mutation of SMU.1398 ("*irvR*") abolished genetic competence and reduced the expression of the late competence genes/operons *comEA*, *comY*, and *dprA* without affecting the expression of the known competence regulators *comC*, *comED*, or *comX*. In addition, *irvR* was found to be a potent negative regulator of dextran-dependent aggregation (DDAG) and *gbpC* expression. Each of these *irvR* mutant phenotypes could be rescued with a double mutation of *irvA* or complemented by introducing a wild-type copy of *irvR* on a shuttle vector. These data indicate that the repression of *irvA* is critically dependent upon *irvR* and that *irvA* repression is essential for the development of genetic competence and the proper control of DDAG in *S. mutans*.

Streptococcus mutans is a gram-positive oral commensal species found in human dental plaque and is primarily associated with the initiation of caries development (tooth decay) (3, 8, 28, 30, 38, 41, 45). Certain species, such as *S. mutans*, have a much greater capacity to both excrete acidic metabolites (acidogenic) and proliferate in an acidic environment (aciduric) and thus, can gain a competitive advantage over nonaciduric species (2, 29). Yet, examinations of oral plaque samples and carious lesions have identified numerous other aciduric species (4, 9), which suggests that the success of *S. mutans* as a dental pathogen cannot be solely attributed to its acid tolerance. Biofilm formation, natural competence, and bacteriocin production are also recognized as virulence factors that are necessary for the persistence of *S. mutans* in the presence of numerous environmental stresses and fierce interspecies competition (2, 21, 29).

Studies of genetic factors that regulate these processes in *S. mutans* have found a surprising variety of genetic mutations that each affect multiple virulence factors and stress tolerances simultaneously (7, 14–18, 20, 22–24, 26, 33, 36, 39, 43). This implies that a large overlap must exist between the pathways responsible for the regulation of persistence-related abilities. For example, our laboratory and others have observed that a mutation in the *S. mutans* ortholog of *luxS* creates altered oxidative and acid stress tolerances, as well as defects in biofilm formation, natural competence, and bacteriocin (mutacin I) production. (25–27, 37, 43, 44).

Previously, our laboratory further investigated the mutacin I phenotype of the *luxS* mutant and identified an uncharacterized transcription regulator, which we referred to as *irvA*, as a mediator of the mutacin I deficiency (25). Following the deletion of *luxS*, this gene was found to be strongly expressed, along with the concomitant loss of mutacin I production. A *luxS irvA* double-deletion strain regained the ability to produce mutacin I, whereas an engineered constitutive *irvA* expression strain was mutacin I deficient, even in a wild-type *luxS* background. Thus, it was concluded that *irvA* was an intermediate component of the pathway responsible for the repression of mutacin I in a *luxS* mutant background. In addition, our laboratory and others have found that the stress-responsive gene *gbpC* is also induced in the *luxS* background (25, 37). Since *gbpC* expression has been found to be highly responsive to numerous environmental stresses (6, 34, 35), we had speculated that the *luxS* mutation may similarly trigger various stress pathways in the cell, which may account for both *gbpC* and *irvA* induction (25). Likewise, *irvA* induction has also been found to be associated with several other genetic mutations known to have multiple stress- and virulence-related phenotypes (40). Moreover, *irvA* has thus far been detected in numerous strains of *S. mutans* (1, 25, 42); therefore, it may be a fundamental component of the basic machinery utilized to modulate multiple virulence-associated functions required for persistence.

In the current study, we report the identification of a putative repressor which is required for preventing *irvA* expression. This gene, which we refer to as *irvR*, is also absolutely required for the development of genetic competence and the proper regulation of dextran-dependent aggregation (DDAG). Fur-

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TABLE 1. Bacterial strains and plasmids used in this study

