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GENETICS AND MOLECULAR BIOLOGY

An IgaA/UmoB Family Protein from *Serratia marcescens* Regulates Motility, Capsular Polysaccharide Biosynthesis, and Secondary Metabolite Production

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ABSTRACT Secondary metabolites are an important source of pharmaceuticals and key modulators of microbe-microbe interactions. The bacterium *Serratia marcescens* is part of the *Enterobacteriaceae* family of eubacteria and produces a number of biologically active secondary metabolites. In this study, we screened for novel regulators of secondary metabolites synthesized by a clinical isolate of *S. marcescens* and found mutations in a gene for an uncharacterized UmoB/IgaA family member here named *gumB*. Mutation of *gumB* conferred a severe loss of the secondary metabolites prodigiosin and serratamolide. The *gumB* mutation conferred pleiotropic phenotypes, including altered biofilm formation, highly increased capsular polysaccharide production, and loss of swimming and swarming motility. These phenotypes corresponded to transcriptional changes in *fimA*, *wecA*, and *flhD*. Unlike other UmoB/IgaA family members, *gumB* was found to be not essential for growth in *S. marcescens*, yet *igaA* from *Salmonella enterica*, *yrfF* from *Escherichia coli*, and an uncharacterized predicted ortholog from *Klebsiella pneumoniae* complemented the *gumB* mutant secondary metabolite defects, suggesting highly conserved function. These data support the idea that UmoB/IgaA family proteins are functionally conserved and extend the known regulatory influence of UmoB/IgaA family proteins to the control of competition-associated secondary metabolites and biofilm formation.

IMPORTANCE IgaA/UmoB family proteins are found in members of the *Enterobacteriaceae* family of bacteria, which are of environmental and public health importance. IgaA/UmoB family proteins are thought to be inner membrane proteins that report extracellular stresses to intracellular signaling pathways that respond to environmental challenge. This study introduces a new member of the IgaA/UmoB family and demonstrates a high degree of functional similarity between IgaA/UmoB family proteins. Moreover, this study extends the phenomena controlled by IgaA/UmoB family proteins to include the biosynthesis of antimicrobial secondary metabolites.

KEYWORDS secondary metabolite, competition, flagella, biofilm, motility, surfactant, capsular polysaccharide

Microorganism-derived secondary metabolites include important interspecies competitive factors and are a major potential source of therapeutic agents (1–6). The bacterium *Serratia marcescens* is found in a wide range of ecological niches and is known for the production of antibiotic secondary metabolites, including the peptide

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antibiotic althiomycin, the red pigment prodigiosin, and the biosurfactant serratamolide, also known as serrawettin W1 (7–11).

Both prodigiosin and serratamolide are reported to have broad-range antimicrobial activity, and serratamolide promotes swarming motility, a behavior that may allow the bacterium to become less susceptible to antibiotics and move into more favorable microenvironments (8, 12–17). Serratamolide from *S. marcescens* is hemolytic and was demonstrated to enable bacteria of different genera to evade phagocytosis by neutrophils *in vitro*, together suggesting that it may be a virulence factor and impact polymicrobial infections (18, 19). Regulation of secondary metabolites by *S. marcescens* occurs at the transcriptional level by several transcription factors, including CopA, cAMP receptor protein (CRP), EepR, HexS, PigP, RssAB, SmaI, and SpnR (19–27).

The goal of this study was to characterize the pleiotropic phenotypes of a newly identified secondary metabolite regulator. The mutations in mutants with reduced prodigiosin and serratamolide in *S. marcescens* mapped to an uncharacterized gene predicted to code for a protein of the IgaA/UmoB family. IgaA/UmoB family proteins were first described from the highly motile bacterium *Proteus mirabilis* by the Hughes group in 1998, where mutation of the *umoB* gene conferred a downregulation of the *flhDC* flagellar master regulator operon (28). Since then, most studies on this family of proteins have been performed using *Salmonella enterica* serovar Typhimurium. The *igaA* gene was found to be a regulator of intracellular growth in *S. enterica* and was later shown to regulate capsular polysaccharide production and pathogenesis (29–33). In *Escherichia coli*, mutations in the IgaA/UmoB-encoding gene, *yrfF*, were found to be enriched following serial coculture with macrophages and were found in a keratitis isolate (34, 35). The *igaA* and *yrfF* genes are essential for growth in *Salmonella* and *E. coli* bacteria (30), but *umoB* is not required for *Proteus* spp. to survive (28). The essential nature of *igaA* and *yrfF* allowed identification of suppressor mutations of the lethality phenotype that mapped to the Rcs regulatory system (30, 31, 36). Subsequent studies using bacteria from several genera indicate that IgaA/UmoB controls the Rcs phosphorelay signaling system that controls hundreds of genes (32, 37–39). Data suggest that IgaA/UmoB, together with RcsF, regulates the Rcs system in response to envelope stress and surface interactions by controlling the phosphorylation state of histidine kinase protein RcsC through an unknown mechanism (39–41). *S. marcescens* has an Rcs phosphorelay system that has been implicated in the regulation of outer membrane vesicle production and could be activated by a mutation of genes involved in biosynthesis of the enterobacterial common antigen outer membrane component (42, 43).

Because *igaA* and *yrfF* are essential genes in *E. coli* and *S. enterica*, there is incomplete knowledge about the range of biological processes that are mediated through this conserved family of proteins. This study characterized pleiotropic phenotypes of a mutant involving the *S. marcescens* IgaA/UmoB family gene, *gumB*, and found novel roles for IgaA/UmoB family proteins in their influence over secondary metabolite prodigiosin and biofilm production.

RESULTS

Isolation of an IgaA/UmoB family gene in *S. marcescens*. Transposon (Tn) mutagenesis was used to identify genes that regulate secondary metabolite production in a clinical keratitis isolate of *S. marcescens*, strain K904 (25). Two mutants with severely reduced red pigmentation and hemolysis were isolated from nonsaturating mutagenesis of ~6,000 mutants (Fig. 1A and B). The coloration of the mutant colonies ranged from white (24 h) to pink over time (≥48 h); similarly, over time, the colonies became elastic (gummy) and recalcitrant to manipulation with a toothpick and developed a rugose colony morphology (Fig. 1A).

