Gene Deletion Strategy To Examine the Involvement of the Two Chondroitin Lyases in *Flavobacterium columnare* Virulence

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Flavobacterium columnare is an important bacterial pathogen of freshwater fish that causes high mortality of infected fish and heavy economic losses in aquaculture. The pathogenesis of this bacterium is poorly understood, in part due to the lack of efficient methods for genetic manipulation. In this study, a gene deletion strategy was developed and used to determine the relationship between the production of chondroitin lyases and virulence. The *F. johnsoniae* *ompA* promoter (*PompA*) was fused to *sacB* to construct a counterselectable marker for *F. columnare*. *F. columnare* carrying *PompA-sacB* failed to grow on media containing 10% sucrose. A suicide vector carrying *PompA-sacB* was constructed, and a gene deletion strategy was developed. Using this approach, the chondroitin lyase-encoding genes, *csIA* and *csIB*, were deleted. The Δ *csIA* and Δ *csIB* mutants were both partially deficient in digestion of chondroitin sulfate A, whereas a double mutant (Δ *csIA* Δ *csIB*) was completely deficient in chondroitin lyase activity. Cells of *F. columnare* wild-type strain G₄ and of the chondroitin lyase-deficient Δ *csIA* Δ *csIB* mutant exhibited similar levels of virulence toward grass carp in single-strain infections. Coinfections, however, revealed a competitive advantage for the wild type over the chondroitin lyase mutant. The results indicate that chondroitin lyases are not essential virulence factors of *F. columnare* but may contribute to the ability of the pathogen to compete and cause disease in natural infections. The gene deletion method developed in this study may be employed to investigate the virulence factors of this bacterium and may have wide application in many other members of the phylum *Bacteroidetes*.

Columnaris disease can affect almost all species of freshwater fish, including cultured and wild fish, often resulting in heavy economic losses in aquaculture worldwide (1, 2). Its causative agent, *Flavobacterium columnare*, has been the focus of microbiological as well as aquaculture research for decades, with respect to diagnosis, epidemiology, pathology, preventive measures, and possible virulence factors (2–4). The genetic diversity of *F. columnare* has been recognized by the presence of at least three distinct genomovars (5–7). However, the pathogenesis of this important pathogen is still rather unclear. Although a few molecules, such as chondroitin AC lyase, have been reported as possible virulence factors (8–11), the lack of genetic manipulation systems suitable for *F. columnare* has been an obstacle in understanding the mechanisms involved in the pathogenesis of this bacterium. Starosick et al. (12) developed techniques to introduce replicative plasmids into *F. columnare* by conjugation and also to construct mutants by insertional mutagenesis and transposon-mediated mutagenesis. However, both processes remain inefficient, and few subsequent reports utilizing these techniques have been published (12, 13).

A technique to construct in-frame deletions was recently developed for the genetically tractable bacterium *Flavobacterium johnsoniae* (14). This involved the isolation of a streptomycin-resistant *rpsL* mutant of *F. johnsoniae* and the use of wild-type *rpsL* as a counterselectable marker (14). While this approach has been very successful, one disadvantage is that deletion mutants must be constructed in an *rpsL* mutant rather than in wild-type cells. In some other Gram-negative bacteria, *sacB*, which confers sensitivity to sucrose, has been used as a counterselectable marker to allow isolation of gene deletion mutants from wild-type cells (15–17). Previous attempts to adapt this approach to *F. johnsoniae* or to other members of the phylum *Bacteroidetes* were not successful (18, 19).

Here we demonstrate techniques to efficiently isolate targeted in-frame gene deletion mutants of *F. columnare*. Deletion mutants were isolated using an *rpsL*-based system similar to the one described for *F. johnsoniae* (14) and using a *sacB*-based system in which a *Flavobacterium* promoter drove expression of *sacB*. Gene deletion coupled with complementation analysis was used to characterize two genes encoding chondroitin lyases and to determine their roles in virulence.

MATERIALS AND METHODS

Bacterial strains and media. Bacterial strains and plasmids used in this study are listed in Table 1. *F. columnare* strain G₄ was grown at 28°C in Shieh medium or on Shieh agar (20), and its genomovar is currently being determined. *Escherichia coli* strains were cultured in Luria broth (LB) liquid medium or on LB agar at 37°C. When necessary, antibiotics (Sigma) were added at the following concentrations: 1 µg/ml tobramycin for *F. columnare*, 100 µg/ml or 10 µg/ml streptomycin for *F. columnare*, 15 µg/ml tetracycline for *F. columnare*, and 100 µg/ml ampicillin for *E. coli*.