Strain/plasmid	Relevant characteristics[a]	Reference/source
Strains		
E. coli JM109	e14$^-$(McrA$^-$) *recA1 endA1 gyrA96 thi-1 hsdR17* (r_K^- m_K^+) *supE44 relA1* Δ(*lac-proAB*) [F′ *traD36 proAB lacI$_q$ZΔM15*]	Cloning strain
S. mutans		
UA159	Wild-type *S. mutans*	1
GN01R	UA159 Δ*irvR* Km^r	This study
GN01Rs	UA159 Δ*irvR* Km^r; pDL278	This study
GN01Rc	UA159 Δ*irvR* Km^r; pDL278-*irvR*	This study
GN01RA	UA159 Δ*irvA* Δ*irvR* Km^r	This study
LZ02C	UA159 *gbpC*::pFW5 Sp^r	This study
GN01RC	UA159 Δ*irvR gbpC*::pFW5 Km^r Sp^r	This study
Plasmids		
pBluescript	Cloning vector; Ap^r	Stratagene
pCR2.1	Cloning vector; Ap^r Km^r	Invitrogen
pSC-A	Cloning vector; Ap^r	Stratagene
pFW5	Suicide vector; Sp^r	32
pLZ02C	pFW5 *gbpC* internal fragment; Sp^r	This work
pGN01R	pFW5 allelic replacement of *irvR*; Sp^r	This work
pDL278	*E. coli-Streptococcus* shuttle vector; Sp^r	11
pGN01Rc	pDL278 *irvR*$^+$ Sp^r	This work
pGNaa3	pSC-A *aphAIII*$^+$ Ap^r Km^r	This work

[a] Ap^r, ampicillin resistance; Km^r, kanamycin resistance; Sp^r, spectinomycin resistance.

TABLE 2. Primers used in this study

Primer	Sequence (5′→3′)	Purpose
Kan F	AGGTGATAGGTAAGATTA TACCG	*irvR*; *irvR irvA* deletions
Kan R	CCCTATCTAGCGAACTTT TAGA	*irvR*; *irvR irvA* deletions
irvR Up F	CGCTGCAGTTTCCTTGTGT GTGACTTTC	*irvR* deletion
irvR Up R	GCGAATTCAGCCTTCATT GCTTCTAAATC	*irvR* deletion
irvR Dn F	GCGAATTCATCGGAACGG TCATTACCTAA	*irvR*; *irvR irvA* deletions
irvR Dn R	CCACTAGTCCTGTAATTTT TCATAAGGAAGAGG	*irvR*; *irvR irvA* deletions
irvR-c F	CATATGAAATACTTTT AATT	*irvR* complementation
irvR-c R	CCGCTCGAGTTAGGTAAT GACCGTTCC	*irvR* complementation
gbpC F	GTCGACATCTGGGCGTGT TGAAAAAG	*gbpC* insertion
gbpC R	GGATCCAATGGCATTATT GCCGCTTA	*gbpC* insertion
irvA RT F	CCCTCAACACACTCTGCTA AGCT	*irvA* RT-PCR
irvA RT R	CCAAATCATTGGCCAGT TGAA	*irvA* RT-PCR
comEA RT F	AGGAACAATCCCTTCAGG TAACC	*comEA* RT-PCR
comEA RT R	CAGTCGTCTGCGTCTTCT TCTG	*comEA* RT-PCR
dprA RT F	GCGGTTTAGCGCGTG GTAT	*dprA* RT-PCR
dprA RT R	GCTCCACCGCTTTTAAGA CTTG	*dprA* RT-PCR
comY RT F	CTTTTTTCTGGACGTCACG ATTT	*comYA* RT-PCR
comY RT R	TCGCCCCTTGATTTCAT TTAA	*comYA* RT-PCR
gbpC RT F	AATTCTGATACTGTTGCA GCACCTA	*gbpC* RT-PCR
gbpC RT R	TTCTGTTGCAGCCGGTTCT	*gbpC* RT-PCR
16S RT F	ATCACTAGTAGATGGA CCTG	RT-PCR normalization
16S RT R	TGTATCGTCGCCTTGG TAAG	RT-PCR normalization

thermore, both of these phenotypes are critically dependent upon the presence of *irvA* as well. Thus, *irvR* and *irvA* may form a regulatory pair that is responsible for controlling important stress responses and virulence factors and may be mediators of a variety of phenotypes found in various mutant strains of *S. mutans*.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. Bacterial strains and plasmids and their relevant characteristics are listed in Table 1. *Escherichia coli* cells were grown in Luria-Bertani (LB; Difco) medium at 37°C. *E. coli* strains carrying plasmids were selected with 100 μg ml^{-1} ampicillin (Fluka), 100 μg ml^{-1} kanamycin (EMD), or 150 μg ml^{-1} spectinomycin (Sigma). All *S. mutans* strains were grown anaerobically (85% N_2, 10% CO_2, and 5% H_2) at 37°C. *S. mutans* strains were cultivated in either brain-heart infusion (BHI) or Todd-Hewitt medium (Difco). For the selection of antibiotic-resistant colonies, BHI plates were supplemented with 800 μg ml^{-1} kanamycin, 15 μg ml^{-1} tetracycline (Sigma), or 900 μg ml^{-1} spectinomycin.