The mutations mapped to an uncharacterized gene in the *S. marcescens* genome, corresponding to open reading frame (ORF) SMDB11_3859 of sequenced strain Db11 (44). The mutations mapped to bp 381 relative to the start codon for gummy mutant 1 and bp 168 for gummy mutant 2 (Fig. 1C). Other mutants defective in both prodigiosin

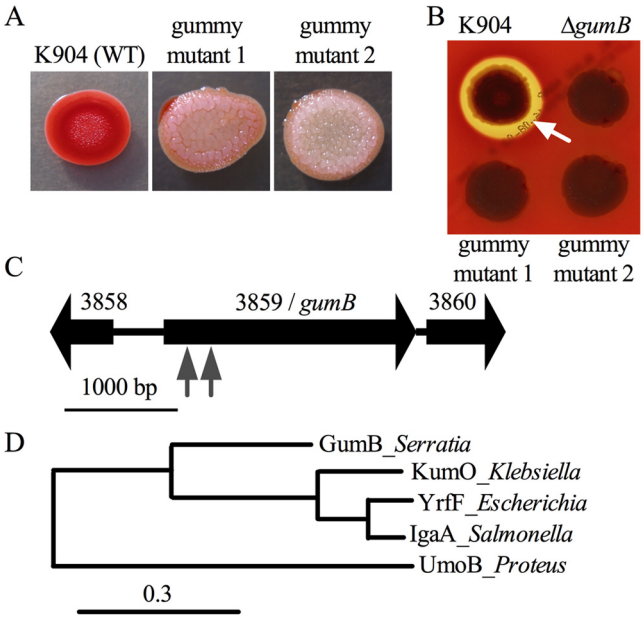

FIG 1 Isolation of mutants with secondary metabolite biosynthesis defects and a distinct colony morphology. (A) Representative images of the wild-type (WT) strain K904 and the two gummy mutants. The colonies were top-lit and demonstrate a lack of prodigiosin and altered colony morphology. (B) Representative images show a hemolysis zone (white arrow) on blood agar plates around K904 and a lack of hemolysis zones around two *gumB* transposon mutants and a defined *gumB* mutant. The plate was lighted from below to highlight the hemolysis zone, obscuring colony color. (C) Genetic map of the *gumB* gene and surrounding genes. Transposon insertion sites are noted by gray arrow points. (D) Phylogram of amino acid sequences of known and predicted IgaA/UmoB family proteins. The dendrogram was made using the "one-click" mode of the tree rendering program from www.Phylogeny.fr, using default settings. The number of substitutions per site are proportional to the branch length.

and serratamolide production mapped to the previously identified *eepS* gene (27) and an uncharacterized transcription factor of the GntR family that will be described elsewhere.

At the amino acid level, the identified gene from strain K904 is predicted to code for a protein that is 99% identical (701/710 amino acids) to the corresponding SMDB11_3859-encoded predicted protein. This protein consists of a Pfam PF07095/IgaA domain and is 42% identical to UmoB from *Proteus mirabilis*, 56% identical to YrfF of *Escherichia coli*, and 57% identical to IgaA from *Salmonella enterica* serovar Typhimurium. The family of proteins is conserved in the *Enterobacteriaceae*, including a putative protein coded by an uncharacterized ORF in *K. pneumoniae* predicted to be 54% identical to GumB at the amino acid level (see Fig. S1 in the supplemental material). Phylogenetic analysis suggests that the SMDB11_3859-encoded protein is structurally intermediate in relatedness between UmoB and the highly similar IgaA and YrfF proteins (Fig. 1D). *In silico* analysis of the GumB primary sequence suggests that it is in the inner membrane with three transmembrane helices (Fig. S1). Published models propose that IgaA/UmoB sits in the inner membrane and responds to RcsF localization or conformation to transmit envelop stress signals, and this is accomplished through IgaA/UmoB control of the Rcs transcription factor system (39–41).

Since *umoB* was the first gene of the IgaA/UmoB family with experimentally defined function, we here refer to the *S. marcescens* SMDB11_3859 as the gummy homolog of *umoB*, *gumB*.

Unlike *igaA* and *yrfF*, the *gumB* gene is not essential for viability. The *gumB* open reading frame appears to be monocistronic (Fig. 1C), although there is a predicted operon 98 bp downstream of the stop codon of *gumB*. The adjacent operon composed of ORFs SMDB11_3960 and SMDB11_3961 is predicted to code for a GMP/IMP nucleotidase and ribosome-associated heat shock protein Hsp15, respectively. As a first step to test whether the observed mutant phenotypes were due to a loss of GumB activity

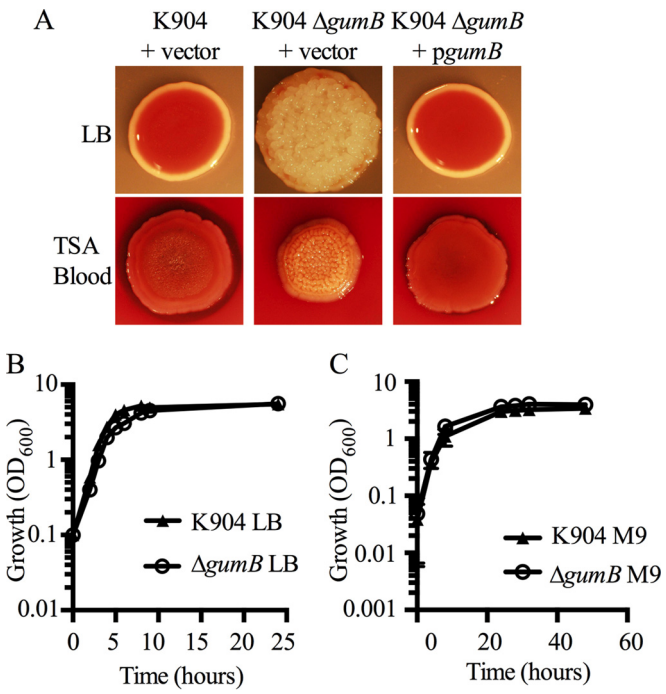

FIG 2 Complementation of *gumB* mutant defects and growth analysis. (A) The *gumB* mutant colony and color phenotypes are complemented by wild-type *gumB* on a plasmid under the control of the P_{lac} promoter. Representative images are of the strain K904 and the Δ*gumB* mutant with vector control plasmid pMQ132 and complementation plasmid p*gumB* (pMQ480). Bacteria were incubated for 48 h at 30°C on LB or blood agar plates and lighted from above. (B and C) Growth curves of the wild-type strain K904 and the Δ*gumB* mutant in LB (B) or M9 minimal medium supplemented with glucose (C). Means ± standard deviation (SD) are shown, $n \geq 3$.

or a polar effect on an adjacent gene, an in-frame deletion mutation of *gumB* was introduced into the K904 chromosome by two-step allelic replacement. Colonies of the resulting mutant strain were similar to the transposon mutants with respect to a lack of pigmentation and the development of an elastic and highly textured colony morphology (Fig. 2A and Movie S1 in the supplemental material). The isolation of transposon insertion mutations in *gumB* and the generation of a *gumB* deletion mutation suggest that unlike what was observed with *igaA* from *S. enterica* and *yrfF* from *E. coli*, the *gumB* gene of *S. marcescens* is not essential for growth.