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TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics or genotype ^a	Reference or source
Strains		
<i>E. coli</i> strain		
S17-1 λ pir	F [−] RP4-2-Tc::Mu <i>aphA</i> ::Tn7 <i>recA</i> λ pir; lysogen	28
<i>F. columnare</i> strains		
G ₄	Wild type; grass carp isolate	29
G _{4S}	<i>rpsL</i> 2 Sm ^r	This study
G _{4S} Δ <i>cslA</i>	<i>rpsL</i> 2 mutant with chondroitin AC lyase gene null mutation; Sm ^r	This study
Δ <i>cslA</i>	Chondroitin AC lyase gene null mutant; strain obtained from G ₄	This study
Δ <i>cslB</i>	Chondroitin ABC lyase gene null mutant; strain obtained from G ₄	This study
Δ <i>cslA</i> Δ <i>cslB</i>	Chondroitin AC and ABC lyase gene null mutant; strain obtained from Δ <i>cslA</i>	This study
Plasmids		
pCP23	<i>E. coli</i> - <i>F. columnare</i> shuttle plasmid; Ap ^r (Tc ^r)	30
pCP29	<i>E. coli</i> - <i>F. columnare</i> shuttle plasmid; Ap ^r (Cf ^r Em ^r)	31
pCP23-rpsl	<i>rpsL</i> gene cloned into KpnI sites of pCP23; Ap ^r (Tc ^r)	This study
pCS	Suicide plasmid derived from pCP23; Ap ^r (Tc ^r)	This study
pCSR	<i>rpsL</i> -containing suicide vector; Ap ^r (Tc ^r)	This study
pCSR-cslA	2.0-kbp region upstream of <i>cslA</i> cloned into BamHI and SalI sites of pCSR; Ap ^r (Tc ^r)	This study
pCSR-csl	Construct used to delete <i>cslA</i> in G _{4S} ; 2.2-kbp region downstream of <i>cslA</i> cloned into SalI and SphI sites of pCSR-cslA; Ap ^r (Tc ^r)	This study
pMS75	<i>sacB</i> -containing suicide vector; Ap ^r (Tc ^r)	This study
pMS-cslA	Construct used to delete <i>cslA</i> in G ₄ ; 2.0-kbp region upstream of <i>cslA</i> fused to 2.2-kbp region downstream of <i>cslA</i> and cloned into BamHI and SphI sites of pMS75; Ap ^r (Tc ^r)	This study
pMS-csl2A	2.6-kbp region downstream of <i>cslB</i> cloned into KpnI and SalI sites of pMS75; Ap ^r (Tc ^r)	This study
pMS-cslB	Construct used to delete <i>cslB</i> in G ₄ or Δ <i>cslA</i> mutant; 2.2-kbp region upstream of <i>cslB</i> cloned into SalI and SphI sites of pMS-csl2A; Ap ^r (Tc ^r)	This study
pCP-A	4.6-kbp fragment spanning <i>cslA</i> and its promoter region cloned into KpnI and PstI sites of pCP23; Ap ^r (Tc ^r)	This study
pCP-B	4.1-kbp fragment spanning <i>cslB</i> and its promoter region cloned into PstI and SphI sites of pCP23; Ap ^r (Tc ^r)	This study
pCP-AB	4.1-kbp fragment spanning <i>cslB</i> and its promoter region cloned into PstI and SphI sites of pCP-A; Ap ^r (Tc ^r)	This study
pAS43	<i>gfp</i> -containing shuttle plasmid; Ap ^r (Em ^r)	12
pCP-gfp	<i>gfp</i> -containing shuttle plasmid originated from pCP23; Ap ^r (Tc ^r)	This study
pDS-RED2-1	<i>rfp</i> -containing shuttle plasmid; Km ^r	Clontech Inc.
pCP-rfp	<i>rfp</i> -containing shuttle plasmid; Ap ^r (Tc ^r)	This study

^a Antibiotic resistance phenotypes: Ap^r, ampicillin resistant; Cf^r, cefoxitin resistant; Em^r, erythromycin resistant; Sm^r, streptomycin resistant; Tc^r, tetracycline resistant. Unless otherwise indicated, the antibiotic resistance phenotypes are those expressed in *E. coli*. The antibiotic resistance phenotypes in parentheses are those expressed in *Flavobacterium* but not in *E. coli*.

Isolation of the streptomycin-resistant *rpsL* mutant G_{4S}. Streptomycin-resistant *F. columnare* cells were obtained by plating 10⁹ wild-type cells on Shieh agar containing 100 μ g/ml streptomycin. Streptomycin-resistant clones were streaked on fresh medium for isolation, and the *rpsL* gene from each clone was PCR amplified using primers rpslKpnF and rpslKpnR (Table 2) before sequencing. Point mutations in *rpsL* conferring streptomycin resistance were identified by comparison to the wild-type *rpsL* sequence.

To determine if wild-type *rpsL* was dominant to the mutant *rpsL* alleles, wild-type *rpsL* was cloned into the shuttle vector pCP23. Specifically, a fragment spanning wild-type *rpsL* and its presumed promoter region was amplified using primers rpslKpnF and rpslKpnR (Table 2), which were designed with engineered KpnI restriction sites. The fragment was digested with KpnI and cloned into the corresponding sites of pCP23 to generate pCP23-rpsl. pCP23-rpsl was introduced into streptomycin-resistant *F. columnare* by conjugation as previously described (12–14). All primers used in this study are listed in Table 2.

Construction of the *rpsL*-containing suicide vector pCSR. To generate a suicide vector carrying *rpsL* as a counterselectable marker, wild-type *rpsL* was amplified using primers rpslSacF and rpslKpnR, which introduced SacI and KpnI restriction sites, respectively. Both the PCR product and the shuttle plasmid pCP23 were digested with SacI and KpnI. The wild-type *rpsL* gene was ligated with the 6.10-kb fragment of pCP23 lacking the replicase gene. The resultant plasmid, pCSR, is an *rpsL*-containing suicide vector (see Fig. S1 in the supplemental material).

