In a previous study, it was shown that Riemerella anatipestifer, a member of Flavobacteriaceae, is naturally competent. However, whether natural competence is universal in Flavobacteriaceae remains unknown. In this study, it was shown for the first time that Riemerella columbina was naturally competent in the laboratory condition; however, Flavobacterium johnsoniae was not naturally competent under the same conditions. The competence of R. columbina was maintained throughout the growth phases, and the transformation frequency was highest during the logarithmic phase. A competition assay revealed that R. columbina preferentially took up its own genomic DNA over heterologous DNA. The natural transformation frequency of R. columbina was significantly increased in GCB medium without peptone or phosphate. Furthermore, natural transformation of R. columbina was inhibited by 0.5 mM EDTA, but could be restored by the addition of CaCl$_2$, MgCl$_2$, ZnCl$_2$, and MnCl$_2$, suggesting that these divalent cations promote the natural transformation of R. columbina. Overall, this study revealed that natural competence is not universal in Flavobacteriaceae members and triggering of competence differs from species to species.

**Keywords:** Flavobacteriaceae, R. columbina, Flavobacterium johnsoniae, natural competence, horizontal gene transfer

**INTRODUCTION**

Naturally competent bacteria can actively take up naked DNA from their environment and integrate it into the genome, which is called natural transformation (Mell and Redfield, 2014). As one of the three horizontal gene transfer mechanisms, natural transformation facilitates bacterial acquisition of virulence genes and antibiotic-resistant cassettes to help bacteria adapt to the environment (Wiedenbeck and Cohan, 2011; Seitz and Blokesch, 2013a). Natural transformation was first discovered in Streptococcus pneumoniae in 1928 (Griffith, 1928). Currently, at least 83 species have been found to have natural competence (Johnston et al., 2014; Liu et al., 2017).

In Gram-positive and Gram-negative bacteria, there are different mechanisms to take up DNA. Naturally competent Gram-negative bacteria, such as Neisseria species and Haemophilus influenzae, use type IV pili (T4P) to take up exogenous double-stranded DNA (dsDNA), in contrast to Helicobacter pylori, which uses a type IV secretion system (T4SS) (Hofreuter et al., 2000), and Campylobacter jejuni, which uses a type II secretion system (T2SS) (Wiesner et al., 2003).
to take up exogenous dsDNA. Gram-positive bacteria, such as *S. pneumoniae* and *Bacillus subtilis*, use a competence pseudopilus, a structure similar to T4P, to take up dsDNA (Hahn et al., 2005). Once dsDNA is transported across the outer membrane in Gram-negative bacteria or the peptidoglycan layer in Gram-positive bacteria, dsDNA is degraded to single-stranded DNA (ssDNA) and transported through the pore protein ComEC into the cytoplasm (Johnston et al., 2014). Internalized ssDNA is presumably bound by DNA-processing protein A (DprA), which recruits the recombinase RecA to mediate homologous recombination by facilitating strand exchange (DprA), which recruits the recombinase RecA to mediate homologous recombination by facilitating strand exchange (DprA), which recruits the recombinase RecA to mediate homologous recombination by facilitating strand exchange.

The homologous gene of *dprA* (C237_RS0105470) in *R. columbina*, which protects ssDNA and loads RecA to facilitate homologous recombination (Mirouze et al., 2013), and the gliding motility gene *gldH* in *F. johnsoniae* were selected as targeted deletion gene, since they are not essential for the growth of bacteria and can be deleted (McBride et al., 2003; Hovland et al., 2017; Huang et al., 2019). Donor DNA was composed of upstream of target gene, an antibiotic resistance cassette and downstream of target gene. Briefly, the ~620 bp upstream sequence and ~620 bp downstream sequence of *C237_RS0105470* were amplified from the genome of *R. columbina* using the primers RC-Up P1 and RC-Up P2, RC-Down P1 and RC-Down P2, respectively. The ~620 bp upstream sequence and ~620 bp downstream sequence of *gldH* were amplified from the genome of *F. johnsoniae* using the primers Up(gldH) P1 and Up(gldH) P2, Down(gldH) P1 and Down(gldH) P2, respectively. An erythromycin resistance cassette was amplified from the genome of *R. anatipestifer* CH-1 using the primers RC-Erm P1 and RC-Erm P2 or Erm(gldH) P1 and Erm(gldH) P2, respectively (Liao et al., 2015; Luo et al., 2015). The three fragments were fused using overlapping PCR (Xiong et al., 2006; Huang et al., 2017, 2019). The fused fragments served as donor DNA for natural transformation.