Construction of mutants. To study the role of *irvR* in *S. mutans*, we constructed an *irvR* deletion mutant via double-crossover homologous recombination. To generate the construct, two fragments corresponding to approximately 1 kb of the upstream and downstream sequences of *irvR* were generated by PCR, using *Pfu* polymerase with the primer pairs irvR Up F/irvR Up R, and irvR Dn F/irvR Dn R (Table 2). Each of the primers incorporated restriction enzyme sites, and the PCR amplicons were subsequently cleaved with the appropriate restriction enzymes and cloned into pFW5. The kanamycin resistance gene *aphAIII* was amplified by PCR from the plasmid pTV1-OK (16) by using the primers Kan F and Kan R (Table 2) and cloned into pSC-A (Stratagene) to create pGNaa3. The kanamycin resistance gene was excised from pGNaa3 with EcoRI and ligated in between the *irvR* upstream and downstream fragments to create the plasmid pGN01R. The plasmid was confirmed via restriction digestion and linearized for transformation into *S. mutans* UA159. For complementation analysis, the complete *irvR* open reading frame and 250 bp of the upstream intergenic region was amplified by PCR with irvR-c F and irvR-c R (Table 2). The addition of 5′-phosphates to the primers with T4 polynucleotide kinase (NEB) allowed the subsequent ligation of the PCR product to the HincII site of pDL278 to create pGN01Rc. The *irvA irvR* double deletion was created by using a strategy similar to that for the *irvR* deletion mutant except that the fragment upstream of *irvA* was obtained from a previously cloned fragment in pCR2.1 (Invitrogen) used for the deletion of *irvA* (25). This fragment was digested by using restriction sites compatible to the kanamycin resistance cassette and ligated along with the *irvR* downstream fragment as described previously (Table 2). The *gbpC* mutant was constructed by single-crossover insertion inactivation using an internal 0.5-kb fragment of *gbpC* amplified by PCR with the primer pairs gbpC F and gbpC R (Table 2). The PCR product was subcloned into the pCR2.1 vector (Invitrogen), digested with BamHI and SalI, and cloned into the suicide vector pFW5. The resulting plasmid, pLZ02C, was confirmed via restriction digestion and transformed into *S. mutans* UA159. The *gbpC irvR* double-mutant strain GN01RC was obtained by transforming linearized pGN01R into the *gbpC* mutant strain. All mutant derivatives of UA159 were confirmed by PCR.

RNA extraction and quantitative real-time RT-PCR. *S. mutans* UA159 and its derivatives were cultivated overnight at 37°C. The overnight cultures were diluted 1:30 in BHI with 0.4% bovine serum albumin in a total volume of 30 ml. The cells were allowed to grow to an optical density at 600 nm of ~0.3 and collected by centrifugation. The pellets were resuspended in 700 μl Tris-EDTA

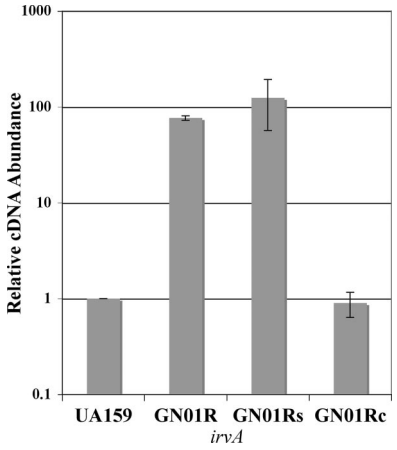

FIG. 1. Expression of *irvA* in various *irvR* (SMU.1398) backgrounds. The relative expression levels of *irvA* were determined by real-time RT-PCR and compared between the wild-type strain UA159 (arbitrarily assigned as 1), the *irvR* mutant (GN01R), the *irvR* mutant strain harboring an empty shuttle vector (GN01Rs), and the complemented mutant strain (GN01Rc). Data are shown as the averages of the results of three independent experiments. All real-time RT-PCR values were normalized according to the abundance of 16S rRNA in each sample. Error bars show standard deviations.