Construction of the *sacB*-containing suicide vector pMS75. In order to use *sacB* as a counterselectable marker in *F. columnare*, the *F. johnsoniae* *ompA* promoter was cloned in front of *sacB* by overlap PCR to replace its original promoter. Briefly, *PompA* was amplified using primers Pom-pASacF and PomPAR, with pAS43 as the template. *sacB* was amplified using primers pr45-sacBF and SacB1KpnR, with pRE112 as the template. To generate the *PompA-sacB* fusion, the PCR products were used as the templates for overlap PCR with primers Pom-pASacF and SacB1KpnR, which were designed with engineered SacI and KpnI sites, respectively. The product was purified, digested with SacI and KpnI, and ligated into pCP23 that had been digested with the same enzymes. The resulting suicide plasmid, pMS75, lacked functions needed for replication in *Flavobacterium* (Fig. 1A).

Construction of the *cslA* deletion mutant in the streptomycin-resistant *F. columnare* strain G_{4S}. The gene deletion strategy is indicated in Fig. 1B. First, a 2.0-kbp fragment including the final 153 bp of *cslA* and 1,841 bp of downstream sequence was amplified using primers csl_delA1BamF (introducing a BamHI site) and csl_delASalR (introducing a SalI site). The fragment was digested with BamHI and SalI and cloned into pCSR which had been digested with the same enzymes to generate pCSR-cslA. A 2.2-kbp fragment including the first 240 bp of *cslA* and 1,973 bp of upstream sequence was amplified with primers csl_delB-SalF (introducing a SalI site) and csl_delB1SphR (introducing an SphI site). The PCR product was digested with SalI and SphI and cloned into

TABLE 2 Primers used in this study

Primer	Sequence (5' → 3') ^a
rpslKpnF	CGGGGTACCTTGGGATGTCTTTTGTAGGAAG
rpslKpnR	CGGGGTACCTTACTTTTGTCTTTAGGGCGT
rpslSacF	CGGCGAGCTCTTGGGATGTCTTTTGTAGGAAG
PompASacF	ACGCGAGCTCGGCAGCGCATACCAAAGAACA
PompAR	TGCAAACTTTTGTATGTTTCATTTTTTAATTACAATTTAGTTAAT
pr45-sacBF	ATTAACATAAATTGTAATTAATAAATGAACATCAAAAAGTTTGCA
SacB1KpnR	CGGGGTACCTTATTGTGTTAACTGTTAATTGTCC
csl_delA1BamF	CGCGGATCCTCATCATACACCCCTCG
csl_delASalR	ACGCGTCGACGATATGCGAAATGTGGAATT
csl_delBSalF	ACGCGTCGACCGCTTGGGCTAAACGAGA
csl_delB1SphR	ACATGCATGCTTGCCCAATAAAATCG
csl_del_ch_inF	AGCGGTTATGCCTTGTTTA
csl_del_ch_inR	AGATGCCCATGATAGAATGTC
csl_del_ch_ouF	AAACCCGTGAGCAAAATCCT
csl_del_ch_ouR1	CTGGCTTCAGGAGGATTAG
csl2_del_AKpnF	CGGGGTACCGCTTACACTTCCGCCACTA
csl2_del_ASaIR	ACGCGTCGACTGGGAATTGCCACAACCTCA
csl2_del_BSaIF	ACGCGTCGACTACTATGGCATTGCTTGGC
csl2_del_BSphR	ACATGCATGCAACAGGAGAAAGTTGGGAGC
csl2_del_chinF	AAAATAGAACCTCCACAA
csl2_del_chinR	GCAATAATACCCTATCAGTT
csl2_del_chouF	TTGTTGCCAGAATATGACC
csl2_del_chouR	CCCATAAAGGGCGTAACAT
csl_cmplKpnF1	CGGGGTACCGTTAAACCTGAAAATAGAATA
csl_cmplPstR	AAAAGTGCAGGCTTGAGCATATTCGCCACA
csl2_comp3PstF	AAAAGTGCAGGCACCGTAAATGAGTCGC
csl2_compSphR	ACATGCATGCCAAAGGGAGAAACCGCATA
Pr44-Bam	CGCGGATCCGGCAGCGCATACCAAAGAACACTTAG
Pr38-Pst	GCTAGCTGCAGCAGATCTATTGTATAGTTCATCCA
PompAKpnF	CGGGGTACCGGCAGCGCATACCAAAGAACA
PompAR	CTTCGCCCTTAGACACCATTTTTAAATTACAATTAGTTAAT
RFPF	ATTAACATAAATTGTAATTAATAAATGGTGTCTAAGGGCGAAG
RFPPstR	AAAAGTGCAGAAGTTAAACAACAACATTGCA

^a Restriction sites in the primer sequences are underlined.

pCSR-cslA which had been digested with the same enzymes to generate the deletion construct pCSR-csl.