**MATERIALS AND METHODS**

**Bacterial Strains, Primers, and Growth Conditions**

*Riemerella columbina* and *Flavobacterium johnsoniae* were purchased from the Culture Collection of the University of Gothenburg (CCUG) and the China General Microbiological Culture Collection Center (CGMCC), respectively. The bacterial strains and primers used in this study are listed in Table 1. The culture conditions for *R. columbina* and *F. johnsoniae* were identical to those used for *R. anatipestifer* described in a previous study (Huang et al., 2019). Briefly, *R. columbina* was cultured in GC broth (GCB) medium with shaking or GCB agar plates and LB plates supplemented with 5% sheep blood (blood plates) at 37°C, however, *F. johnsoniae* was cultured in GCB medium with shaking or GCB plates at 25°C. When required, erythromycin was added into the medium at a final concentration of 1 µg/ml for *R. columbina* and 50 µg/ml for *F. johnsoniae*.

**Preparation of Donor DNA**

The homologous gene of *dprA* (C237_RS0105470) in *R. columbina*, which protects ssDNA and loads RecA to facilitate homologous recombination (Mirouze et al., 2013), and the gliding motility gene *gldH* in *F. johnsoniae* were selected as targeted deletion gene, since they are not essential for the growth of bacteria and can be deleted (McBride et al., 2003; Hovland et al., 2017; Huang et al., 2019). Donor DNA was composed of upstream of target gene, an antibiotic resistance cassette and downstream of target gene. Briefly, the ~620 bp upstream sequence and ~620 bp downstream sequence of *C237_RS0105470* were amplified from the genome of *R. columbina* using the primers RC-Up P1 and RC-Up P2, RC-Down P1 and RC-Down P2, respectively. The ~620 bp upstream sequence and ~620 bp downstream sequence of *gldH* were amplified from the genome of *F. johnsoniae* using the primers Up(gldH) P1 and Up(gldH) P2, Down(gldH) P1 and Down(gldH) P2, respectively. An erythromycin resistance cassette was amplified from the genome of *R. anatipestifer* CH-1 using the primers RC-Erm P1 and RC-Erm P2 or Erm(gldH) P1 and Erm(gldH) P2, respectively (Liao et al., 2015; Luo et al., 2015). The three fragments were fused using overlapping PCR (Xiong et al., 2006; Huang et al., 2017, 2019). The fused fragments served as donor DNA for natural transformation.

**Natural Transformation Procedure**

The procedure of natural transformation was similar to that used for *R. anatipestifer* described in a previous study (Liu et al., 2017; Huang et al., 2019). Briefly, *R. columbina* and *F. johnsoniae* were cultured in GCB liquid with shaking at 37°C for *R. columbina* and at 25°C for *F. johnsoniae*. The bacteria were collected during the logarithmic phase (OD$_{600}$ of 3–4 for *R. columbina*, OD$_{600}$ of 1–1.5 for *F. johnsoniae*) and adjusted to an optical density (OD) of 1. The growth curve of *F. johnsoniae* in GCB was shown in Supplementary Figure 1. The donor DNA was added to the bacterial cells and incubated for 1 h at 37°C for *R. columbina* and at 25°C for *F. johnsoniae*. Then, 100 µl of cells were plated on GCB agar plates supplemented with erythromycin (1 µg/ml for *R. columbina*; 50 µg/ml for *F. johnsoniae*) to count transformants.

Then, 10 µl of cells were serially diluted with PBS and plated on GCB agar plates to count viable bacteria. The transformation frequency (TF) was calculated as transformants divided by viable bacteria. Then, 100 µl of cells were plated on GCB supplemented with the corresponding concentration of erythromycin to check for spontaneous mutants.

**Determination of Growth Curves**

The bacteria were streaked on blood plates or GCB agar plates. A single colony was cultured in 5 ml of GCB liquid medium with shaking at 37°C for 14 h. The bacterial cells were transferred into 20 ml of GCB with or without peptone, phosphate or iron at an OD of 0.05 and cultured at 37°C with shaking. The OD$_{600}$ was
TABLE 1 | Strains and primers used in this study.

| Strain                          | Genotype or description    | Source          |
|---------------------------------|----------------------------|-----------------|
| F. johnsoniae                   | ATCC 17061                 | CGMCC           |
| R. columbina                    |                            | CGCUG           |
| R. columbinaΔC237_RS0105470-Erm | ATCC 10105470, EmR         | This study      |
| R. anatipestifer                | ATCC11845, KanR            | This study      |

