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Development and Application of an Insertional System for Gene Delivery and Expression in *Campylobacter jejuni*

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The genetic investigation of *Campylobacter jejuni*, an important gastrointestinal pathogen, has been hampered by the lack of an efficient system for introduction of exogenous genetic information, as commonly used vectors designed for *Escherichia coli* and other bacteria cannot be maintained in *Campylobacter* cells. Additionally, gene expression in *Campylobacter* requires the presence of species-specific promoters. In this study we exploited the availability of several conserved copies of rRNA gene clusters for insertion of various genes into the chromosome by homologous recombination. The high conservation of the rRNA sequences means that the procedure can be applied to other *Campylobacter* strains. The presence of a *Campylobacter*-derived promoter in this vector ensures expression of exogenous genes in target cells. The efficiency of the procedure was demonstrated by complementation of mutations in two strains of *Campylobacter*. In addition, we applied the system for introduction and expression of a green fluorescent protein (GFP). GFP-expressing *Campylobacter* allowed visualization of sessile bacteria attached to a glass surface in stationary liquid culture. The study demonstrated that the attached bacteria contained an assemblage of coccoid and spiral forms with liquid channels preserving viable highly motile cells. We demonstrate a novel universal procedure for gene delivery and expression that can be used as an efficient tool to study this poorly understood pathogen. The principles developed in this study could be more widely applied for the manipulation of other bacteria that are refractory to genetic analysis.

Despite some reduction in the number of cases linked to infection with *Campylobacter*, it remains one of the major causes of gastrointestinal disease worldwide (3). Genetic studies on the physiology and virulence of *Campylobacter* have become more efficient since the publication of the complete genomic sequence of *Campylobacter jejuni* strain NCTC 11168 (25). Subsequent studies resulted in the discovery of heterogeneity in gene content and genetic variations among various strains of *C. jejuni* (10). One of the key mechanisms of such diversity is a remarkable propensity of the bacterium to undergo phase variation and recombination (13, 15), combined with many strains being naturally transformable with exogenous DNA (31). The marker rescue technique developed for *Campylobacter* cells (20) has now become the most common approach used in mutagenesis of these bacteria.

Although insertional mutagenesis is now routine, the introduction and expression of exogenous genes into *Campylobacter* cells remain a major problem, as many strains, including the sequenced strain NCTC 11168, are refractive to complementation and gene expression. Although some reports described shuttle vectors for the complementation of mutations in *trans* and for introduction and expression of a *gfp* gene, their use is often inefficient and is limited to certain strains (23, 24). For example we have been unable to introduce shuttle vectors pRY112 (34), pMW10 (32), pGUO0202 (1), and pMEK91 (24) into various *C. jejuni* strains, including the sequenced strain NCTC 11168 and its derivative 11168H.

In this study we describe an efficient procedure for gene delivery and expression, which overcomes shuttle vector-related limitations. The method has been successfully tested in a number of applications that have facilitated our understanding of this important pathogen.

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MATERIALS AND METHODS

Bacterial strains and growth conditions. The following *C. jejuni* strains were used in this study: 11168H (HS:2), which is a hypermotile derivative of strain NCTC 11168 (15), and 81-176 (HS:23/36, enteritis isolate used in human challenge studies) (4). *Escherichia coli* strain XL2-MRF′ (Stratagene, La Jolla, CA) was used in cloning experiments. *C. jejuni* was grown under microaerobic conditions in an incubator (85% N_2, 10% CO_2, 5% O_2) for 2 days at 37°C on Columbia base agar (Oxoid, Basingstoke, United Kingdom) supplemented with 6% horse blood. *E. coli* was grown on Luria-Bertani agar (Oxoid, Basingstoke, United Kingdom). Where necessary, kanamycin or ampicillin was added to a concentration of 50 μg ml^{-1} or 100 μg ml^{-1}, respectively. Motility was tested on 0.4% Mueller-Hinton agar (Oxoid, Basingstoke, United Kingdom). For investigation of bacteria attached to glass cover slides in stationary cultures, a 11168H strain expressing a green fluorescent protein (GFP) was first grown in brucella broth medium (Oxoid, Basingstoke, United Kingdom) supplemented with chloramphenicol at 15 μg ml^{-1} on a rotatory platform at 90 rpm for 2 days and then diluted 1:20 with the same medium and incubated for 4 days without shaking in flasks containing glass coverslips. The coverslips were removed and mounted on a glass slide for visualization under a confocal microscope.

Electroporation. The electroporation procedure was carried out as follows. A 2-day bacterial plate culture was resuspended in buffer containing 272 mM sucrose and 15% glycerol at 0°C and washed three times with the same buffer, and 50-μl aliquots were used for each transformation. After addition of DNA (0.5 μg in 5 μl), the mixture was transferred to an ice-cold electroporation cuvette. Electroporation was performed at 2.5 kV, 200 Ω, and 25 μF, after which 100 μl of SOC buffer were added to the cuvette, and the bacterial suspension was transferred onto a nonselective blood agar plate. After overnight incubation at 37°C under microaerobic conditions, bacteria were spread onto a selective plate and incubated for a further 3 to 4 days.