Plasmid pCSR-csl was introduced into the streptomycin-resistant wild-type *F. columnare* strain G_{4S} by conjugation (21). Briefly, 20 ml of *E. coli* S17-1 λpir with pCSR-cslA or G_{4S} was cultured overnight at 37°C or

28°C, respectively. The cells were collected by centrifugation at 5,000 rpm for 10 min at room temperature. Pellets were washed twice with antibiotic-free Shieh medium and suspended in 100 μl Shieh medium. The cell suspensions were mixed, placed as spots on nitrocellulose membranes which had been placed on Shieh agar, and incubated at 28°C for 24 h. After

FIG 1 Map of the suicide plasmid pMS75 (A) and gene deletion strategy in *Flavobacterium columnare* (B). *PompA*, *ompA* promoter of *F. johnsoniae*; *cslA*, chondroitin AC lyase gene of *F. columnare*; Sm^r, Suc^s, and Tc^r, streptomycin sensitive, sucrose sensitive, and tetracycline resistant, respectively.

incubation, selection for a positive clone whose genomic DNA was integrated with pCSR-csl after the first recombination was conducted by spreading the conjugation product on Shieh plates containing 15 µg/ml tetracycline. A tetracycline-resistant (streptomycin-sensitive) clone was streaked on Shieh agar with 15 µg/ml tetracycline. An isolated colony was inoculated into 1 ml antibiotic-free Shieh liquid medium and shaken at 28°C overnight. One milliliter of culture was spread on Shieh agar which contained 100 µg/ml streptomycin to screen for mutants that had lost the plasmid by a second recombination event. The colonies were streaked on Shieh agar with or without tetracycline, separately. Colonies which grew on Shieh agar but not on Shieh agar containing tetracycline were inoculated into liquid culture for further characterization. The mutants were confirmed by PCR amplification using primers csl_del_ch_inF and csl_del_ch_inR, which resulted in a 409-bp product from wild-type cells but produced no PCR product from cells of the deletion mutant. The mutant strain was further verified by PCR amplification using primers csl_del_ch_ouF and csl_del_ch_ouR1, which resulted in a 3.0-kb product from wild-type cells and a 1.1-kb product from cells of the deletion mutant.

Construction of a *cslA* deletion mutant in wild-type *F. columnare* strain G₄. A 4.2-kb fragment containing regions upstream and downstream of *cslA* was isolated from pCSR-csl by digestion with BamHI and SphI and was inserted into the same sites of pMS75 to generate the deletion construct pMS-cslA. This plasmid was introduced into wild-type *F. columnare* strain G₄ by conjugation followed by growth on Shieh agar containing 15 µg/ml tetracycline to select colonies that contained the integrated plasmid after homologous recombination. The tetracycline-resistant colonies were inoculated into liquid Shieh medium without antibiotics and incubated with shaking overnight at 28°C to allow for loss of the plasmid by a second recombination event. The Δ *cslA* deletion mutant was selected by growth on Shieh agar medium containing 10% sucrose and was confirmed by PCR as described above.

Deletion of *cslB* in wild-type *F. columnare* strain G₄ and in the Δ *cslA* mutant. A 2.7-kb gene predicted to encode a chondroitin ABC lyase was identified and named *cslB*. To identify the function of this gene, a deletion mutant was constructed. A 2.6-kb fragment including the final 381 bp of *cslB* and 2,192 bp downstream of *cslB* was amplified using primers csl2_del_AKpnF (introducing a KpnI site) and csl2_del_ASaIR (introducing a SalI site). The product was digested with KpnI and SalI and ligated into the corresponding sites of pMS75 to generate pMS-csl2A. A 2.2-kb fragment including the first 36 bp of *cslB* and 2,180 bp of upstream sequence was amplified by PCR with primers csl2_del_BSaIF (introducing a SalI site) and csl2_del_BSphR (introducing an SphI site). The product was digested with SalI and SphI and cloned into the corresponding sites of pMS-csl2A to generate the deletion construct pMS-cslB.

Plasmid pMS-cslB was introduced into wild-type *F. columnare* strain G₄ and into the Δ *cslA* mutant by conjugation, and deletion mutants were obtained as described above. Deletion of *cslB* in each strain was confirmed by PCR amplification using primers csl2_del_ch_inF and csl2_del_ch_inR, resulting in a 631-bp product from the parent cells but no PCR product from cells of the deletion mutants. The mutants were further confirmed by PCR amplification using primers csl2_del_ch_ouF and csl2_del_ch_ouR, which resulted in products of 5,008 bp from the parent cells and products of 2,663 bp from cells of the deletion mutants.

Construction of complementation strains. Plasmids carrying *cslA* and *cslB* and their promoter regions were constructed using the shuttle vector pCP23. Primers csl_cmplKpnF1 (introducing a KpnI site) and csl_cmplPstR (introducing a PstI site) were used to amplify a 4,641-bp fragment spanning *cslA* and its putative promoter region. The product was digested with KpnI and PstI and cloned into the corresponding sites of pCP23 to generate pCP-A. Primers csl2_comp3PstF (introducing a PstI site) and csl2_compSphR (introducing an SphI site) were used to amplify a 4,075-bp fragment spanning *cslB* and its putative promoter region. The product was digested with PstI and SphI and ligated into the same sites of pCP23 to generate pCP-B. To construct a plasmid containing both *cslA*

and *cslB*, the fragment spanning *cslB* was isolated from pCP-B by digestion with PstI and SphI and cloned into the corresponding sites of pCP-A to generate pCP-AB. The plasmids were transferred into the corresponding mutants of *F. columnare* by conjugation for complementation studies.