| Primer                          | Sequence                   | Source          |
|---------------------------------|----------------------------|-----------------|
| Up(gldH) P1                     | TAGCCGGGACAAATGTGGTAAACTAAATGCT | F. johnsoniae   |
| Up(gldH) P2                     | GACTGGAAAGTGGTTTTTTGTGATAATTATAGGTTTT | F. johnsoniae   |
| Erm(gldH) P1                    | ATTTATCTCACAAGACCGAGTCTCTACGAAA | R. anatipestifer CH-1 |
| Erm(gldH) P2                    | ATAATCTTTTTTCAGACTTAGCAAGGGAAGGATGAAA | R. anatipestifer CH-1 |
| Down(gldH) P1                   | GTAGTTCAAAGTCTTGCTGTGCTAATTTTGC | R. anatipestifer CH-1 |
| Down(gldH) P2                   | TTTTAGAGAAATAGGTTTGTGCTGCTGAGCT | R. anatipestifer CH-1 |
| RC-Up P1                        | CCCACCATAGTTTGCAGTAGTATTATGGCC | R. anatipestifer |
| RC-Up P2                        | CGGGAAATGTTGAGAAACGATATAAAATTTCCTCG | R. anatipestifer |
| RC-Erm P1                       | TTAATATAACGTTCTATCACACTTTCAGCTTCATAGCA | R. anatipestifer |
| RC-Erm P2                       | GATTTATATAGACAAGTGCAAAATACGCTTTTTGCAAA | R. anatipestifer |
| RC-Down P1                      | TGGTCAAAATGAGCGCCCATAAATACGCTTTTTGCAAA | R. anatipestifer |
| RC-Down P2                      | TAGTTCAAAGTGGGCTGCTGCTGCTGAGCT | R. anatipestifer |
| RC-16S rRNA P1                  | ATGGAATTAATACAGCACCATTTTGA | R. anatipestifer |
| RC-16S rRNA P2                  | TCAAATATGCCTTTTATGAAAAGGTA | R. anatipestifer |
| C237_RS0105470 P1               | AGTTAATACGAAAGAATTTATATGCTA | R. anatipestifer |
| C237_RS0105470 P2               | AAGTTAATACGAAAGAAGGTCCTC | R. anatipestifer |

The Effect of Components of GCB on Natural Transformation in R. columbina

Bacterial cells were cultured to the logarithmic phase (OD600 = 3–4) and adjusted to an OD600 of 1. The bacterial cells were collected and resuspended in GCB medium depleted of vitamin B1 (VB1), glucose, L-glutamine, NaCl, peptone, or phosphate. After the bacteria were incubated at 37°C for 30 min, donor DNA was added to the cultured cells, and natural transformation was performed. Iron is essential for the growth of most bacteria (Liao et al., 2016). To investigate whether iron affects the growth and natural transformation of R. columbina, the growth curve of R. columbina in GCB supplemented with different concentrations of iron chelator ethylenediamine-N,N'-bis(2-hydroxyphenylacetic acid) (EDDHA) according to the method mentioned previously (Press et al., 2001; Liu et al., 2016, 2019) and natural transformation were performed after the bacteria were incubated into GCB supplemented with the corresponding concentration of EDDHA at 37°C for 30 min. The viable bacteria and transformants were counted, and the TF was calculated.

EDTA Treatment

Bacterial cells were cultured until the logarithmic phase (OD600 = 3–4) at 37°C with shaking and adjusted to an OD600 of 1. Three hundred microliters of bacteria were collected and resuspended in GCB medium supplemented with 0.5 mM EDTA. Natural transformation was performed after the bacteria were incubated at 37°C for 30 min. The TF was calculated according to the method described previously. To investigate which divalent cation affects the natural transformation of R. columbina, different concentrations of CaCl2, MgCl2, ZnCl2, MnCl2, or CuCl2 were added into the GCB medium supplemented with the corresponding concentration of EDTA. The bacterial cells were first incubated in the above medium at 37°C for 30 min, and natural transformation was then performed. The TF was calculated as described previously.

Statistics

Statistical analysis was performed using GraphPad Prism 8.0 (GraphPad Software Inc., La Jolla, United States). An unpaired two-tailed Student’s t-test was used to compare two groups, and a value of P < 0.05 was considered significant. Data represent the mean and standard deviation (SD) from at least three independent experiments.

RESULTS

R. columbina, but Not F. johnsoniae, Is Naturally Competent Under the Same Conditions

To assay whether other members of Flavobacteriaceae were able to undergo natural transformation, R. columbina and F. johnsoniae were selected. We used the same method as described in a previous study for R. anatipestifer to determine the natural competence of these two species.
After *R. columbina* incubated with donor DNA which contains the upstream sequence of C237_RS0105470, an erythromycin resistance cassette and the downstream sequence of C237_RS0105470 (Figure 1A), many resistant colonies grew on the plate with erythromycin. However, no resistant colonies appeared in the control group without donor DNA (the spontaneous mutation rate of erythromycin resistance was lower than the detection limitation). Random single colonies were verified using PCR to ensure that the target gene was replaced by the erythromycin resistance cassette through homologous recombination. As shown in Figure 1B, compared to the wild-type strain, the resistant colonies contained an erythromycin resistance gene but not a target gene. It was suggested that the target gene has been replaced by the erythromycin resistance cassette and that the target sequence of the *R. columbina* strain was lost. It was strongly supported that *R. columbina* was naturally competent and that natural transformation could be used to efficiently generate targeted gene disruptions in *R. columbina*, with a TF of 4.14 (±0.5) × 10⁻⁶.