Growth competition test. The growth competition index (CI) was determined as described previously (8). Two-day agar cultures of the *Campylobacter* Cam^r derivative 11168H/pRRC4 and the wild-type recipient 11168H, grown with or without chloramphenicol (10 μg/ml), respectively, were inoculated into separate tissue culture flasks containing 10 ml of brucella broth without chloramphenicol.

TABLE 1. Plasmids

Plasmid	Description	Source (reference)
pRY112	Shuttle vector	P. Guerry (34)
pMW10-13	Shuttle vector containing wild-type *gfp* gene	E. Gaynor (unpublished)
pMEK91	Shuttle vector containing *egfp-pMEK* under control of *ompE* promoter	M. E. Konkel (24)
pAV35	Source of Cam^r gene cassette	A. van Vliet (29)
pJMK30	Source of Kan^r gene cassette	A. van Vliet (29)
pGUO0202	Shuttle vector	V. Korolik (1)
pGEM-T Easy	Cloning vector	Promega, Southampton, United Kingdom
pEGFP	Contains *egfp* gene	BD Biosciences Clontech, Palo Alto, CA
pRR	Fragment of rRNA gene cluster cloned into pGEM-T Easy	This study
pRRC	Cam^r cassette cloned into pRR	This study
pRPGL	*pglH* gene in pRRC	This study
pRMAF	*maf5* gene in pRRC	This study
pRGW	Wild-type *gfp* gene in pRRC	This study
pRED	*egfp* gene from pEGFP with added optimal SD sequence cloned into pRRC	This study
pREM	P_{ompE}-*egfp* fusion from pMEK91 in pRRC	This study

After incubation for a further 2 days on a rotary platform at 90 rpm under microaerobic conditions, the optical density at 600 nm was measured and serial dilutions were plated onto agar plates with (11168H/pRRC4) or without (11168H) chloramphenicol. A 100-μl aliquot of liquid culture of the Cam^r derivative (2.8×10^4 CFU) was mixed with an equal volume of wild-type strain 11168H (3.5×10^4 CFU) in a flask with fresh brucella broth (10 ml), which corresponded to an input Cam^r/Cam^s ratio of 1.25. After incubation for two days, serial dilutions were plated onto blood agar plates with and without chloramphenicol in triplicates. The colonies were counted, and the output Cam^r/Cam^s ratio was calculated. The CI was determined as output Cam^r/Cam^s ratio divided by input Cam^r/Cam^s ratio.

General cloning techniques. Plasmids used in this study are listed in Table 1. Restriction enzymes were purchased from either Promega (Southampton, United Kingdom) or New England Biolabs (Hitchin, United Kingdom). T4 DNA ligase, T4 DNA polymerase, and calf thymus alkaline phosphatase were purchased from Promega (Southampton, United Kingdom). Oligonucleotides (Table 2) were from Sigma-Genosys (Pampisford, United Kingdom). Standard restriction digestion, DNA ligation, and cloning procedures were essentially as described elsewhere (26). T4 DNA polymerase treatment for generation of blunt ends was performed according to the manufacturer's protocol (Promega, Southampton, United Kingdom).

Construction of the delivery vector pRRC. The rRNA region containing fragments of genes encoding 16S and 28S rRNAs (*rrs* and *rrl* genes, respectively) was PCR amplified using primers ak231 and ak232 (Fig. 1) and cloned into pGEM-T Easy vector (Promega, Southampton, United Kingdom) to produce pRR. The pRR plasmid was digested with XbaI, made blunt ended using T4 DNA polymerase, and ligated with a blunt-ended BamHI fragment of plasmid pAV35 (29) containing a Cam^r gene cassette. After transformation into *E. coli*, a recombinant plasmid containing a chloramphenicol resistance gene inserted in the same orientation as the rRNA genes was selected and designated pRRC.

Construction of delivery vectors for gene expression in *C. jejuni*. (i) *pglH* gene. The *pglH* gene was PCR amplified using primers ak248 and ak249, and the product was digested with XbaI enzyme and cloned into XbaI-digested pRRC plasmid to produce pRPGL in such a way that *pglH* gene was transcribed in the same orientation as the Cam^r gene (Table 1; Fig. 1), which was verified via restriction analysis.

(ii) *maf5* gene. The *maf5* gene was PCR amplified using primers ak244 M and ak245 M, and the product was digested with XbaI enzyme and cloned into XbaI-digested pRRC plasmid to produce pRMAF in such a way that the *maf5* gene was transcribed in the same orientation as the Cam^r gene, which was verified via restriction analysis. The forward primer (ak244 M) contained a Shine-Dalgarno (SD) region optimized for *Campylobacter* (Fig. 1; Table 1).