Construction of RFP-labeled wild-type and GFP-labeled mutant strains. To construct a red fluorescent protein (RFP)-labeled wild-type G₄ strain, a plasmid containing a fused fragment carrying *PompA* and *rfp* was constructed. Briefly, the promoter region of *F. johnsoniae ompA* was amplified from pAS43 with primers PompAKpnF and PompAR. The *rfp* gene was amplified by using pDS-RED2-1 as the template, with primers RFPF and RFPstR. The fused fragment carrying *PompA* and *rfp* was obtained using overlap PCR with primers PompAKpnF and RFPstR. The fused fragment was digested with KpnI and PstI and ligated into the corresponding sites of pCP23 to generate pCP-rfp. This plasmid was introduced into G₄ by conjugation. The RFP-labeled strains were screened on Shieh agar containing tobramycin and tetracycline.

To construct green fluorescent protein (GFP)-labeled mutants, including Δ *cslA*, Δ *cslB*, and Δ *cslA* Δ *cslB* mutants, a fused fragment carrying *PompA* and *gfp* was obtained by PCR using the primers pr44-Bam and pr38-Pst, with pAS43 as the template. The fragment was cloned into pCP23 to generate plasmid pCP-gfp. The plasmid was introduced into mutants by conjugation. Colonies containing pCP-gfp were screened using Shieh agar with tobramycin and tetracycline.

In vitro growth rate determination. *In vitro* growth rates of wild-type G₄ and its mutants were determined by measuring the optical density at 600 nm (OD₆₀₀) with a spectrophotometer (Eppendorf Biophotometer, Germany). Briefly, 100 µl of early-exponential-phase culture (OD₆₀₀ = 0.3) was inoculated into 20 ml Shieh medium and incubated at 28°C with shaking at 150 rpm. The OD₆₀₀ was determined every 30 min for 7.5 h.

ECP preparation and CslA and CslB identification. The extracellular products (ECPs) of wild-type G₄, deletion mutants, and complemented strains were prepared as described by Abbass et al. (22). Briefly, *F. columnare* cells were cultured to the early logarithmic growth phase in 30 ml Shieh medium at 28°C and 150 rpm. Cells were removed by centrifugation (3,000 × g for 10 min), and the supernatants were passed through 0.22-µm membrane filters to remove residual cells. The filtered supernatants (30 ml) were concentrated to 100 µl by using Amicon Ultra-15 concentrators (Millipore). The samples were diluted with 1 ml phosphate-buffered saline (PBS; pH 7.4) and concentrated again to 100 µl. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of the ECPs was performed according to standard procedures, and proteins were detected by staining with Coomassie blue. Proteins that were present in the culture fluid of wild-type G₄ but not in the culture fluid of the mutants were characterized by matrix-assisted laser desorption ionization–tandem time of flight mass spectrometry (MALDI-TOF/TOF MS) by using an Applied Biosystems model 4800 Plus MALDI-TOF/TOF analyzer.

Generation of antibodies against CslA and CslB and Western blot analyses. Affinity-purified polyclonal antibodies were prepared from whole rabbit serum by immunization with synthetic peptides (SEGKLI ESKDMRN for CslA and CNPDGLGPKRSFDS for CslB) and were stored at –20°C (GenScript, Nanjing, China).

The ECPs were separated by SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes. Detection of CslA and CslB was carried out at room temperature as previously described (23). Briefly, the membranes were blocked with 5% skim milk in Tris-buffered saline (TBS; 25 mM Tris-Cl, 500 mM NaCl, pH 7.5) for 1 h at room temperature, followed by three washes in TBS containing 0.05% Tween 20 (TBST). The membranes were then incubated with antibody against CslA or CslB in 1% skim milk in TBS for 1.5 h, followed by three washes in TBST. The washed membranes were incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG secondary antibody for 0.5 h, followed by three washes with TBST, and bound antibodies were visualized using chemiluminescence detection (ChemiDoc MP imaging system).

FIG 2 Comparison of chondroitin sulfate A digestion activities among *Flavobacterium columnare* wild-type, mutant, and complemented strains. (A) Chondroitin lyase activity can be seen as a clear zone around bacterial colonies against a cloudy background. (B) The chondroitin lyase activity of each strain was determined by measuring the amount of residual chondroitin sulfate A after incubation of this compound with the strains for 30 min in a 96-well flat-bottom microplate. The values are means and standard deviations (SD) ($n = 3$). Asterisks indicate significant differences ($P < 0.05$).

Chondroitin lyase assay. As previously described (23, 24), sterilized chondroitin sulfate A (Sigma-Aldrich) and bovine serum albumin (BSA) were added to Shieh agar, to final concentrations of 150 $\mu\text{g}/\text{ml}$ and 4%, respectively. Two-milliliter mid-log-phase cultures of bacteria ($\text{OD}_{600} = 0.4$) were placed on the plates as spots and incubated at 28°C for 9 h. Degradation of chondroitin was visualized by flooding the plates with acetic acid followed by incubation for 5 min. Chondroitin lyase activity was identified as the clear zone around bacterial colonies.