After *F. johnsoniae* incubated with donor DNA containing the upstream sequence of *gldH*, an erythromycin resistance cassette and the downstream sequence of *gldH*, the transformants were selected on GCB plates supplemented with 50 µg/ml erythromycin (the MIC of erythromycin for *F. johnsoniae* is 16 µg/ml). However, no resistant colony appeared with or without donor DNA. It has been shown that *gldH* can be deleted in *F. johnsoniae* through other methods (McBride et al., 2003), suggesting that this gene is not essential for the survival of the bacteria. Overall, it was suggested that *F. johnsoniae* could not perform natural transformation using the same method as *R. columbina* under the same conditions.

**Searching for the Components of the Natural Transformation Machinery in the Genome of *F. johnsoniae***

To investigate whether *F. johnsoniae* contains the homologous proteins that involved in natural transformation. We aligned the amino acids sequences of T4SS from *H. pylori* and *Agrobacterium*.
tumefaciens, T4P from V. cholerae, T2SS from C. jejuni, and other hypothetical competence proteins from R. anatipestifer with genome of F. johnsoniae. As shown in Table 2, only the homolog of ComB11 in T4SS, which is a putative VirB11-homologous ATPase (Karnholz et al., 2006), was found in F. johnsoniae and showed 40.8% identity with the ComB11 of H. pylori. Based on the T4P of V. cholerae (Seitz and Blokesch, 2013b), only the homologs of PilB, PilC, PilF, PilQ and PilT were discovered.

| Homologs in F. johnsoniae. |
|-----------------------------|
| Identity² |
| Homologs in F. johnsoniae. |
| Identity² |

| T4SS Protein ID | Homologs in F. johnsoniae. |
|-----------------|-----------------------------|
| VirB1 AAZ50518.1 None None |
| ComB2 HP_0015 None None |
| ComB3 HP_0016 None None |
| ComB4 HP_0017 None None |
| VirB5 AAZ50522.1 None None |
| ComB6 HP_0037 None None |
| VirB7 AAZ50524.1 None None |
| ComB8 HP_0038 None None |
| ComB9 HP_0039/40 None None |
| ComB10 HP_0041/42 None None |
| ComB11 HP_1421 WP_012022707.1 40.80% |
| VirD4 HP_0524 None None |

| T4P Protein ID | Homologs in F. johnsoniae. |
|----------------|-----------------------------|
| PiA VC_2423 None None |
| PiB VC_2424 WP_012022707.1 48.41% |
| PiE VC_0857 None None |
| FimT VC_0858 None None |
| VC_0859 VC_0859 None None |
| VC_0860 VC_0860 None None |
| PiV VC_0861 None None |
| PiF VC_1612 WP_012024651.1 25.98% |
| PiQ VC_2630 WP_012022708.1 27.43% |
| PiP VC_2631 None None |
| PIO VC_2632 None None |
| PiN VC_2633 None None |
| PiM VC_2634 None None |
| PiC VC_2425 WP_012022704.1 29.62% |
| PiT VC_0462 WP_012022707.1 43.33% |

| T2SS Protein ID | Homologs in F. johnsoniae. |
|-----------------|-----------------------------|
| CtsD Cj1474c WP_012022708.1 23.51% |
| CtsF AAP87276.1 WP_012022704 24.21% |
| CtsP Cj1473c None None |
| CtsR Cj1475c None None |
| CtsW Cj1028c None None |
| CtsG Cj1343c None None |
| CtsE Cj1471c None None |

| Others Protein ID | Homologs in F. johnsoniae. |
|-------------------|-----------------------------|
| DprA RA0C_RS05130 WP_012023081.1 37.91% |
| ComEC RA0C_RS04895 WP_012023505.1 24.45% |
| RecA RA0C_RS04870 WP_012023074.1 77.08% |
| ComM RA0C_RS07335 WP_012024210.1 74.56% |
| Ssb RA0C_RS02530 WP_012022955.1 65.71% |
| RadC RA0C_RS02540 WP_012022955.1 56.89% |