(iii) Wild-type *gfp* gene. Plasmid pMW10-13 (Table 1) containing the wild-type *gfp* gene was digested with XbaI and ClaI. The fragments were made blunt ended using T4 DNA polymerase and ligated to blunt-ended vector pRRC after digestion with XbaI (Fig. 1; Table 1). A recombinant clone containing pRRC with the wild-type *gfp* gene transcribed in the same direction as the Cam^r gene cassette was selected. A plasmid with the correct orientation of the wild-type *gfp* gene was selected via restriction analysis. Expression of GFP was confirmed via visualization of colonies with a fluorescence microscope.

TABLE 2. Primers used[a]

Primer	Sequence[a]	Description[b]
ak231	CTGGAACTCAACTGACGCTAAG	*rrs* (16S rRNA) (dir)
ak232	CTCTTGCACATTGCAGTCCTAC	*rrl* (23S rRNA) (rev)
ak233	GCAAGAGTTTTGCTTATGTTAGCAC	Cj*0029* (dir)
ak234	GAAATGGGCAGAGTGTATTCTCCG	Cj*0431* (dir)
ak235	GTGCGGATAATGTTGTTTCTG	Cj*0742* (dir)
ak237	TCCTGAACTCTTCATGTCGATTG	Cam^r gene cassette (up)
ak248	GCTCTAGACTTAAAGAGGAGAA**ATG**ATGAAAATAAGC	*pglH* (dir)
ak249	GCTCTAGATCATTAGGCATTTTTAACCTCGGCTATAAGC	*pglH* (rev)
ak261	GGACTAGTAGGAGATTTAAA**ATG**GTGAGCAAGGGCGAGGAGCTGTTCAC	*egfp* from pEGFP (dir)
ak262	GCTCTAGAAGGCCTTTACTTGTACAGCTCGTCCATGCCGAGAGTG	*egfp* from pEGFP (rev)
ak244M	GCTCTAGAAAGGAAGATAAA**ATG**GATGGAAAGGGTGAGAAGGTG	*maf5* (dir)
ak245M	GCTCTAGATTAAAGTGCTTTTTTCTTTTCTAAGAAG	*maf5* (rev)
ak238	GCTTGCATCTGATAAAGCACCTG	Cj*0863* (*xerD*) primer, reverse
DL3	ACCCAGCGAACCATTTGAGG	Kan^r gene cassette-specific primer
ak290	GCTTTTAGTTTATAAGACAAAAACCAAAAGAG	*rrs* (16S rRNA) gene-derived primer for sequencing of insertion sites

[a] The start codons are shown in boldface; the regions complementary to *Campylobacter* 16S rRNA (SD sequences) are underlined.
[b] dir, direct primer; rev, reverse primer; up, upward primer.