The chondroitin sulfate degradation activity of each strain was also determined in 96-well flat-bottom microplates (10, 25). Briefly, bacterial cultures in the early logarithmic growth phase were collected by centrifugation at $1,000 \times g$ for 10 min. Bacterial pellets were suspended and diluted with Shieh medium until the OD_{600} reached 0.3, and then the chondroitin sulfate A solution was added to the suspension, to a final concentration of 1.5 mg/ml. Next, 20 μl of bacterial suspension containing chondroitin sulfate A and 150 μl basal medium (0.2% tryptone, 0.05% yeast extract, pH 7.0) were added to each well of the 96-well microplate for 30 min of incubation at 28°C. Finally, 0.5% BSA (dissolved in 0.45 M acetate buffer, pH 4.0) was added to each well, and the turbidity of each well was measured at 405 nm, using an ELx800 microplate reader (BioTek Instruments, Inc.). The Student t test was used to evaluate the statistical significance of differences.

LD_{50} determination. In order to ensure that the infection dose was optimal, 50% lethal dose (LD_{50}) values were determined by using intraperitoneal infection. Healthy grass carp (*Ctenopharyngodon idella*), weighing 25 to 30 g each and with no disease symptoms, were obtained from a local fish farm, where columnaris disease has not been recorded, in Wuhan, China. Fish were kept at 25°C in an 8- m^3 flowthrough tank with aeration and fed once a day with commercial fish feed pellets for more than 1 week before each experiment. The bacterial strains were cultured overnight in 5 ml Shieh medium with shaking at 28°C until the OD_{600} reached 0.4. The cultures were diluted in 10-fold series or 2-fold series in Shieh medium, and the number of cells per milliliter was determined by plating dilutions on Shieh agar and counting the colonies after 48 h of incubation at 28°C. For wild-type G_4 and each mutant strain, bacterial suspensions used for injection were all adjusted to the following concentrations: 4×10^6 , 6×10^6 , 8×10^6 , 1×10^7 , 1.5×10^7 , and 2×10^7 CFU/ml. The use of fish in the present study (i.e., the use of animals in the

laboratory) was approved by the Institute of Hydrobiology, Chinese Academy of Sciences.

Fish were divided into seven groups, with 10 individuals in each group, and were further reared at 25°C for 1 week in a 10-liter tank with continuous aeration, water changes at 1-day intervals, and feeding with commercial pellets. Six groups of fish were injected correspondingly with bacterial suspensions at the six different concentrations indicated above, at a dose of 100 μl bacterial suspension per fish for the six experimental groups and 100 μl Shieh medium for the control. Mortality was recorded every 6 h for 7 days following infection. The LD_{50} values were calculated by using the method of Reed and Muench (26). The Student t test was used to evaluate the statistical significance.

CI analysis. To compare infection abilities between each mutant and the wild type, competitive index (CI) analysis was used. GFP-labeled mutants and the RFP-labeled wild-type strain were grown in Shieh medium containing 1 $\mu\text{g}/\text{ml}$ tobramycin and 15 $\mu\text{g}/\text{ml}$ tetracycline at 28°C. When the OD_{600} value for the bacteria reached 0.4, the cultures were mixed at a 1:1 ratio, and 7 ml of the mixture was diluted in 4 liters of water in a plastic container that contained 10 fingerling grass carp at 26°C. After 30 min, an additional 10 liters of water was added, and the fish were maintained for 7 days at 26°C. For fish that died during this period, gills were collected and homogenized in Shieh medium. The homogenized liquid was serially diluted in buffer (0.3 g NaCl, 0.1 g KCl, 0.02 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.04 g $\text{MgCl}_2 \cdot 7\text{H}_2\text{O}$, pH 7.4) and spread on Shieh agar containing tobramycin and tetracycline, and the numbers of wild-type and mutant cells were determined using a stereo fluorescence microscope. The CI was defined as the output-to-input ratio for the mutant compared to the wild-type strain. A CI of <1 indicates a competitive virulence defect. The mean CI values were calculated for 12 fish in each group. The data were analyzed by the Student t test, with P values of <0.05 considered statistically significant.

Nucleotide sequence accession number. Sequence data for *cslB* were deposited in GenBank under accession number [KR083049](https://www.ncbi.nlm.nih.gov/nuclot/KR083049).

RESULTS

Development of *rpsL* and *sacB* as counterselectable markers for *F. columnare*. Ten spontaneously streptomycin-resistant mu-

FIG 3 Extracellular products of wild-type *G*₄ and the $\Delta cslA$, $\Delta cslA$ (pCP-A), $\Delta cslB$, and $\Delta cslB$ (pCP-B) mutants as determined by SDS-PAGE. The upper arrows indicate CslB, and the lower arrows indicate CslA.

tants of wild-type *F. columnare* *G*₄ were isolated, and their *rpsL* genes were amplified and sequenced. Two of the mutants had an adenine-to-guanine point mutation at bp 128 of the *rpsL* coding sequence that resulted in a K43R substitution in RpsL, which is similar to that in streptomycin-resistant *rpsL* mutants of *F. johnsoniae* (14). The wild-type *F. columnare* *rpsL* gene was inserted into the shuttle vector pCP23 and introduced into *G*_{4S}. *G*_{4S} with the plasmid containing wild-type *rpsL* failed to grow on Shieh agar with 10 μ g/ml streptomycin, whereas *G*_{4S} without the plasmid grew well in the presence of streptomycin. These results suggest that wild-type *rpsL* can function as a counterselectable marker in *F. columnare*.