³Homologs in F. johnsoniae. ²Amino acids identity between F. johnsoniae and the example in the table.
in *F. johnsoniae* and shared 48.41, 29.62, 25.98, 27.43, and 43.33% with each relative protein of *V. cholerae*, respectively. PilB and PilT are polymerization and depolymerization ATPases, respectively (Seitz and Blokesch, 2013b). PilC is an inner membrane platform protein which interacts with PilB and PilT to control both pilus assembly and disassembly (Takhar et al., 2013). PilF is pilolin protein which is essential for pilus biogenesis (Matthey and Blokesch, 2016). PilQ is a secretion pore, which plays a role in translocating pilus on the cell surface (Wolfgang et al., 2000). Furthermore, only the homologs of CtsD and CtsF were found in *F. johnsoniae* based on the T2SS of *C. jejuni* (Wiesner et al., 2003). CtsF is an outer membrane protein which has homology to the PilQ protein (Wiesner et al., 2003). CtsD is an inner membrane protein and shares similarity to PilG of *N. gonorrhoeae* which has homology to the PilC of *V. cholerae* (Tønjum et al., 1995). Other hypothetical competence protein of *R. anatipestifer*, like DprA, ComEC, RecA, Ssb, ComM and RadC is also present in *F. johnsoniae* (Liu et al., 2017). These results indicated that these homologs of *F. johnsoniae* may be sufficient to encode a T4P-type DNA uptake system in addition to the proteins usually needed for DNA translocation and cytoplasmic processing.

**Natural Transformation of *R. columbina* Increases During the Logarithmic Phase**

We were wondering whether natural transformation was able to occur in all growth phases in *R. columbina*, the TF was assayed. Natural transformation was performed at each time point by adding the same amount of donor DNA. As shown in Figure 2, natural transformation of *R. columbina* occurred in all growth phases, and the TF was the highest during the logarithmic phase \( [TF = 6.45 \pm 0.55] \times 10^{-6} \) and lowest in the lag phase \( [TF = 6.35 \pm 0.5] \times 10^{-8} \). The number of transformants in different growth phases were included in the Supplementary Data Sheet 2. To investigate the saturated concentration of donor DNA for logarithmic growth period bacteria, *R. columbina* was cultured to the logarithmic phase and mixed with different amounts of donor DNA (0.1, 1, 10, 100, 200, 500, 1,000, 2,000, or 4,000 ng). As shown in Figure 3, the TF increased with increasing DNA concentration when the amount was lower than 1,000 ng. However, when the DNA amount was higher than 1,000 ng, the TF no longer increased. The number of transformants were included in the Supplementary Data Sheet 2. These results suggested that 1,000 ng of donor DNA was saturating for natural transformation in *R. columbina*.

**R. columbina Preferentially Takes Up Its Own DNA Over Heterologous DNA**

It has been reported that some bacteria, such as *H. influenzae* and *Neisseria*, preferentially take up DNA containing short motifs known as uptake signal sequences (USSs) or DNA uptake sequences (DUSs) (Scocca et al., 1974; Sisco and Smith, 1979). These short motifs have accumulated in the genome to high densities over evolutionary time (Mell and Redfield, 2014). To determine whether *R. columbina* also preferentially takes up its own DNA, a natural transformation competition experiment was performed. In this experiment, the genome of *R. columbina* ΔC237_RS0105470 was used as the donor DNA. As shown in Figure 4, when 1 µg of donor DNA was mixed with 1 µg of genomic DNA of *R. columbina*, the TF was decreased two-fold compared to the control in which without competition DNA was added; Moreover, the TF was decreased with the increase of competing DNA. However, the TF showed no significant changes when 1 µg donor of DNA was mixed with 1 µg of *R. anatipestifer* or *E. coli* genomic DNA compared to that when only 1 µg of donor DNA was added. The TF was decreased significantly only when the *R. anatipestifer* or *E. coli* genomic DNA was increased to 10 µg, which can be considered as unspecific effect. The
number of transformants were included in the Supplementary Data Sheet 2. To further investigate whether *R. columbina*, *F. johnsoniae* or *R. anatipestifer* contain putative DUSs or USSs, Jellyfish was to be used to count the numbers of occurrences of individual kmers in both strands of their genome, respectively, with a parameter that limited the length of kmers to less than 10 bp. As shown in Table 3, sequences with the top three repeats for 10 bp, 9 bp, 8 bp, and 7 bp were listed, respectively. It was found that hundreds of repeat sequences or its complement were present in their genomes. The frequency of the 9-bp repeat sequence is 0.6/kb for *R. anatipestifer*, 0.5/kb for *R. columbina* and 0.5/kb for *F. johnsoniae*, respectively, which is much higher than the frequency of 0.1/kb expected for a random sequence of this base composition for them. Whether this sequence has the function of DUSs or USSs needs to be further investigated.