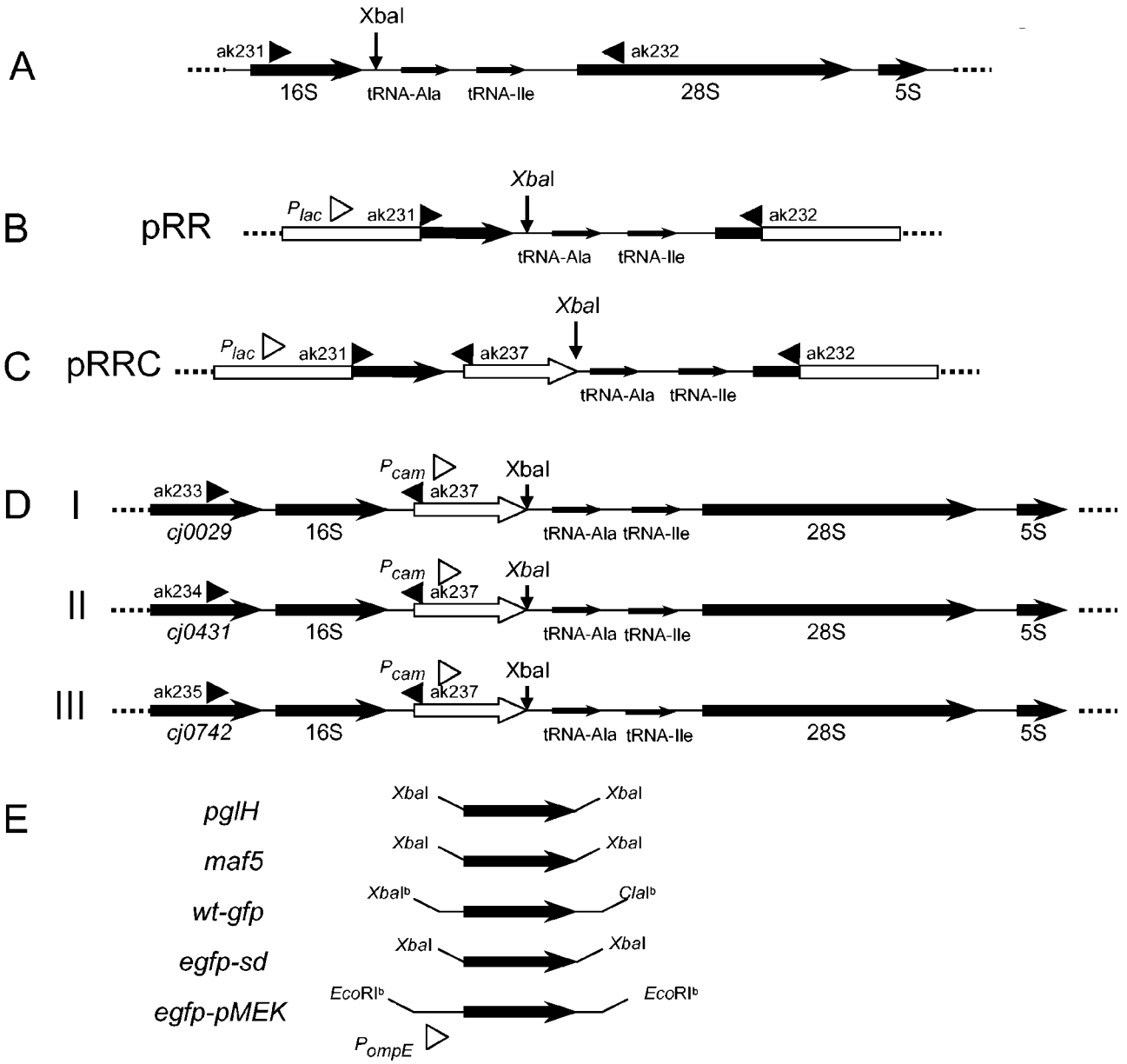

FIG. 1. A, Organization of the three rRNA gene clusters in strain NCTC 11168; the location of the intergenic XbaI site and positions of primers ak231 and ak232 used for PCR amplification are shown. B, A fragment of plasmid pRR containing the ak231/ak232 PCR product cloned into pGEM-T Easy vector. C, A fragment of plasmid pRRC derived from pRR via insertion of the Cam^r gene cassette. D, Three possible products of allelic replacement resulting from recombination of pRRC with the genome; the primers used for localization of insertion sites (ak233, ak234, ak235, and ak237) are shown. E, Genes inserted into the chromosome in this study. Blunt-ended DNA fragments are indicated by a "b" in the name of a restriction site; the arrowhead in the *egfp-pMEK* construct represents and additional promoter (P_{ompE}). The genes are shown as thick black arrows (not to scale). Open arrows represent the Cam^r gene. Open boxes indicate vector regions. Locations and directions of PCR primers and promoters are indicated by solid and open arrowheads, respectively.

(iv) *egfp-SD* gene. Primers ak261 and ak262 were used to PCR amplify the enhanced GFP gene (*egfp*) from the pEGFP plasmid (Fig. 1; Table 1). The primer ak261 contained a *Campylobacter* SD sequence. The PCR product was digested with XbaI enzyme and cloned into vector pRRC so that the gene encoding the enhanced green fluorescent protein with the *Campylobacter*-specific SD sequence (*egfp-SD*) was under the control of Cam^r gene promoter as verified by restriction analysis.

(v) *egfp-pMEK* gene. The EcoRI fragment of plasmid pMEK91 (24) containing the *efgp* gene (23) under the control of the P_{ompE} promoter of *C. jejuni* was blunt ended and inserted into XbaI-digested and blunt-ended vector pRRC (Fig. 1; Table 1). By using restriction analysis, a recombinant plasmid containing the gene encoding enhanced green fluorescent protein from plasmid pMEK91 (*egfp-pMEK*) under the control of two promoters, P_{cam} and P_{ompE}, was selected. *E. coli* colonies harboring this plasmid appeared yellow when visualized by eye under daylight and appeared bright green under the fluorescence microscope (excitation at 488 nm).

Construction of *xerD* mutant. Plasmid cam71a9, constructed during the course of the *C. jejuni* genome sequencing project (25), contains a 1.5-kb DNA insert with a fragment of gene *xerD* (Cj*0863*). After insertion of a blunt-ended BamHI fragment of pJMK30 (29) containing a Kan^r gene cassette into the unique SwaI

site within the *xerD* gene on plasmid cam71a9 and transformation into 11168H, Kan^r clones were selected. Insertion of the Kan^r gene cassette in a nonpolar orientation was confirmed by PCR with primers ak238 and DL3 (Table 2).