The *F. johnsoniae* *ompA* promoter was used to express *sacB* in

F. columnare. Wild-type cells carrying pCP23-*sacB* failed to grow on Shieh agar with 10% sucrose, whereas cells without the plasmid grew well, indicating that *sacB* can also function as a counterselectable marker in *F. columnare*.

Construction of *cslA* deletion mutants of *F. columnare* strains *G*_{4S} and *G*₄. To investigate the feasibility of constructing in-frame deletion mutants of *F. columnare* by using *rpsL* or *sacB* as a counterselectable marker, the suicide vectors pCSR, carrying *rpsL* (see Fig. S1 in the supplemental material), and pMS75, carrying *PompA-sacB* (Fig. 1), were used. Fused fragments of upstream and downstream regions of *cslA* (GenBank accession no. AY912281) were cloned into pCSR and pMS75 and introduced by conjugation into *G*_{4S} and *G*₄, respectively. After two rounds of recombination events, hundreds of streptomycin-resistant colonies were obtained on the Shieh plate containing 100 μ g/ml streptomycin (pCSR-mediated deletion), and dozens of sucrose-resistant colonies were obtained on the Shieh plate containing 10% sucrose (pMS75-mediated deletion). About 50% of the streptomycin-resistant colonies and about 20% of the sucrose-resistant colonies were identified as *cslA* deletion mutants by PCR analysis. These results demonstrate that both *rpsL* and *sacB* can function as counterselectable markers to isolate deletion mutants of *F. columnare*. Although *sacB* was less effective as a counterselectable marker, it has a significant advantage over *rpsL* in that mutations are constructed in the wild-type strain rather than in a streptomycin-resistant *rpsL* mutant. For this reason, *sacB*-mediated gene deletion was used to construct most of the in-frame deletion mutants analyzed in this study.

Deletion of *cslA* resulted in a partial reduction in chondroitin lyase activity, which was restored following the introduction of the wild-type gene on pCP-A (Fig. 2). The residual chondroitin lyase activity exhibited by the mutant suggested the presence of an additional chondroitin lyase that had not previously been recognized in wild-type *F. columnare* cells.

***F. columnare* *cslB* encodes a second chondroitin lyase.** Chondroitin lyases typically have catalytic polysaccharide lyase family 8 (PL8) domains corresponding to Pfam 02278 (27). Analysis of the *F. columnare* *G*₄ genome (unpublished data) revealed two genes encoding proteins with PL8 domains: *cslA* and a gene that we labeled *cslB* (GenBank accession no. KR083049). To verify the function of *F. columnare* *cslB*, in-frame deletions were made in *G*₄

FIG 4 Extracellular products of wild-type *G*₄ and the $\Delta cslA$, $\Delta cslA$ (pCP-A), $\Delta cslB$, $\Delta cslB$ (pCP-B), $\Delta cslA \Delta cslB$, and $\Delta cslA \Delta cslB$ (pCP-AB) mutants, separated by SDS-PAGE and recognized by rabbit anti-CslA (A) or rabbit anti-CslB (B) polyclonal antiserum. M, molecular size marker.

FIG 5 Growth curves for wild-type *G*₄ and the $\Delta cslA$, $\Delta cslB$, and $\Delta cslA \Delta cslB$ mutants. Cells were grown in Shieh medium at 28°C.

and in the $\Delta cslA$ mutant. The $\Delta cslB$ deletion mutant had little change in chondroitin sulfate digestion compared to the wild type (Fig. 2A). However, the mutant lacking both *cslA* and *cslB* failed to digest chondroitin sulfate. The ability to digest chondroitin sulfate was restored to the $\Delta cslA$ mutant, the $\Delta cslB$ mutant, and the double deletion mutant by complementation with plasmids expressing *CslA*, *CslB*, and both *CslA* and *CslB*, respectively (Fig. 2A).

To quantify the decrease of chondroitin sulfate degradation activity, chondroitin sulfate was incubated with bacterial cells for 30 min, and the turbidity of the mixture was measured. As shown in Fig. 2B, deletion of *cslB* resulted in a slight decrease in chondroitin lyase activity, whereas deletion of *cslA* resulted in a much larger decrease in chondroitin lyase activity. Deletion of both *cslB* and *cslA* had a cumulative effect, essentially eliminating chondroitin lyase activity. Complementation of the mutants with plasmids expressing the appropriate chondroitin lyase genes restored the ability to digest chondroitin sulfate (Fig. 2).

Extracellular products (ECPs) of wild-type, mutant, and complemented cells were collected and separated by SDS-PAGE. A band corresponding to approximately 82 kDa was missing in the ECP fraction from the $\Delta cslA$ mutant, and a band corresponding to approximately 96 kDa was missing in the ECP fraction from the

$\Delta cslB$ mutant (Fig. 3). Both bands were missing from the double deletion mutant (data not shown). The protein bands at 82 and 96 kDa for wild-type cells were identified by MALDI-TOF/TOF MS as *CslA* and *CslB*, respectively.