Complementation analysis of the Δ*gumB* mutation with wild-type *gumB* on a multicopy plasmid further supports that the observed mutant defects, loss of pigmentation, and rugose phenotypes are due to a mutation of *gumB* and not another mutation or a polar effect on adjacent genes (Fig. 2A).

Growth analysis in rich medium demonstrates the slightly retarded growth of the Δ*gumB* mutant in log phase between 4 and 8 h; however, the *gumB* mutant achieved a final optical density similar to that of the wild type by stationary phase (~9 h) (Fig. 2B). Given the slight growth reduction of the *gumB* mutant in rich medium, we tested whether it was defective in primary metabolite production by assessing growth in M9 minimal medium. The Δ*gumB* mutant grew slightly better than the wild type in M9 medium supplemented with glucose (Fig. 2C). These data indicate that despite the pleiotropic phenotypes imparted by a mutation of *gumB*, the mutant strain is able to generate all of the primary metabolites necessary for growth in minimal medium.

Δ*gumB* mutant phenotypes are complemented by the *igaA* gene from *S. enterica*, *yrfF* from *E. coli*, and *kumO* from *K. pneumoniae*. To genetically test whether the GumB protein shares functionality with the IgaA/UmoB family proteins noted above, genes from *E. coli* (*yrfF*), *K. pneumoniae* (*kumO*), and *S. enterica* (*igaA*) were cloned and introduced into the Δ*gumB* mutant strain on a multicopy plasmid. The pigment and

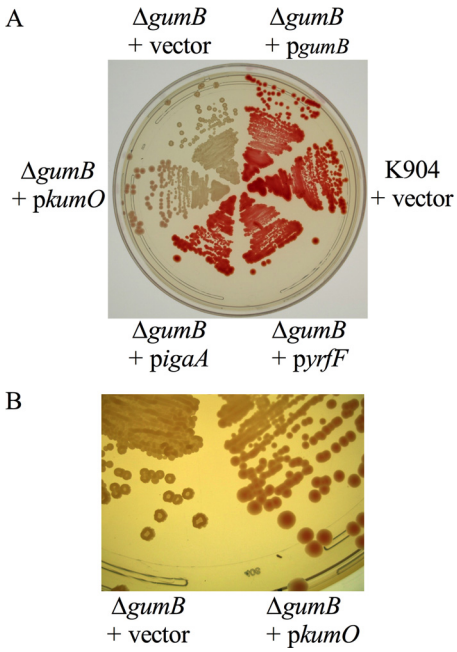

FIG 3 Complementation of *gumB* mutant defects by IgaA/UmoB family genes from several genera. (A) Image of the Δ*gumB* mutant with vector alone (pMQ132) or pMQ132 with various IgaA/UmoB family genes under the control of P_{lac} to test for complementation. (B) Zoomed-in view of panel A to highlight the colony morphology complementation of the *gumB* mutant with a predicted IgaA/UmoB gene from *K. pneumoniae* (*kumO*).

colony morphology phenotypes of the Δ*gumB* mutant were restored to those of the wild type by the expression of *igaA* and *yrfF* (Fig. 3A). The *K. pneumoniae* gene complemented the colony morphology phenotype and partially complemented the pigment phenotype that is clear in the larger colonies (Fig. 3B). These results support the idea that the UmoB/IgaA family proteins are highly conserved among opportunistic pathogens from the *Enterobacteriaceae*.

Motility defects were conferred by mutation of *gumB*. Tests were done to determine whether GumB shared function with IgaA and UmoB in the regulation of flagellum-based motility. The Δ*gumB* mutant did not produce a swimming zone when measured at 24 h, unlike the wild type (Fig. 4A). GumB was also required for surface swarming motility (Fig. 4A). The *gumB* mutant swarming defects (Fig. 4B) and swimming defects (Fig. 4C) were complemented by the wild-type *gumB* gene on a plasmid.

To test whether expression of the flagellar master regulator operon *flhDC* was altered in the Δ*gumB* mutant, quantitative-reverse transcriptase PCR (qRT-PCR) was employed. The transcript level of the *flhD* gene was reduced ($P < 0.05$, Mann-Whitney test) by several orders of magnitude in the Δ*gumB* mutant compared to the wild-type K904 strain when measured at an optical density at 600 nm (OD_{600}) of 3 (Fig. 4D). This reduction in *flhDC* operon expression correlated with an ~10,000-fold reduction of flagellar gene (*fliC*) expression when measured by qRT-PCR (Fig. 4D). Transmission electron microscopy (TEM) analysis of the Δ*gumB* mutant cells for stationary-phase liquid cultures also demonstrated a loss of flagella (Fig. S2), with 71% of the wild-type cells having a flagellum, and <1% of Δ*gumB* mutant cells with a flagellum ($n = 105$ cells for each genotype; $P < 0.001$, Fisher's exact test). Together, these data suggest that GumB plays a role in flagellum-based motility through positive control of the *flhDC* flagellum master regulator operon.