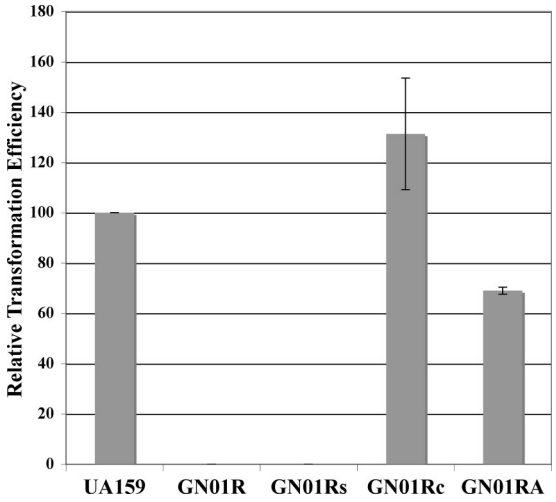

FIG. 2. Results of transformation efficiency assays in *irvR* mutant strains. The transformation efficiency values are presented relative to the wild-type value (6.0×10^{-7}), which was arbitrarily assigned as 100% and compared to the values for the *irvR* mutant (GN01R), the mutant strain harboring an empty shuttle vector (GN01Rs), the complemented mutant strain (GN01Rc), and the *irvR irvA* double mutant (GN01RA). Data are presented as the averages of the results of three independent experiments performed in triplicate. Error bars show standard deviations.

buffer (pH 8.0) and transferred to a 2-ml screw-cap tube containing 500 μl 0.1-mm silica beads (Biospec). Six hundred microliters of Trizol (Sigma) was added to the tube, vortexed, and submitted to three consecutive 30-s homogenization cycles with a FastPrep-24 system (MP Biomedicals) set at a speed of 6.0 M/s. After homogenization, 200 μl chloroform (Sigma) was added, and the solution was centrifuged for 10 min at full speed. The supernatant was extracted three times with 450 μl acidic phenol (Sigma) and 200 μl chloroform. RNA was precipitated with isopropyl alcohol and washed with 70% ethanol. After drying, the RNA pellet was resuspended in 87 μl RNase-free water–10 μl 10× DNase buffer–3 μl RNase-free DNase (Ambion). The mixture was incubated at 37°C for 45 min. After incubation, samples were further purified with an RNeasy spin column (Qiagen) and eluted in 30 μl RNase-free water. Five hundred nanograms of total RNA was used for cDNA synthesis using SuperScript II (Invitrogen) according to the manufacturer's protocol. For real-time reverse transcription-PCR (RT-PCR), oligonucleotide primers were designed with Primer Express 3.0 software (Applied Biosystems), which selects primers optimized for "delta-delta threshold cycle" ($\Delta\Delta C_T$) method analysis. Real-time PCR was performed using an Applied Biosystems 7300 system, and the reaction mixtures were prepared using Applied Biosystems Sybr green PCR master mix. Changes in levels of gene expression were calculated automatically with the Applied Biosystems 7300 system software using the $\Delta\Delta C_T$ method, which is briefly described as follows: $\Delta C_T = C_T$(target) $- C_T$(housekeeping gene); $\Delta\Delta C_T = \Delta C_{T1} - \Delta C_{T2}$; the levels of change are calculated as $2^{-\Delta\Delta CT}$. The 16S rRNA gene was used as the housekeeping gene reference, and all cDNA synthesis reactions included a replicate reaction without added reverse transcriptase to assess genomic DNA contamination. The primers used for real-time RT-PCR are listed in Table 2.

Transformation assay. Genetic competence was determined by a transformation efficiency assay with genomic DNA and the *E. coli-Streptococcus* shuttle vector pDL278. Cells were grown as mentioned above to an optical density at 600 nm of ~0.3. Genomic DNA (10 μg ml^{-1}) or plasmid DNA (1 μg ml^{-1}) was added to each reaction mixture, and the cultures were incubated for an additional 2 h. After the incubation, the cultures were briefly sonicated (Misonix) to disperse the cells and plated on antibiotic-containing BHI agar plates, as well as on nonselective BHI plates. Successful transformation was scored based on antibiotic resistance, and the total viable cell population was determined by counting the number of colonies growing on nonselective plates. The transformation efficiency was determined by calculating the ratio of transformants to total viable cells.