Antibodies raised against peptides corresponding to *CslA* and *CslB* were used for Western blot analyses of the ECPs. The results confirmed that *CslA* was missing from the *cslA* deletion mutants and that *CslB* was missing from the *cslB* deletion mutants (Fig. 4). They also demonstrated a restoration of production of each protein by complementation with the appropriate plasmid (Fig. 4).

The wild-type and mutant strains grew at similar rates in Shieh medium (Fig. 5), indicating that deletion of *cslA* or *cslB* was not detrimental to the growth of cells *in vitro*.

LD₅₀ and CI of *G*₄ and mutants. The LD₅₀s of wild-type *G*₄ and its mutants were determined by injection into grass carp. The results showed that the LD₅₀ values for *G*₄ and the $\Delta cslA$, $\Delta cslB$, and double mutants were 5.1×10^4 , 6.3×10^4 , 3.2×10^4 , and 4.4×10^4 CFU/fish, respectively (Fig. 6A). Compared with wild-type *G*₄, the $\Delta cslA$ mutant had a higher LD₅₀. Unexpectedly, the LD₅₀ of the $\Delta cslB$ mutant was reduced, and the LD₅₀ of the double mutant was similar to the LD₅₀ exhibited by wild-type cells (Fig. 6A).

FIG 6 LD₅₀s (A) and CIs (B) for *F. columnare* wild-type *G*₄ and its mutants, determined in grass carp by using intraperitoneal infection. LD₅₀ values are expressed as means and SD for three replicate experiments. Asterisks indicate significant differences ($P < 0.05$). The CI values were obtained 36 h after infection with wild-type *G*₄ and its mutants. A CI of < 1 indicates a virulence defect. Each data point represents the result for an individual animal, and the mean $\pm$ SD of CIs for all fish in each competitive experiment is shown as a horizontal line and error bars.

The CI for the wild type versus each mutant strain was obtained through coinfection with the wild type and each mutant in fingerling grass carp by immersion. The $\Delta cslA$, $\Delta cslB$, and $\Delta cslA \Delta cslB$ mutants each exhibited a decreased ability to compete with the wild type, with CI values of 0.34 ± 0.21 , 0.22 ± 0.22 , and 0.27 ± 0.25 , respectively (Fig. 6B). These values indicate that the $\Delta cslA$, $\Delta cslB$, and $\Delta cslA \Delta cslB$ mutants had 2.94-, 4.55-, and 3.70-fold decreases, respectively, in the number of CFU obtained from grass carp gills compared to the number of CFU of the wild type. The results suggest that chondroitin lyases may have roles in competition with other strains and in the ability to cause disease in nature.

DISCUSSION

Although *F. columnare* is an important pathogen of almost all species of freshwater fish, many details regarding its pathogenesis have not been elucidated, in part because of the lack of suitable genetic techniques. We constructed two suicide vectors, with *rpsL* and *sacB* as counterselectable markers, and used these to construct targeted gene deletions. Both *rpsL* and *sacB* were effective counterselectable markers that allowed efficient isolation of deletion mutants. Because *rpsL*-based gene deletion only works in a streptomycin-resistant mutant strain, *sacB* was established as the preferred counterselectable marker for construction of in-frame deletion mutants. Previous attempts to use *sacB* as a counterselectable marker in members of the phylum *Bacteroidetes* failed, perhaps because of a lack of expression of the *sacB* gene (18, 19). We eliminated this problem by introducing a *Bacteroidetes* promoter that is known to function in many members of the phylum. It is likely that this approach will allow *sacB* to function as a counterselectable marker in many other members of the large and diverse phylum *Bacteroidetes* and will thus facilitate genetic analysis of these important bacteria.

In this study, two genes encoding chondroitin lyases were deleted: *cslA*, which was identified previously (4), and *cslB*. Similar lyases are also present in many other members of the *Bacteroidetes* phylum (see Fig. S2 in the supplemental material). With the deletion of both *F. columnare cslA* and *cslB*, the degradation of chondroitin was completely eliminated. *CslA* may be more important than *CslB* for degradation of chondroitin, because *CslA* was more abundant than *CslB* and deletion of *cslA* caused a more dramatic reduction of chondroitin lyase activity than did deletion of *cslB*.

Chondroitin lyases have been suggested to be important virulence factors of *F. columnare* (9–11). However, in this study, deletion of *cslA* and *cslB* resulted in little change in virulence as measured by the LD₅₀ following infection by injection. This indicates that chondroitin lyases are not critical virulence factors for bacterial cells introduced by injection. In contrast, deletion of the chondroitin lyase genes resulted in a decreased competitive index with respect to the wild type during coinfection by immersion, a very different and perhaps more natural challenge method. This suggests that chondroitin lyases may play a role in the process of natural infection. The exact step at which these enzymes confer their competitive advantage remains to be determined.

The genetic techniques developed in this study can be applied to examine other predicted *F. columnare* virulence factors and should help to elucidate their roles in infection and pathogenesis in fish. The techniques may also be used to construct rationally designed avirulent mutants as candidate vaccine strains to protect freshwater aquaculture fish from columnaris disease.

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