Secondary metabolite defects of the *gumB* mutant. The red pigment prodigiosin is a secondary metabolite made by a subset of *S. marcescens* strains and is thought to be involved in competition between microbes and to modulate energy levels during stationary phase. IgaA family proteins have not been implicated in the control of

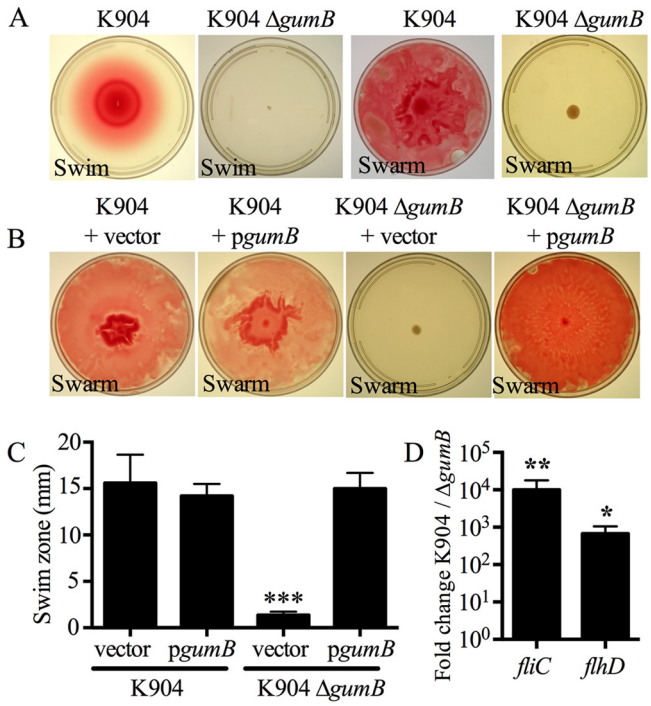

FIG 4 *S. marcescens* GumB is necessary for swimming and swarming motility. (A) Representative images of the swimming and swarming defects of the Δ*gumB* mutant. (B) Representative images of complementation of the Δ*gumB* swarming defect. (C) Complementation of the Δ*gumB* swimming phenotype. $n \geq 5$ independent plates per genotype from 2 separate days. Asterisks indicate a significant difference from all of the other genotypes ($P < 0.001$). Mean and SD are shown. Vector, pMQ132; p*gumB*, pMQ480. (D) Fold change of both flagellum-related gene transcript levels. qRT-PCR of *fliC* and *flhD* gene expression, $n \geq 4$. Mean and SD are shown. *, $P < 0.05$; **, $P < 0.01$.

secondary metabolites, so the obvious defect in pigmentation of the Δ*gumB* mutant was quantified. Prodigiosin was extracted and measured from stationary-phase cultures of the wild-type and Δ*gumB* mutant strains with complementation plasmid, pMQ480, or vector negative control. Prodigiosin levels were significantly lower in the Δ*gumB* mutant and could be complemented in *trans* (Fig. 5A).

This effect could stem from reduced transcriptional expression of the prodigiosin biosynthesis operon. Reverse transcriptase PCR (RT-PCR) analysis was used on stationary-phase cultures, as prodigiosin is produced during stationary phase (OD_{600}, 3). A clear ~100-fold reduction in the *pigA* transcript level was observed in the Δ*gumB* mutant, suggesting that the *gumB* mutant pigment defect is largely at the transcriptional level (Fig. 5B).

The biosurfactant serratamolide (45), also called serrawettin W1 (8), is antimicrobial and required for the hemolytic activity of *S. marcescens* strain K904 on blood agar plates and for growth inhibition of *Staphylococcus aureus* and other microbes (14, 19). The Δ*gumB* mutant was defective in hemolysis and antistaphylococcal inhibition zones, suggesting a serratamolide defect (Fig. 6A and B). The Δ*gumB* mutant was correspondingly defective in the biosynthesis of serratamolide when relative amounts were measured by mass spectrometry (Fig. 6C) and in expression of the serratamolide biosynthesis gene *swrW* at an OD_{600} of 3.0 (Fig. 6D). Hemolysis, an indirect measurement of serratamolide production, was used in a complementation analysis, and *gumB* on a plasmid was able to restore serratamolide production to the Δ*gumB* mutant (Fig. 6A).

The quantitative RT-PCR-generated expression levels of *pswP*, a gene necessary for both prodigiosin and serratamolide production (46), were similar for the K904 (0.0014 ± 0.0027) and the K904 Δ*gumB* (0.0012 ± 0.0024) strains ($P = 0.84$, Student's *t* test). Similarly, multicopy expression of *pswP* did not alter the pigment or serratamolide defects of the

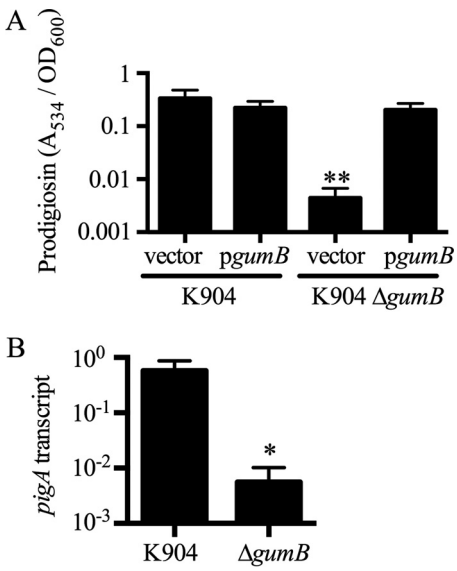

FIG 5 Prodigiosin pigmentation is severely reduced in a Δ*gumB* mutant. (A) The *gumB* mutant grown in LB medium is defective in prodigiosin production (75-fold reduction compared to strain K904; $n = 3$, $P < 0.01$). Extracted prodigiosin was measured by absorbance, and the defect could be complemented by wild-type *gumB* in *trans*. The vector control plasmid is pMQ132, and p*gumB* is pMQ480. (B) qRT-PCR analysis revealed reduced expression from the prodigiosin biosynthetic locus; *pigA* gene expression in the Δ*gumB* mutant was down 103-fold compared to the wild type ($P = 0.029$, $n = 4$). Means and standard deviations are shown. *, $P < 0.05$; **, $P < 0.01$.

Δ*gumB* mutant, suggesting that the *gumB* secondary metabolite defect was PswP independent.