Analysis of DDAG. The DDAG assay was performed using the BTR-G medium described by Sato et al. (34), as well as with BHI. In brief, *S. mutans* colonies were picked from BHI plates and incubated anaerobically in BHI broth overnight at 37°C. The cells were then diluted (1:100) into 3 ml fresh BHI or BTR-G broth. The cultures were incubated for an additional 24 h and then divided into two 1-ml portions with or without 100 μg ml^{-1} dextran T2000 from *Leuconostoc* spp. Each pair of tubes was swirled briefly, and aggregation was observed as obvious clumping and cell precipitation. Generally, DDAG was obvious within 1 to 2 min of swirling the cultures.

RESULTS

***irvR* is required to repress *irvA* transcription.** Since the results of previous studies suggested that *irvA* itself appeared to be strongly regulated at the transcriptional level, we aimed to identify and characterize its upstream regulator(s). Previously, we found through a BLASTP analysis of the putative sequence of IrvA that it matched strongly to a variety of Cro repressors from various gram-positive bacteriophages (25). Thus, we speculated that perhaps IrvA was itself controlled by a "CI-like" repressor, as is also the case with numerous temperate bacteriophages. Surprisingly, the most promising candidate was located directly adjacent to *irvA* in the genome (NCBI annotation, SMU.1398; Oralgen annotation, SMu1275). Its predicted amino acid sequence matched well with a variety of gram-positive bacteriophage CI repressors, with the highest homology to the streptococcal phage EJ-1 (37% identity; 50% similar overall). Based upon its putative role as a transcription repressor, we surmised that this gene would be required to maintain the low, basal-level expression of *irvA* normally seen during typical growth conditions. Therefore, we deleted SMU.1398 and measured the expression of *irvA* by real-time RT-PCR to determine whether this mutation could cause a derepression of *irvA*. As shown by the results in Fig. 1, the expression of *irvA* in the SMU.1398 deletion background was increased 76-fold over the level in the wild type and could be fully complemented by providing a wild-type copy of SMU.1398 in *trans*. As expected, the empty shuttle vector used for complementation had no effect upon *irvA* expression in the