Gel electrophoresis and Western blotting. Bacteria were resuspended in sample buffer and incubated at 100°C for 10 min, and the lysate was analyzed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis in 12.5% precast polyacrylamide gels (Invitrogen, Paisley, United Kingdom). Gels were blotted onto a polyvinylidene difluoride membrane (Millipore, Watford, United Kingdom), blocked in phosphate-buffered saline containing 0.5% Tween 20 (PBST) for at least 30 min, and incubated with biotinylated soybean agglutinin (Vector Laboratories, Burlingame, CA) at a concentration of 10 to 20 $\mu g\ ml^{-1}$ in PBST for 1 hour. Following three brief washes in PBST, blots were incubated with extravidin peroxidase (Sigma-Aldrich, Poole, United Kingdom) diluted 1 in 1,000 in PBST for 30 min. Following a further three brief washes in PBST, blots were developed using a diaminobenzidine staining kit with nickel enhancement according to the manufacturer's instructions (Vector Laboratories, Burlingame, CA). Alternatively, the blots were probed with Penner 6 typing antiserum (1:100 dilution), followed by treatment with anti-rabbit immunoglobulin G peroxidase conjugate (Sigma-Aldrich, Poole, United Kingdom) at a 1:1000 dilution. All antibody dilutions were made using Tris-buffered saline containing 0.01% Tween 20 and supplemented with 1% bovine serum albumin (Sigma). The blots were developed using the diaminobenzidine staining kit with nickel enhancement according to the manufacturer's instructions (Vector Laboratories, Burlingame, CA). Broad-range molecular weight standards were supplied by New England Biolabs (Hitchin, United Kingdom).

Confocal microscopy. Bacterial cells were visualized using a Carl Zeiss LSM 510 confocal microscope according to the manufacturer's manual. The laser excitation wavelength was 488 nm, and the detection wavelength was 505 nm.

RESULTS

Transformation of *C. jejuni* by using shuttle vectors. The shuttle vectors pMW10-13, pRY112, pMEK91, and pGUO0202 (Table 1) were used for transformation into *C. jejuni* NCTC 11168 via natural transformation and electroporation, using standard protocols (31). Both electroporation and natural transformation protocols are very efficient tools for making insertional mutants via allelic replacement in many strains of *C. jejuni*, including NCTC 11168. However, repeated attempts with any of the available shuttle vectors and strain NCTC 11168 (or its derivative 1118H) resulted in no colonies on selective media.

Construction of the delivery vector. In order to minimize the disruption of rRNA genes, a spacer region between the 16S and 28S rRNA genes was selected. The genome of *C. jejuni* strain NCTC 11168 carries three identical clusters of rRNA genes. An XbaI site located immediately downstream from 16S rRNA genes was selected as an insertion site (Fig. 1A). A region containing this site with long flanking regions was PCR amplified and cloned into pGEM-T Easy vector to produce plasmid pRR (Fig. 1B). Insertion of the blunt-ended BamHI fragment containing the Cam^r gene into the blunt-ended XbaI site of pRR resulted in plasmid pRRC (Fig. 1C). A unique XbaI site of pRRC downstream from the Cam^r gene was used for cloning other genes, so that, if inserted in the correct orientation, these genes would be under the control of the constitutively expressed Cam^r gene promoter.

The Cam^r gene can be inserted into any of the three rRNA clusters. The pRRC vector was transformed into *C. jejuni* strain 11168H via electroporation, and Cam^r colonies were selected. The transformants were analyzed using the Cam^r gene-specific PCR primer ak237 and three other primers (ak233, ak234, and ak235) corresponding to the regions adjacent to the three potential insertion sites on the chromosome

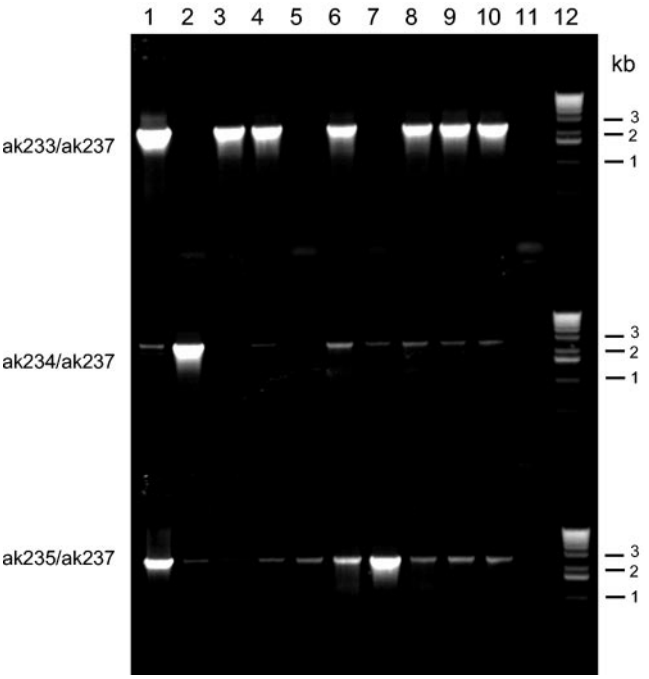

FIG. 2. Insertion of the Cam^r cassette into various rRNA clusters. PCR analysis with primers ak233/ak237, ak234/ak237, and ak235/ak237 of 10 insertion derivatives (lanes 1 to 10) resulting from transformation of strain 11168H with pRRC is shown. Lane 11, negative control (no DNA); lane 12, molecular size standards.