GumB inhibits exopolysaccharide biosynthesis. To determine the mechanism by which GumB-deficient mutant colonies become elastic, suppressor mutant analysis was performed. Transposon mutagenesis was performed on the Δ*gumB* mutant strain, and colonies that had lost the rugose colony morphology were chosen for analysis. Multiple independent transposon mutations in the *gumB* deletion mutant strain yielded smooth nongummy colonies (Fig. 7A). These transposons mapped to adjacent genes in the formerly described capsular polysaccharide/enterobacterial common antigen biosynthetic locus, which has recently been shown to be important for virulence (47, 48). Five mutations were in the *wza* polysaccharide exporter gene, and two others were found upstream of the *wecA* exopolysaccharide biosynthesis gene; these genes correspond to strain Db11 ORFs SMDB11_2151 and SMDB11_2152, respectively. TEM analysis of bacteria grown on plates had staining consistent with excessive extracellular polysaccharide surrounding Δ*gumB* mutant bacteria that is absent in the Δ*gumB wza* double mutant (Fig. S2). These data suggested that capsular polysaccharide transcription in the Δ*gumB* mutant was increased.

Total extracellular polysaccharide production was measured from the liquid cultures of the wild-type K904 strain, the Δ*gumB* mutant, and the Δ*gumB wza* double mutant. The amount of extracellular polysaccharide extracted from the Δ*gumB* mutant was ~2-fold higher than that from the wild type and the Δ*gumB wza* double mutant (Fig. 7B). Dry weight polysaccharide measurements showed a similar trend, with 3.2 ± 0.8 mg/ml derived from K904, 5.6 ± 0.7 mg/ml from the Δ*gumB* mutant, and 2.1 ± 0.3 mg/ml from the Δ*gumB wza* mutant.

Transcriptional analysis of a gene from the capsular polysaccharide biosynthesis operon, *wecA*, supports the prediction that there is a deregulation of capsular polysaccharide production in the Δ*gumB* mutant. There was an approximate 100-fold increase in the *wecA* transcript level in the Δ*gumB* mutant strain compared to the K904 strain, at an OD_{600} of 3 ($P < 0.05$; Fig. 7C). These data suggest that the gummy and rugose colony phenotype of the *gumB* mutant is a result of deregulated capsular polysaccharide production.

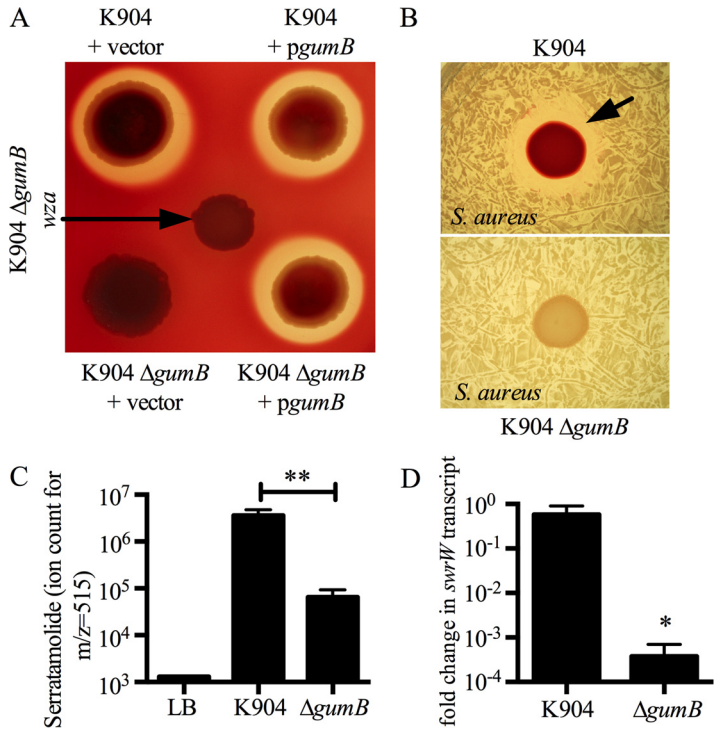

FIG 6 Serratamolide is severely reduced in a Δ*gumB* mutant. (A) Image of blood agar plate, lit from below. Serratamolide is responsible for hemolysis zones generated by strain K904. The Δ*gumB* mutant was defective for hemolysis and could be complemented by wild-type *gumB* in *trans*. The vector control plasmid is pMQ132, and p*gumB* is pMQ480. (B) Representative top-lit image. Inhibition of *S. aureus* bacterial growth (black arrow) by *S. marcescens* is serratamolide dependent and is eliminated in the Δ*gumB* mutant. (C) Mass spectrometry reveals a >50-fold significant reduction of serratamolide in the Δ*gumB* mutant ($P < 0.01$, $n = 3$). (D) qRT-PCR analysis demonstrates reduced expression of the serratamolide biosynthetic gene *swrW*, which was reduced >1,000-fold in the relative transcript levels for the mutant compared to the wild type ($P = 0.029$, $n = 4$). Mean and SD are shown. *, $P < 0.05$; **, $P < 0.01$.

Mutation of the capsule gene *wza* in the Δ*gumB* mutant did not alter hemolysis, motility, or pigmentation phenotypes, suggesting that the excessive capsular polysaccharide of the Δ*gumB* mutant is not responsible for most of its phenotypes (Fig. 6A, 7A, and S3).

Because extracellular polysaccharides can promote biofilm formation, we tested whether the *gumB* mutant had altered biofilm formation. Biofilms were formed on borosilicate glass under high-sheer conditions (rotated at ~62 rpm) in LB medium and then stained with crystal violet. At 6 h, the Δ*gumB* mutant had a minor increase in biofilm formation compared to K904, whereas the Δ*gumB wza*::Tn double mutant had a 2.3-fold reduction in biofilm formation compared to K904 (Fig. 7D). At 20 h, the Δ*gumB wza*::Tn double mutant was defective (6.5-fold; $P < 0.01$, analysis of variance [ANOVA] with Tukey's posttest) in biofilm formation compared to the Δ*gumB* mutant, suggesting that under the tested conditions, the extracellular polysaccharide regulated by GumB contributes to biofilm formation (Fig. 7D).

GumB mutant phenotypes are not strain specific. To test whether Δ*gumB* mutant phenotypes were specific to strain K904, the *gumB* gene was deleted in *S. marcescens* strains Db11, a nonpigmented insect pathogen, and CHASM, a pigmented environmental isolate. These deletions conferred a similar pigment phenotype in CHASM (Fig. S4A) and similar swarming defects in both CHASM and Db11 (Fig. S4B), indicating that the role of GumB in regulating secondary metabolite production and motility is not strain specific.