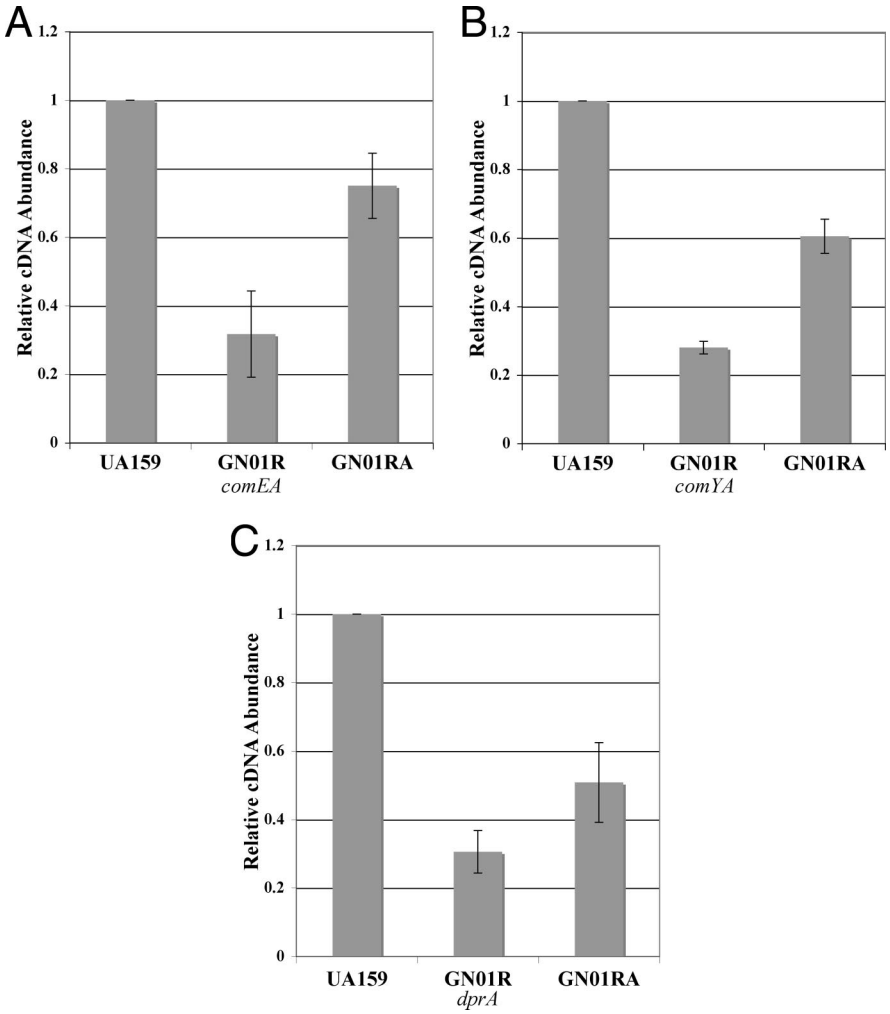

FIG. 3. Results of real-time RT-PCR analysis of late competence genes. The expression levels of late competence genes are presented relative to that of the wild type (UA159), which was arbitrarily assigned a value of 1. (A) Expression level of *comEA* in the *irvR* mutant strain GN01R compared to the levels in the wild-type strain UA159 ($P < 0.001$) and the *irvR irvA* double-mutant strain GN01RA ($P < 0.01$). (B) Expression level of *comYA* in strain GN01R compared to the levels in the wild type ($P < 0.0001$) and GN01RA ($P < 0.0001$). (C) Expression level of *dprA* in strain GN01R compared to the levels in the wild type ($P < 0.0001$) and GN01RA ($P < 0.01$) All data are presented as the averages of the results of five independent experiments. Statistical analysis was performed by using the two-tailed Student's *t* test. Error bars show standard deviations.

SMU.1398 mutant (Fig. 1). Thereafter, SMU.1398 was designated *irvR*, a repressor of *irvA*.

***irvR* is essential for the development of genetic competence.** Previously, we had determined that a *luxS* mutant has a reduced capacity for genetic competence (27). Likewise, we had observed a similar result in several other *irvA*-inducing mutants as well (unpublished observations). Therefore, we were curious to determine whether *irvR* could also affect genetic competence, since it seemed to be a major regulator of *irvA* expression. Interestingly, the *irvR* mutant strain was found to be severely deficient in its genetic competence ability, as we were unable to transform this strain after repeated attempts (Fig. 2). We also scaled up the transformation assay by 10-fold in order to sample a larger number of cells, but we were still unable to detect any transformants. Thus, the transformation efficiency of the *irvR* mutant was $<4 \times 10^{-10}$. In addition, we assayed competence over a range of optical densities, as well as in the presence of added synthetic competence-stimulating peptide, and found that the *irvR* mutant remained untransformable (data not shown). However, despite the various failed attempts to transform the *irvR* mutant, we were able to fully restore the transformation defect with a *trans*-complementation of *irvR* (Fig. 2). Furthermore, since the results of our previous studies suggested that *irvA* expression seemed to be correlated with reduced genetic competence, we created a double mutant of both *irvA* and *irvR* to determine whether the competence defect of the *irvR* mutant could be suppressed. As shown by the results in Fig. 2, this did appear to be the case, which suggests that both *irvR* and *irvA* function in the same pathway responsible for regulating genetic competence.

***irvR* affects the expression of late competence genes via *irvA*.** The regulation of genetic competence occurs through a well-characterized cascade of transcriptional regulation, ultimately resulting in the expression of numerous late competence genes required for the uptake and recombination of transforming DNA. Given that both *irvR* and *irvA* seemed to have a major

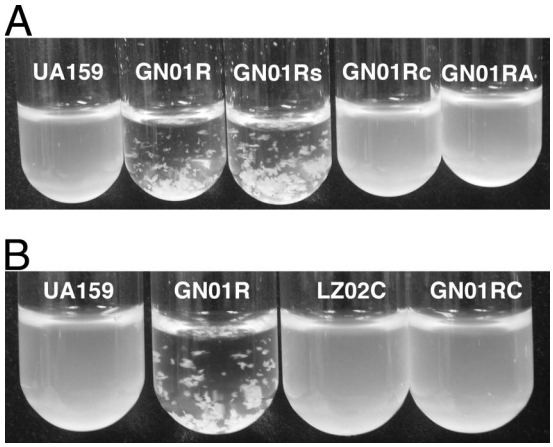

FIG. 4. Results of DDAG assays in various *irvR* backgrounds. (A) Dextran T2000 was added to the wild-type strain UA159, the *irvR* mutant (GN01R), the *irvR* mutant strain harboring an empty shuttle vector (GN01Rs), the complemented *irvR* mutant (GN01Rc), and the *irvR irvA* double mutant (GN01RA). (B) The same experiment was performed using the wild-type strain UA159, the *irvR* mutant (GN01R), the *gbpC* mutant (LZ02C), and the *gbpC irvR* double mutant (GN01RC). Shown here are representative results seen after approximately 1 to 2 min of gentle agitation. These experiments were performed three times with similar results.