(Fig. 1D). The results of the analysis demonstrated random recombination with any of the three rRNA clusters (Fig. 2).

The minor bands present in some insertion derivatives possibly result from the migration of the cassette between the rRNA clusters. As can be seen in Fig. 2, in some cases PCR products with more than one primer pair could be detected. In order to find out whether the additional bands resulted from the simultaneous presence of the Cam^r gene cassette in two or more clusters in the genome or from the heterogenous population of the cell culture, we selected three typical derivatives and performed subcloning. Three subclones of these derivatives were analyzed by PCR with three primer pairs (Fig. 1), each specific to one of the possible integration sites.

The subclones of isolate A (Fig. 3) produced the same band patterns as the original strains, with one major band with the primer pair ak234/ak237 and minor bands of variable yields with the two other primer pairs. The presence of the minor bands suggested occasional migration of the Cam^r gene cassette from the primary insertion site into one of the two other rRNA clusters after subcloning.

In the case of isolate B, it was possible to select subclones producing a PCR product with just one primer pair (ak235/ak237) (Fig. 3), indicating that the minor bands were not due to insertion of the Cam^r gene cassette into multiple sites of the same cell but were due to heterogeneity of bacterial population.

In contrast, all subclones of isolate C produced strong bands with two primer pairs, ak234/ak235 and ak235/ak237, indicating that both the original isolate and its subclones contained the cassette integrated into two rRNA clusters on the same

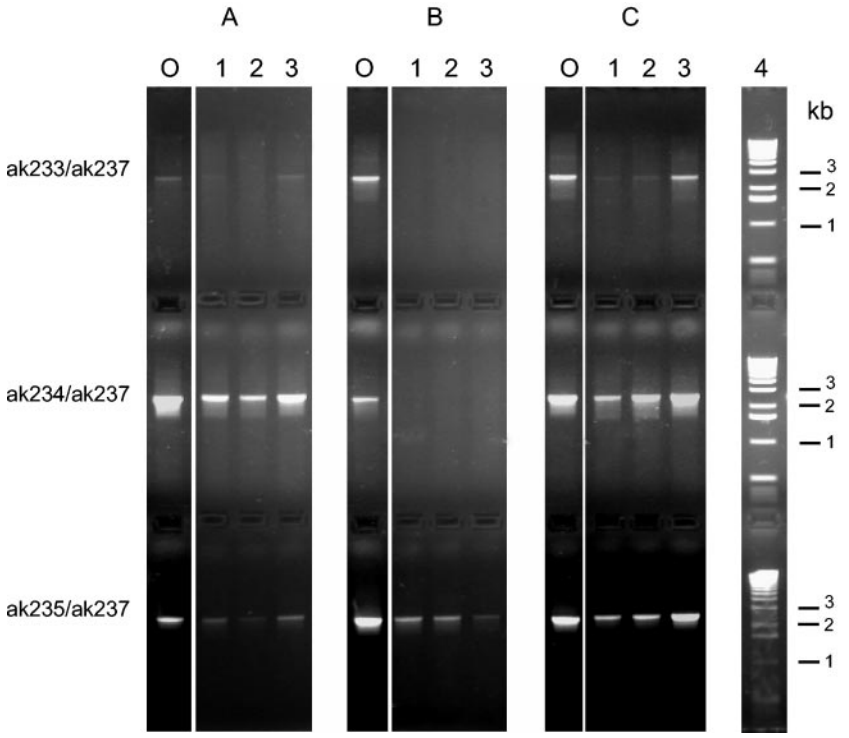

FIG. 3. PCR analysis of three insertion derivatives A, B, and C (lanes O) and respective subclones (lanes 1 to 3) with primers ak233/ak237, ak234/ak237, and ak235/ak237. Lane 4, molecular size standards.

chromosome. The presence of minor bands with the ak233/ak237 primer pair also suggested migration of the cassette into another rRNA cluster (Fig. 1) in a subpopulation of bacterial cells, similarly to what was observed with derivatives A and B (see above). The authenticity of these minor bands to the respective rRNA cluster was confirmed by gel extraction followed by sequencing with Cam^r primer ak290 (Table 2) (data not shown).