DISCUSSION

This study, designed to identify new regulators of secondary metabolism in *S. marcescens*, reports a new member of the IgaA/UmoB family of proteins, named GumB.

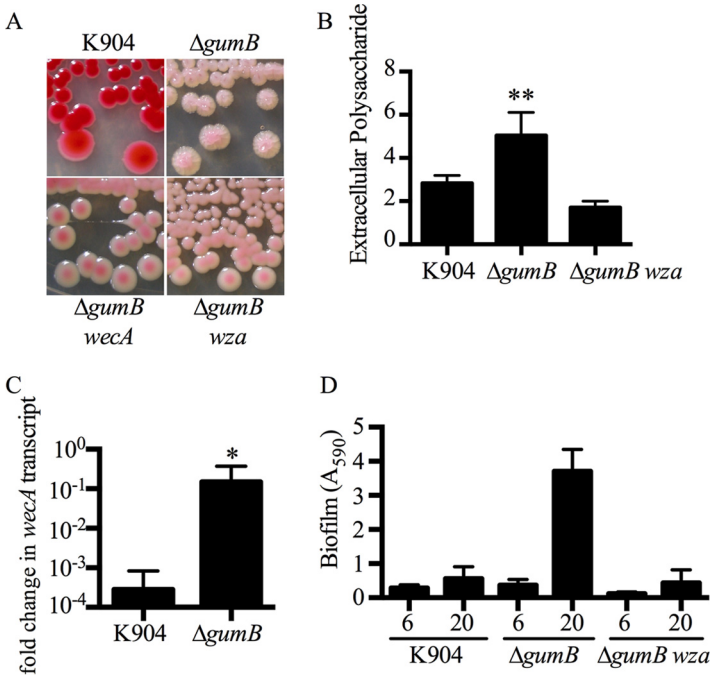

FIG 7 Gummy phenotype of the Δ*gumB* mutant requires the capsular polysaccharide operon. (A) Suppressor mutants of the rugose colony morphology phenotype of the Δ*gumB* mutant map to and *wza* genes of the capsular polysaccharide operon. (B) Extracellular polysaccharide (EPS) was extracted and measured from liquid cultures. Mean and SD are shown, $n = 4$. Asterisks indicate a significant increase relative to other groups (ANOVA, Tukey's posttest, $P < 0.01$). (C) qRT-PCR analysis revealed elevated relative *wecA* gene expression levels in the Δ*gumB* mutant ($P = 0.016$, $n = 5$). Mean and SD are shown. *, $P < 0.05$. (D) Biofilm formation in LB medium on borosilicate glass tubes grown under high-sheer conditions for 6 and 20 h and stained with crystal violet. The Δ*gumB* mutant had an elevated biofilm that was absent in a Δ*gumB wza* mutant. Mean and SD are shown, $n = 12$ biological replicates.

Mutation of the *gumB* gene introduced pleiotropic phenotypes, most notably a severe reduction in secondary metabolite production.

The genetic results presented here indicate that IgaA/UmoB family genes from several genera of *Enterobacteriaceae*, including an uncharacterized ortholog from *K. pneumoniae*, are functionally and structurally conserved. Therefore, it was somewhat surprising that the *gumB* gene is not essential for viability in *S. marcescens* as *yrfF* and *igaA* are in *E. coli* and *S. enterica*, respectively. Analysis of the *S. marcescens* genome suggests that there is not another obvious IgaA/UmoB gene, so the essential requirement for IgaA/UmoB family proteins found in *E. coli* and *S. enterica* must be downstream of GumB and absent or redundant in *S. marcescens*. Similar to the situation in *S. marcescens*, the *umoB* gene in *P. mirabilis* is not necessary for viability (28). While *gumB* is not essential, there was a slight growth delay in the Δ*gumB* mutant grown in LB medium compared to the wild type, although the final culture densities were the same. This growth delay may be due to altered expression of cell division genes in Rcs system and IgaA/UmoB gene mutants, as seen in *E. coli* and *S. enterica* (36, 49–51).

The polysaccharide phenotype of the *gumB* mutant is similar to those of *S. enterica* with partial-function *igaA* mutations, where increased activation of the Rcs system is required for the highly mucoid phenotype (29–33). This hypercapsule phenotype conferred by mutation of IgaA/UmoB genes is predicted to be the reason why *E. coli* cells with nonlethal mutations in *yrfF* had a selective advantage in a model in which they were serially cocultured with a phagocytic cell line (34). The hypercapsule suggests that IgaA/UmoB proteins may negatively regulate virulence; however, studies with *S. enterica* bearing nonlethal mutations in *igaA* indicate that IgaA, at least, is required for virulence in a rodent infection model (31). Importantly, data presented indicated that, other than biofilm formation and colony morphology, excess polysaccharide production is not responsible for the other *gumB* mutant phenotypes.

This study provides evidence for polysaccharides contributing to *S. marcescens* biofilm formation, whereas previous studies suggest a dominant role for type I fimbriae (52, 53). This is a new role for IgaA/UmoB family proteins, although it has been demonstrated that the Rcs system can control biofilms (54), and it is likely that GumB works through regulation of the Rcs system in *S. marcescens*. This is predicted because known IgaA/UmoB proteins, such as IgaA and YrfF, regulate the Rcs system, and here, we showed that the *igaA* and *yrfF* genes could complement *gumB* defects, implying structural and functional similarity between GumB, IgaA, and YrfF. In-progress genetic studies further support that *gumB* mutant phenotypes require the *S. marcescens* Rcs system genes (N. A. Stella and R. M. Q. Shanks, unpublished data).

Whereas GumB shares function with known IgaA/UmoB family proteins with respect to flagellar and polysaccharide biosynthesis, it was unknown whether this family of proteins mediates secondary metabolite function. Here, we measured a severe reduction in both prodigiosin and serratamolide. Given the ability of these two compounds to prevent the growth of other microbes, promote motility, promote resistance to antimicrobials via swarming motility, provide resistance to phagocytosis, and cause lysis of mammalian cells, a *gumB* mutant of *S. marcescens* would be expected to be at a competitive disadvantage in many niches. A loss of secondary metabolite production was observed when the *gumB* gene was mutated in three different strains. This is important, as a given gene does not necessarily confer the same phenotypes when mutated in different strains, as has been reported for cyclic-AMP-regulated pathways in *S. marcescens* (27).