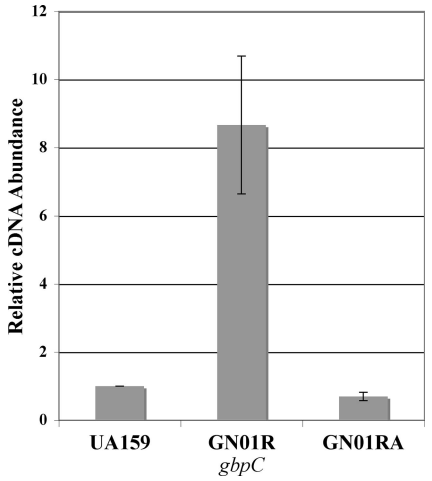

FIG. 5. Results of real-time RT-PCR analysis of *gbpC*. The expression level of *gbpC* in the wild type (arbitrarily assigned as 1) is compared to the levels in the *irvR* (GN01R) and *irvR irvA* (GN01RA) mutant backgrounds. The data are the averages of the results of three independent experiments. Statistical analysis was performed by using the two-tailed Student's *t* test. Error bars show standard deviations.

role in competence development, we were curious to determine whether the competence defect seen in the *irvR* mutant strain would also correlate with the altered expression of any of the known competence genes. Therefore, we tested the expression of the well-characterized competence gene regulators *comC*, *comED*, and *comX*, as well as genes required during late competence, such as *comYA*, *comEA*, *comEB*, *comFA*, *cinA*, *coiA*, *dprA*, *endA*, *mecA*, and *recA*. Of these, the expression levels of three late competence genes essential for natural transformability (*comEA*, *comYA*, and *dprA*) were significantly reduced in the *irvR* background ($P < 0.001$) and rescued by an *irvR irvA* double mutation ($P < 0.01$) (Fig. 3). Surprisingly, no effect was seen upon the expression of the late competence gene regulators *comC*, *comED*, and *comX* (data not shown). Thus, the *irvA*-dependent pathway appears to circumvent the upstream components of the competence cascade to affect these genes through an alternate mechanism.

Mutation of *irvR* induces DDAG via *gbpC*. As mentioned previously, in our prior studies of a *luxS* mutant, we had observed increased expression of *gbpC* (25), an essential mediator of DDAG (34, 35). Conspicuously, we had noted increased *gbpC* expression in several other *irvA*-inducing mutations as well (unpublished observations). Therefore, we reasoned that perhaps the *irvR* mutant might exhibit phenotypes related to DDAG. Upon the addition of 100 μg ml^{-1} dextran T2000, we found that the *irvR* mutant strain displayed rapid aggregation, with the cells visibly clumped and fully precipitated within 1 to 2 min (Fig. 4A). Furthermore, this phenotype appeared independent of the growth medium, as it occurred similarly with both the published medium for DDAG assays (BTR-G) (35) and BHI medium. Interestingly, this response also did not require pretreatment with any of the environmental stress conditions normally used to induce DDAG, such as the addition of a sub-MIC concentration of tetracycline or heat shock (6, 35). As expected, no aggregation was observed in the absence of dextran (data not shown). Similar to the competence phenotype, full complementation was observed with the introduction of a wild-type copy of *irvR* on a shuttle plasmid, and a double mutant of *irvR* and *irvA* was also able to suppress the *irvR* phenotype (Fig. 4A). This suggests that both *irvR* and *irvA* are components of the pathway responsible for the DDAG phenotype. In order to further characterize this pathway, we examined the role of *gbpC* as well. Consistent with previous suggestions of *gbpC* as a mediator of DDAG, a double mutation of *gbpC* and *irvR* potently suppressed the DDAG phenotype of the *irvR* mutant (Fig. 4B). As a further confirmation of this result, we also compared the expression of *gbpC* in the wild-type, *irvR* mutant, and *irvR irvA* double-mutant strains. In the *irvR* background, *gbpC* expression was significantly increased over its level in both the wild type ($P = 0.022$) and the *irvR irvA* double mutant ($P = 0.018$). In addition, the *irvR irvA* double mutant had *gbpC* expression nearly identical to that of the wild type (Fig. 5). These results suggest that both *irvA* and *gbpC* are essential mediators of the DDAG phenotype in the *irvR* mutant strain and that *irvA* is apparently upstream of *gbpC* in this pathway.