The TF of *R. columbina* Is Increased Under Peptone-Restrictive or Phosphate-Restrictive Conditions

To investigate the effect of the nutrients on natural transformation, natural transformation was conducted in GCB depleted for each component, including vitamin B1 (VB$_1$), glucose, L-glutamine, NaCl, peptone and phosphate. As shown in Figure 5A, the TF of *R. columbina* was 1.9 ($\pm$0.1) $\times$ 10$^{-5}$ in GCB depleted of peptone, which increased five-fold compared to that in GCB. The TF of *R. columbina* was 9.05 ($\pm$0.5) $\times$ 10$^{-6}$ in GCB depleted of phosphate, which increased approximately two-fold compared to that in GCB. However, compared to the TF of *R. columbina* in GCB, there was no significant difference when VB$_1$, glucose, L-glutamine or NaCl was removed from GCB (Figure 5A). The number of transformants were included in the Supplementary Data Sheet 2.

Next, we investigated whether the change in TF was associated with the growth ability of bacteria. Therefore, the growth curve of bacteria was determined when peptone, phosphate, NaCl, glucose, L-glutamine or VB$_1$ was removed from GCB. The results showed that bacteria did not grow in GCB without peptone or VB$_1$, whereas the growth of bacteria was significantly decreased in GCB without phosphate; however, there were no significant differences when NaCl, glucose, or L-glutamine was removed from GCB (Figure 5B). To investigate whether iron affects the natural transformation of *R. columbina*, different concentrations of iron chelator EDDHA were supplemented into the GCB medium. As shown in Figure 5A, the TF did not change compared to that of the control (without EDDHA). The number of transformants were included in the Supplementary Data Sheet 2. However, the growth of *R. columbina* was significantly inhibited in iron-depleted medium (GCB supplemented with 200 $\mu$M EDDHA), suggesting that iron is essential for the growth of *R. columbina* (Figure 5B). Overall, these results suggested that peptone-restrictive or phosphate-restrictive medium had an effect on the natural transformation and this effect is not directly related to the growth ability.

Ca$^{2+}$, Mg$^{2+}$, Zn$^{2+}$, and Mn$^{2+}$ but Not Cu$^{2+}$ Promote the Natural Transformation of *R. columbina*

Iron has no effect on natural transformation, and we wondered if other divalent cations influence the natural transformation of
TABLE 3 | Analysis of putative DUSs or USSs in *R. anatipestifer*, *R. columbina* and *F. johnsoniae*.

| kmer | *R. anatipestifer* | *R. columbina* | *F. johnsoniae* |
|------|-------------------|----------------|-----------------|
|      | Sequence | Repeats | Expected repeats | Sequence | Repeats | Expected repeats | Sequence | Repeats | Expected repeats |
| 10   | AAAATAAAA | 301 | 56 | AAAAGAAAAA | 203 | 30 | AAAAATAAAAA | 759 | 183 |
|      | AAAATAAAA | 235 | 56 | AAAAATAAA | 196 | 54 | AAAACAAAAAA | 743 | 95 |
|      | AAAATAAA | 201 | 56 | AAAATTTAAA | 196 | 54 | AAAAATATAT | 479 | 183 |
| 9    | AAAATAAAA | 650 | 175 | AAAATAAA | 666 | 171 | TTATATAAA | 1769 | 558 |
|      | AAAATAAA | 534 | 175 | AAAATAAA | 551 | 171 | AAAATAAA | 1591 | 558 |
|      | AAAATAAA | 520 | 175 | AAAATAAA | 514 | 171 | AACCAAA | 1569 | 288 |
| 8    | AAAATAAA | 1428 | 538 | AAAATAAA | 1534 | 535 | TTATTTAAAA | 4593 | 1694 |
|      | AAAATAAA | 1424 | 538 | AAAATAAA | 1522 | 535 | AAAATAA | 4095 | 1694 |
|      | AAAATAAA | 1412 | 538 | AAAATAAA | 1510 | 535 | AACAAAT | 3910 | 1694 |
| 7    | AAAATAAA | 3872 | 1657 | AAAATAA | 4210 | 1674 | TTATTTAA | 13724 | 5141 |
|      | AAAATAAA | 3185 | 1657 | AAAATAA | 4114 | 1674 | AAAATAA | 11435 | 5141 |
|      | AAAATAAA | 2881 | 1657 | AAAATAA | 3445 | 1674 | AAAATAA | 11213 | 5141 |

*Expected repeats is calculated directly from the base composition and length of the genome.*

*R. anatipestifer* (2436790 bp) and *F. johnsoniae* (6096872 bp) and the GC content of each strain: *R. anatipestifer* (35%), *R. columbina* (36%) and *F. johnsoniae* (34.1%).