IgaA family proteins are thought to regulate the Rcs system in response to cellular stress (39, 42, 55, 56). Based on this model, GumB likely senses extracellular stress and modifies the activity of the RcsC and or RcsD histidine kinases that in turn influence the expression of many genes by the RcsB response regulator. One may thus consider a Δ*gumB* mutant cell to be in a constitutively "stressed-out" state. The production of excess capsular polysaccharide is thought to relieve outer membrane stress (57) and promote biofilm formation, both of which may enhance survival. In this stressed state, the bacteria increase the expression of capsular polysaccharide genes but stop the production of flagella and competition-promoting secondary metabolites, perhaps to conserve energy and reduce activation of host immune systems through eliminating the production of pathogen-associated molecular patterns. How GumB interacts with other regulators is unknown at this time; however, it is notable that regulation of pigment, serratamolide, and flagellum by GumB follows a directly opposite trend from that of cAMP-CRP (19, 25, 53, 58), so it could be that GumB-associated phenotypes are mediated through control of intracellular cAMP levels and the corresponding activity of the CRP. Indeed, there is evidence in *P. mirabilis* that the Rcs system regulates CRP expression (54).

In summary, this report describes a new regulator of secondary metabolites, motility, and biofilm formation in the bacterium *S. marcescens*. The GumB gene is predicted to be highly important for *S. marcescens* strains to compete in a variety of environments, given the biological functions that it controls. These could include the use of *S. marcescens* as a biological control agent, where its GumB-regulated behaviors would be necessary, and in the human gut, where successful competition of *S. marcescens* is associated with Crohn's disease (59). Last, this study extends the known role of the conserved IgaA/GumB family proteins to the control of secondary metabolites and biofilm formation.

MATERIALS AND METHODS

Strains and growth medium used in this study. Bacteria were grown in lysogeny broth (LB) (60) or M9 minimal medium (61) supplemented with glucose at 0.4% (wt/vol). *Saccharomyces cerevisiae* was grown in yeast extract-peptone-dextrose (YPD) broth or synthetic complete (SC)-uracil medium (62). Swimming and swarming agars consisted of LB medium supplemented with 0.3% and 0.6% (wt/vol) agar, respectively. Cultures were grown with aeration using a TC-7 tissue culture roller. All incubations were performed at 30°C. The strains used are listed in Table 1.

Genetic manipulations and plasmid construction. Transposon mutagenesis was performed as previously described (52) using transposon delivery plasmids pBT20 (63) and pSC189 (64). Insertion sites

TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
Strains		
SM10 λpir	*E. coli* conjugation strain	70
S17-1 λpir	*E. coli* conjugation strain	70
EC100D	*E. coli* cloning strain, *pir-116* version	Epicentre
K746	*E. coli* keratitis isolate	71
LT2	*S. enterica* serovar Typhimurium	72
883	*K. pneumoniae*, clinical isolate	73
CHASM	*S. marcescens*, environmental isolate	25
K904	*S. marcescens*, keratitis isolate	25
Db11	*S. marcescens*, insect isolate	74
CMS2265	K904 gummy mutant 1, K904 *gumB*::Tn	This study
CMS4001	K904 Δ*gumB*	This study
CMS4124	K904 Δ*gumB wza*::Tn	This study
CMS4225	K904 *fimC*-kan	75
CMS4619	K904 Δ*gumB wecA*::Tn	This study
CMS4320	K904 gummy mutant 2, K904 *gumB*::Tn	This study
CMS4462	Db11 Δ*gumB*	This study
CMS4662	CHASM Δ*gumB*	This study
Plasmids		
pBT20	Transposon delivery plasmid	63
pSC189	Transposon delivery plasmid	64
pMQ132	pBBR1-replicon shuttle vector, *aacC-1*	68
pMQ200	oriR6K plasmid with P_{BAD} promoter, *nptII*	68
pMQ460	Allelic replacement vector	76
pMQ480	pMQ132 plus *gumB* from *S. marcescens* strain K904	This study
pMQ507	pMQ460 plus Δ*gumB*	This study
pMQ530	pMQ132 plus *igaA* from *S. enterica* strain LT2	This study
pMQ529	pMQ132 plus *kumO* from *K. pneumoniae* strain 883	This study
pMQ531	pMQ132 plus *yrfF* from *E. coli* strain K746	This study

were mapped by marker rescue (64) or arbitrary PCR (65) followed by sequencing. Mutants of *S. marcescens* strain K904 were plated on LB or blood agar and were visually screened for a loss of pigmentation and hemolysis zones, as previously described (27).

Allelic replacement of the *gumB* open reading frame (ORF) was performed as previously described but using plasmid pMQ507 (all plasmids used are listed in Table 1) (66). The mutation deletes 2,044 bp of the 2,133-bp gene, starting at the ninth codon. To make pMQ507, 470 bp of DNA upstream of *gumB* and 662 bp of DNA downstream of *gumB* were amplified with primers that have additional sequence such that the amplicons can recombine with each other and with allelic replacement vector pMQ460. This and other plasmids were made by homologous recombination using yeast *in vivo* recombination (67, 68). The oligonucleotide primers used to make pMQ507 were primers 3419 to 3422 and are listed in Table 2. Plasmids were verified by PCR and sequencing of cloned junctions, and deletion mutations were verified by PCR.

To make complementation plasmids, the *igaA-umoB* family genes from *E. coli* (*yrfF*), *Klebsiella pneumoniae* (*kumO*), *S. marcescens* (*gumB*), and *S. enterica* (*igaA*) were amplified with primers 3655/3656, 3661/3662, 3417/3418, and 3658/3659, respectively. These ORFs were placed under transcriptional control of the *E. coli* P_{lac} promoter on pMQ132 (68).

Transmission electron microscopy. TEM of bacteria was performed using cultures grown overnight in LB medium for 18 to 20 h or taken from agar plates after 48 h of growth, washed in phosphate-buffered saline (PBS), and processed and observed as previously described (53). At least four independent preparations were made for each group.

Secondary metabolite assays. Prodigiosin was extracted from cultures grown for 18 to 20 h in LB medium using acidified ethanol and the absorbance measured, as previously described (25).

Serratamolide was measured by mass spectrometry from cultures grown for 20 h in LB medium and normalized to an OD_{600} of 2.0, as previously described (27). Zones of growth inhibition for *Staphylococcus aureus* were measured as previously described (14).