DISCUSSION

In the current study, we describe a novel regulator, *irvR*, which comprises an essential portion of the upstream regulatory pathway required for the repression of *irvA*, a recently characterized transcription regulator induced by a variety of genetic mutations (25, 40). Based upon our previous experience with phenotypes associated with *irvA* induction, we tested both genetic competence and DDAG in the *irvR* background. Competence was found to be severely inhibited, whereas DDAG appeared to be hyperactive. Both of these phenotypes could be complemented efficiently by the introduction of a wild-type copy of *irvR* on a shuttle plasmid or by creating a

double mutant of both *irvR* and *irvA*. These data implicate *irvR* and *irvA* as forming a regulatory pair that functions to mediate a variety of phenotypes. Based upon the genetic data, it appears that the function of *irvR* is largely devoted to preventing *irvA* transcription, whereas IrvA appears to be the major mediator of the associated phenotypes.

Both the competence and DDAG phenotypes were associated with the altered expression of effector genes. However, given the severity of the competence phenotype, it was somewhat surprising that only a subset of competence genes/operons exhibited reduced expression. Additionally, each of the differentially expressed genes/operons in the *irvR* mutant has been shown to be absolutely required for genetic competence, but none appeared to be affected to an extent that might be expected for a competence-negative phenotype. There are several possible explanations for this result. First, it may be simply that the combined effect of a reduction in transcription for all of these genes is sufficient to fully disrupt genetic competence. For example, both *comEA* and *comYA* are located in operons that contain a total of at least 11 genes between them, and almost all of their individual operon components play an essential role in natural transformation (5, 27, 31). Furthermore, in the *irvR irvA* double-mutant strain, late competence gene expression was restored to about 60% of the wild-type level on average (Fig. 3), which seems to correlate reasonably well with the transformation efficiency seen in this strain (Fig. 2). This result may be expected if reduced competence gene transcription was the source of the transformation deficiency. Alternatively, there may be an as-yet-unrecognized level of regulation of the competence system. For example, this could occur at the level of protein-protein interaction. Indeed, the *comY* operon gene products are thought to form a complex with each other, as well as with additional competence proteins (10). Disruption of this complex will result in a total loss of transformability (12, 13, 27). Therefore, it may be possible that in the *irvR* background one or more of these interactions are disrupted. Similarly, any loss in the ability to bind exogenous DNA or to recombine transforming DNA would also result in a loss of transformability. Further studies are necessary to fully reconcile the connection between *irvA* and competence. However, the transcription data do suggest that the reduction in gene expression of these late competence genes is *irvA* dependent. Studies are currently under way to determine the scope of the IrvA regulon, which should eventually determine whether IrvA affects these genes through direct regulation of their promoters. If this is indeed the case, it may explain how these late competence genes could exhibit reduced expression without any noticeable changes in the level of expression of *comC*, *comED*, or *comX*. However, there are some indications that *S. mutans* regulates the late competence genes in a manner that differs from the *Streptococcus pneumoniae* paradigm (19). Therefore, an alternate possibility is that IrvA regulates an uncharacterized component of the competence cascade. Further analysis of the IrvA regulon should help to determine whether this is the case, as well as identify any additional components of the pathway between *irvA* and *gbpC*.

The data presented in this study suggest that *irvR* and *irvA* are closely associated and probably function as a regulatory pair. This is consistent with the homology both putative gene products have with the CI and Cro proteins from numerous bacteriophages. If IrvR and IrvA also function similarly to CI and Cro, we would predict that stress signals influence IrvR transcription factor activity, which would subsequently modulate the expression status of *irvA*. The stress hypothesis is also consistent with our observation that increased *gbpC* expression seems to correlate with *irvA* expression in various genetic backgrounds (unpublished observations). As previously mentioned, *gbpC* induction and DDAG in *S. mutans* have been shown to occur in the presence of various environmental stress conditions (6, 35). Thus, an interesting possibility is that *irvA*-inducing genetic mutations create stress signals in the cell and/or simulate an intracellular stress state similar to that encountered during unfavorable growth conditions. Furthermore, *irvR* may be one of the downstream targets of these stress-regulated pathways. Studies are currently under way to examine whether there is any evidence to support this hypothesis.

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