**Figure 5** | The effect of GCB ingredients on natural transformation and growth in *R. columbina*. (A) TFs of *R. columbina* in GCB, GCB depleted of VB$_1$, glucose, L-glutamine, NaCl, peptone or phosphate and GCB supplemented with different concentrations of EDDHA. The *P*-value refers to the difference between the TF in GCB and in GCB depleted of peptone or phosphate, respectively. Insignificance *p*-value (*P* > 0.05) was not shown. (B) Growth curves of *R. columbina* in GCB, GCB depleted of VB$_1$, glucose, L-glutamine, NaCl, peptone, or phosphate and GCB supplemented with different concentrations of EDDHA. The bacteria were cultured in 20 ml of the above medium inoculated at an OD$_{600}$ of 0.05, and the OD$_{600}$ value was determined every 2 h. All the results are representative of three independent experiments. Error bars denote standard deviation.

*R. columbina*. Natural transformation was conducted in GCB medium with 0.5 mM EDTA, which had no effect on the survival of bacteria (Supplementary Figure 2). The results showed that the TF in GCB with 0.5 mM EDTA was 5.75 (±0.75) × 10$^{-7}$, which was decreased approximately 4-fold compared to that in GCB [TF = 2.5(±0.1) × 10$^{-6}$], suggesting that 0.5 mM EDTA had a significant inhibitory effect on natural transformation in *R. columbina* (Figure 6A). To investigate which divalent cation has an effect on natural transformation, different concentrations of CaCl$_2$, MgCl$_2$, ZnCl$_2$, MnCl$_2$, or CuCl$_2$ were supplemented into the cell culture after incubation with EDTA. The addition of 0.5 mM Ca$^{2+}$ basically restored transformation, and the TF increased as the concentration of Ca$^{2+}$ increased (Figure 6B). The addition of 0.5 mM Mg$^{2+}$ completely restored the TF; however, the frequency did not increase as the concentration of Mg$^{2+}$ increased (Figure 6B), suggesting that 0.5 mM was likely a saturating concentration of Mg$^{2+}$ for natural transformation in *R. columbina*. The TF gradually increased as the concentration of Zn$^{2+}$ increased from 0.1 mM to 0.5 mM. The TF was the highest at 0.5 mM Mn$^{2+}$ (Figure 6B). However, the addition of different concentrations of Cu$^{2+}$ did not restore the natural transformation but instead inhibited natural transformation.
which preferentially take up DNA with
which takes up only methylated DNA but not PCR
C. jejuni
PCR fragments are not suitable substrates, such as occurs with
process (Mirouze et al., 2013). Another possibility is that the
assembly of the uptake apparatus is a transient and regulated
which natural transformation is not constitutive, as synthesis and
fragments (Beauchamp et al., 2017), and
third possibility is that natural transformation in
Berry et al., 2013; Frye et al., 2013; Mell and Redfield, 2014). The
an USS or DUS over other sources of DNA (Mell et al., 2012;
choose the correct isolates. It has been reported that even for the
transformation of Vibrio cholerae
must be induced by special substrates, such as occurs with natural
transformation of
that the natural transformation of
was not. One possibility is that the natural transformation of
F. johnsoniae does not occur at all growth phases but only at a certain time point, for
example natural transformation happens to S. pneumoniae, in which natural transformation is not constitutive, as synthesis and
assembly of the uptake apparatus is a transient and regulated process (Miroouze et al., 2013). Another possibility is that the
PCR fragments are not suitable substrates, such as occurs with
C. jejuni, which takes up only methylated DNA but not PCR
fragments (Beauchamp et al., 2017), and H. influenzae and
Neisseria gonorrhoeae, which preferentially take up DNA with an
USS or DUS over other sources of DNA (Mell et al., 2012;
Berry et al., 2013; Frye et al., 2013; Mell and Redfield, 2014). The
third possibility is that natural transformation in F. johnsoniae
must be induced by special substrates, such as occurs with natural transformation of Vibrio cholerae, which is induced by chitin
(Meibom et al., 2005). The fourth possibility is that we did not choose the correct isolates. It has been reported that even for the
competent bacteria, some isolates are non-transformable (Evans and Rozen, 2013; Dalia et al., 2015). The last possibility is that
F. johnsoniae does not undergo natural transformation because of the lack of some essential genes for natural transformation.
Consistent with the natural transformation of R. anatipestifer
(Liu et al., 2017), the natural transformation of R. columbina
is also constitutive, although the TF is different at the different
growth phases. This phenomenon might occur because the expression of genes involved in natural transformation in
R. columbina is different at the different growth phases. Similar to R. anatipestifer, R. columbina preferentially takes up self-
sourced genomic DNA, suggesting that each bacterium might use a certain mechanism, such as a restriction modification (R-M)
system (Aras et al., 2002; Zhang and Blaser, 2012) or other systems, to prevent the uptake of excessive extracellular DNA that
may overload the bacteria, subverting the bacterial genome with extracellular DNA from competing strains.
Originally, the function of natural transformation was hypothesized as “DNA for food” (Redfield, 2001), because the natural competence of H. influenzae and B. subtilis was activated under nutrient-limited condition (Bobb, 1963; Herriott et al., 1970). However, this hypothesis is questionable, since the natural competence of some other bacteria, such as A. baumannii, requires a nutrient-rich condition (Traglia et al., 2016). In the case of R. columbina, we showed that the TF of R. columbina was significantly increased under peptone-restrictive or phosphate-restrictive conditions, suggesting that the uptake of DNA may be “food” for R. columbina to supplement the nitrogen and phosphorus.
A more plausible hypothesis for the function of natural transformation is “DNA for repair” (Michod et al., 2008; Engelmoer et al., 2013), since the natural transformation of some