Extracellular polysaccharide quantitation. *S. marcescens* polysaccharides were isolated as described by Anderson et al. (48) and Masuko et al. (69). Briefly, bacteria were grown overnight in LB medium for 16 to 20 h and pelleted by centrifugation at 25,000 × *g* and 15°C for 15 min. The supernatants were discarded, and the bacterial pellets were suspended in 30 ml of PBS and 6 ml of 1% Zwittergent 3-14 (EMD Millipore) in 100 mM citric acid (pH 2) and incubated at 50°C for 20 min. Following incubation, the bacteria were pelleted by centrifugation at 25,000 × *g* and 15°C for 30 min, and the cell-free supernatants were transferred to 250-ml centrifuge bottles. Four volumes of cold (−20°C) ethanol were added to each and the samples placed at −20°C overnight. After overnight precipitation, the samples were centrifuged at 27,000 × *g* and 4°C for 45 min, and the supernatants were discarded. The precipitated polysaccharides were allowed to air-dry in a chemical fume hood. The polysaccharides

TABLE 2 Oligonucleotide primers used in this study

Primer no.	Purpose[a]	Sequence (5′ to 3′)[b]
1055	*fimA*-RT	ACTACACCCTGCGTTTCGAC
1056	*fimA*-RT	GCGTTAGAGTTTGCCTGACC
2638	16S-RT	AACTGGAGGAAGGTGGGGAT
2639	16S-RT	AGGAGGTGATCCAACCGCA
2891	*pswP*-RT	CGTGACATCGTCACCTTCACG
2892	*pswP*-RT	GCCAAAGAGAGCCTGTTCAAG
2911	*pigA*-RT	GGAGCGAACTGACCTTCAAC
2912	*pigA*-RT	CTGTTCCAGACGCAGTTTCA
3417	*gumB* cloning	ggccagtgccaagcttgcatgcctgcaggtcgactctaTTGAAGCAGCTGTCGTAGTAAC
3418	*gumB* cloning	tgtgagcggataacaatttcacacaggaaacagctATGAGCACAATAGTGTTGATATTGG
3419	*gumB* deletion	cggccagtgccaagcttgcatgcctgcaggtcgactctaGATTGCCTCAAAGAGGTTACC
3420	*gumB* deletion	gaagcagctgtcgtagtaacgctggCCAATATCAACACTATTGTGCTCAT
3421	*gumB* deletion	atgagcacaatagtgttgatattggCCAGCGTTACTACGACAGCTGCTTC
3422	*gumB* deletion	attgtgagcggataacaatttcacacaggaaacagctGGTTGAACCCGGTGTGCTGTTGC
3570	*wecA*-RT	ACCGAGCATCACTTCCTGAT
3571	*wecA*-RT	GTACCTGACGCTGATGCTGA
3575	*flhD*-RT	AATGTTTCGCCTGGGTATTG
3576	*flhD*-RT	ATAGCAAAATGCCGGTATGG
3582	*swrW*-RT	AATTAGGCGAGATCGAGCAA
3583	*swrW*-RT	AACAGGACGGCACCATAAAG
3655	*yrfF* cloning	aaattctgttttatcagaccgcttctgcgttctgatTTATTCGATAAGGCTTTCTGAAGG
3656	*yrfF* cloning	gtgagcggataacaatttcacacaggaaacagctATGAGCACCATTGTGATTTTTTTAGC
3658	*igaA* cloning	aattctgttttatcagaccgcttctgcgttctgatTCAGATGAGATTTTCCGGAGAGACG
3659	*igaA* cloning	gtgagcggataacaatttcacacaggaaacagctATGAGCACCATTCTGATTTTTATAGC
3661	*kumO* cloning	caaattctgttttatcagaccgcttctgcgttctgatTTAATCTTGCGAGTCAGATGAGG
3662	*kumO* cloning	aattgtgagcggataacaatttcacacaggaaacagctATGGGCACCTTTCTGATATTCC
3809	*fliC*-RT	GTATCTCTCTGGCGCAGACC
3810	*fliC*-RT	ATGGTTTCACCGTCGTTAGC

[a]"-RT" indicates that primers are used for qRT-PCR.
[b]Uppercase letters indicate sequences that prime amplification of the desired DNA, whereas lowercase letters target homologous recombination with a plasmid.

were weighed, and the total carbohydrate content was quantitated using a microplate phenol-sulfuric assay (48, 69).

Biofilm assay. Biofilm formation on borosilicate glass was performed as previously described (52, 53). Biofilms were stained with crystal violet (0.1% [wt/vol]) and solubilized with glacial acetic acid (33%), and the absorbance at 590 nm was measured using a plate reader (BioTek Synergy 2). Biofilms were generated for 6 and 20 h in LB medium in tubes rotated on a TC-7 tissue culture roller (New Brunswick Scientific).

qRT-PCR and RNA preparation. RNA preparation from cultures with an OD_{600} of 3.0 and qRT-PCR were performed as previously described (27). Briefly, cultures were treated with RNAprotect reagent (Qiagen), and RNA was purified using an RNeasy kit (Qiagen) and concentrated using a spin column (RNA Clean and Concentrator; Zymo Research). Two rounds of DNase treatment were performed (10 units for 15 min at room temperature on column with Qiagen DNase I, and 1 unit for 30 min at 37°C with Promega RQ1 DNase after purification and prior to the concentration step). Controls for cDNA preparation included no-reverse transcriptase reactions for each RNA sample, included to validate the absence of chromosomal DNA in RNA samples; any samples with detectable chromosomal DNA contamination were excluded prior to experimentation. The coefficient of variance was less than 10% between experiments.

Statistical analysis. Experiments were done at least twice, with a minimum of three biological replicates. The GraphPad Prism software was used to perform Student's *t* tests, Mann-Whitney U tests, Fisher's exact test, and one-way ANOVA with Tukey's posttest. Significance was set at a *P* value of <0.05.

Accession number(s). The sequences of the *gumB* gene from *S. marcescens* K904 and the *kumO* gene from *K. pneumoniae* were deposited in GenBank under accession numbers KY098906 and KY098907, respectively.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.02575-17.

SUPPLEMENTAL FILE 1, PDF file, 6.8 MB.

SUPPLEMENTAL FILE 2, MOV file, 15.8 MB.

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