Allelic replacement in *C. jejuni* is not affected by the product of the *xerD* gene. Many strains of *C. jejuni* are capable of highly efficient double recombination events; the mechanism involved is unknown. One of the candidate genes that might be involved in such recombination was the *xerD* (Cj*0863*) gene. In *E. coli* XerD acts in concert with XerC and is involved in segregation of chromosome strands after replication (12). It was also shown that the XerC and XerD proteins of *E. coli* are required for filamentous phage integration (14).

The *xerD* mutant of 11168H was transformed via electroporation by plasmid pRRC, and Kan^r clones were selected and allelic replacement confirmed by PCR. The mutant was then transformed with pRRC and, following selection of Cam^r Kan^r clones, the chromosomal DNA was analyzed for the presence of Cam^r gene- and vector-specific sequences using PCR. The Cam^r gene was found to be inserted into an rRNA gene cluster near gene Cj*0029* (Fig. 1D). However, no vector-derived sequences could be detected (data not shown), indicating that the allelic replacement does not require expression of the XerD protein.

Integration of the Cam^r gene cassette into the spacer region of the rRNA gene cluster does not affect growth in liquid culture. Preliminary experiments demonstrated no difference in growth rates in liquid cultures (brucella broth) or in colony sizes on solid medium (blood agar plates) between the recipient strain 11168H and the Cam^r derivatives. For a more detailed comparative analysis of growth rates, in vitro competition studies were carried out as described elsewhere (8). One randomly selected Cam^r derivative, 11168H/pRRC4, with the Cam^r gene inserted into cluster III (Fig. 1) was grown in a mixture with the wild-type strain 11168H. The CI determined after 2 days of incubation (CI = 0.89) indicated that the insertion of the Cam^r gene cassette into the XbaI site of the rRNA cluster does not have a dramatic effect on cell viability under the conditions used. Similar results were obtained with the derivatives containing the Cam^r gene cassette inserted into clusters I and III (Fig. 1) and even with the derivative containing a Cam^r-*gfp* (wild-type *gfp*) fusion inserted into two clusters (II and III) simultaneously (data not shown).

Complementation of the *pglH* mutation in strain 11168H. It was shown previously that insertional inactivation of the *pglH* gene affects protein glycosylation (21). In order to check whether the integration system described in this study can restore protein glycosylation in this mutant, the *pglH* gene was PCR amplified and inserted into the pRRC vector to produce pRPGL (Table 1). After transformation of the 11168H/*pglH*:: Kan^r mutant with plasmid pRPGL, Kan^r Cam^r clones were selected. PCR analysis confirmed integration of the *pglH* gene into one of the rRNA gene clusters. Protein glycosylation in the derivatives was found to be completely restored (Fig. 4), indicating expression of a functionally active PglH protein.

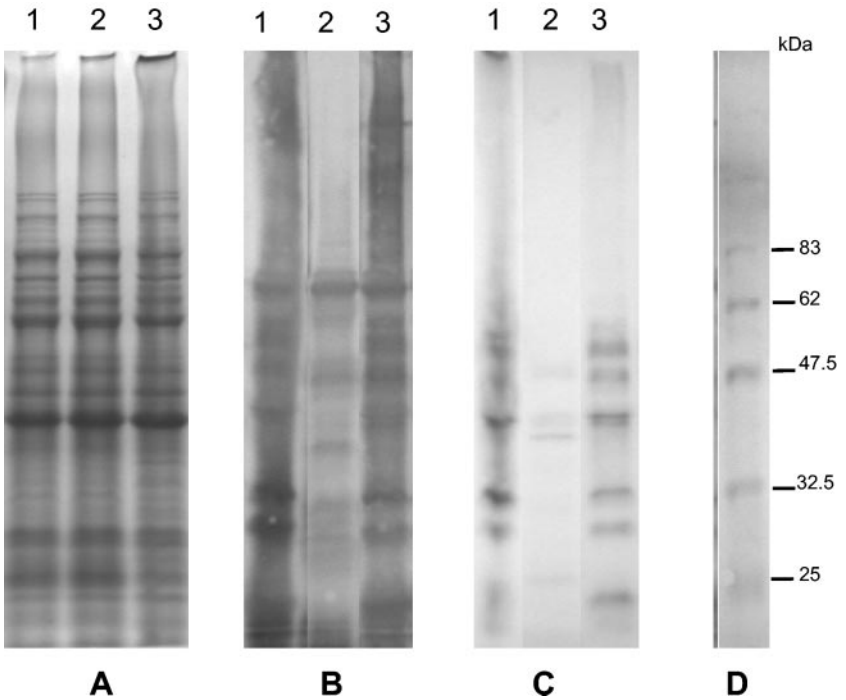

FIG. 4. Restoration of protein glycosylation after complementation of the *pglH* mutation. Lanes 1, wild-type strain 11168H; lanes 2, 11168H/*pglH*::Kan^r mutant; lanes 3, complementation derivative 11168H/*pglH*::Kan^r/pRPGLH1. After electrophoresis of whole-cell lysates, the gels were stained with Coomassie blue (A) or probed with either Pen2 antiserum (B) or biotinylated soybean agglutinin (C). D, molecular size standards.