![Figure 6](https://example.com/figure6.png)

**Figure 6** Effect of EDTA and different divalent cations on the natural transformation of R. columbina. (A) Viable bacteria were counted after treatment with or without 0.5 mM EDTA for 1 h, and the effect of 0.5 mM EDTA on the natural transformation of R. columbina was determined. The P-value refers to the difference between the TF in GCB and in GCB containing 0.5 mM EDTA. (B) The natural TF of R. columbina in GCB supplemented with 0.5 mM EDTA and different supplemented concentrations of divalent cation. The concentration of Ca$^{2+}$ are 0.5, 1, and 5 mM, respectively. The concentration of Mg$^{2+}$ are 0.5, 1, and 5 mM, respectively. The concentration of Zn$^{2+}$ are 0.1, 0.2, and 0.5 mM, respectively. The concentration of Mn$^{2+}$ are 0.2, 0.5, and 1 mM, respectively. The concentration of Cu$^{2+}$ are 0.1, 0.2, and 0.5 mM, respectively. The P-value refers to the difference between the TF in GCB containing 0.5 mM EDTA and GCB containing 0.5 mM EDTA supplemented by Ca$^{2+}$, Mg$^{2+}$, Mn$^{2+}$, or Cu$^{2+}$, respectively. All the results are representative of three independent experiments. Error bars denote standard deviation.

**DISCUSSION**

*Riemerella anatipestifer* is the first bacterium of Flavobacteriaceae to be reported to have natural competence (Liu et al., 2017). To check whether other bacteria in Flavobacteriaceae are also naturally competent, F. johnsoniae and R. columbina were selected. The results showed that R. columbina was able to undergo natural transformation; however, F. johnsoniae was not competent under the same conditions. One possibility is that the natural transformation of F. johnsoniae does not occur at all growth phases but only at a certain time point, for example natural transformation happens to S. pneumoniae, in which natural transformation is not constitutive, as synthesis and assembly of the uptake apparatus is a transient and regulated process (Miroouze et al., 2013). Another possibility is that the PCR fragments are not suitable substrates, such as occurs with C. jejuni, which takes up only methylated DNA but not PCR fragments (Beauchamp et al., 2017), and H. influenzae and Neisseria gonorrhoeae, which preferentially take up DNA with an USS or DUS over other sources of DNA (Mell et al., 2012; Berry et al., 2013; Frye et al., 2013; Mell and Redfield, 2014). The third possibility is that natural transformation in F. johnsoniae must be induced by special substrates, such as occurs with natural transformation of Vibrio cholerae, which is induced by chitin (Meibom et al., 2005). The fourth possibility is that we did not choose the correct isolates. It has been reported that even for the
bacteria, such as *H. pylori* (Dorer et al., 2010), *S. pneumoniae* (Prudhomme et al., 2006) and *B. subtilis* (Zhang et al., 2018), was activated by antibiotics or DNA damage reagent. Here, we also investigated the effects of antibiotics such as ampicillin (inhibitor of cell wall biosynthesis), kanamycin (inhibitor of protein biosynthesis), nalidixic acid (inhibitor of DNA replication) and mitomycin C (intercalation with DNA) during the natural transformation of *R. columbina*. We showed that none of the antibiotics affected natural TF of *R. columbina* did not change after treatment with antibiotics (Supplementary Figure 3), suggesting that the antibiotics used here cannot trigger natural transformation of *R. columbina*.

Our systematic investigation of natural transformation in the Flavobacteriaceae family shows that it is widely distributed. However, the environmental conditions that trigger natural transformation vary from species to species. In this family, natural transformation appears to play a major role in HGT. The discovery of natural transformation in *R. columbina* represents the basis for the establishment of gene editing and cloning system in this bacterium.

**DATA AVAILABILITY STATEMENT**

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author/s.

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AUTHOR CONTRIBUTIONS

ML, DZ, and AC conceived and designed the experiments. LH, LX, MH, CX, SZ, QG, DS, and BT performed the experiments. MW, RJ, SC, ZX, QY, and YW analyzed the data. JH, XO, and SM contributed to reagents, materials, and analysis tools. ML, FB, and AC wrote the manuscript. All authors have reviewed the manuscript.

**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.634895/full#supplementary-material
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 Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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