Complementation of the *maf5* (Cj*1337*) mutation in strain 11168H. It was shown previously that mutation of the *maf5* gene results in the loss of motility (15). However, the putative Maf5 protein does not show significant sequence similarity to any other protein with a known function, and the role of this protein in motility remains uncertain. Remarkably, in strain NCTC 11168 there are six other genes with close similarity of the respected putative products to Maf5. Some of these genes contain variable poly(G) tracts and are involved in phase variation of motility. Expression of a functionally active Maf5 protein, which could be simply monitored via the restoration of motility in the *maf5* mutant, both in the intact form and as His-tagged derivatives, would assist in understanding the function of this new family of motility-related proteins.

Complementation of mutants in motility studies is especially important because of a large number of genes involved in flagellar formation and motility (33). Spontaneous mutations in these genes may also result in reduced motility in a specific mutant, thus skewing the effect of a primary mutation. In our previous study we overcame this problem by analyzing several independent clones of the *maf5* mutation in various strains (15). However, a possibility of direct complementation of the mutation would be advantageous.

The *maf5* gene was cloned into the pRRC vector, and the resulting plasmid pRMAF (Table 1) was transformed into the *maf5* mutant described previously (15). The integration of the *maf5* gene into an rRNA cluster was confirmed by PCR. The motility of the Kan^r Cam^r derivatives was restored (although partially [see Discussion]) compared with that of the mutant (Fig. 5), indicating that the cells produced a functionally active Maf5 protein.

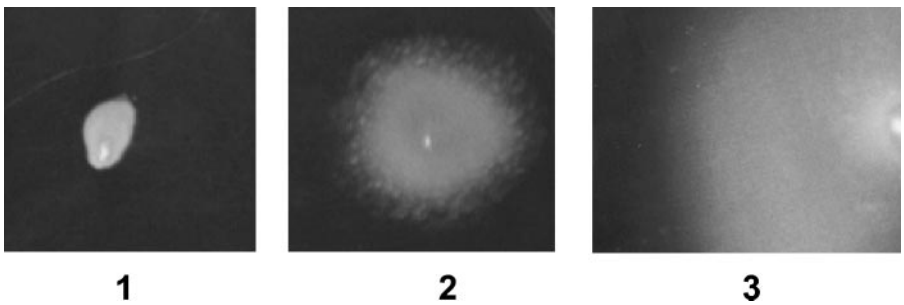

FIG. 5. Restoration of motility after complementation of the *maf5* mutation after growth for 3 days on 0.4% Mueller-Hinton agar. 1, 11168H/*maf5*::Kan^r; 2,11168H/*maf5*::Kan^r/pRMAF; 3, 11168H. A similar picture of restoration of motility was observed after complementation of such mutations in strain 81-176.

Complementation of a *maf5*-like mutation in strain 81-176. In order to demonstrate the applicability of the integration system to other strains of *C. jejuni*, we have used complementation of the *maf5*-like mutation in strain 81-176. The amino acid sequences of the respective gene products from strains NCTC 11168 and 81-176 reveal 55% identity (GenBank accession number AY102622). The *maf5* mutant of 81-176 was created using a protocol similar to that for constructing the *maf5* mutant of strain 11168H (15) (A. V. Karlyshev et al., unpublished). As expected, the 81-176/*maf5*::Kan^r mutant was nonmotile. Complementation of this mutation using the pRMAF5 delivery vector resulted in partial restoration of the motility, as in the case of strain 11168H (Fig. 5). The results indicate that the integration method for introduction and expression of exogenous genes is not limited to strain 11168H. The finding also demonstrates that despite the sequence difference, the product of the *maf5* gene of 11168H strain performs a function similar to the product of the *maf5*-like gene of strain 81-176.

Expression of wild-type GFP. The gene encoding wild-type GFP was PCR amplified and cloned into pRRC to produce pRGW (Table 1). Integration of the Cam^r–wild-type *gfp* cassette into various rRNA gene clusters was confirmed by PCR. No fluorescence of the Cam^r colonies could be detected with the fluorescence microscope (excitation at 488 nm). Similarly, only very weakly fluorescent cells could be seen using a confocal microscope. Importantly, this fluorescence was the same as that in the recipient strain 11168H, indicating some background fluorescence. Since both the clones and cells of the control strain *E. coli*/pRGW were strongly fluorescent, the lack of fluorescence in *C. jejuni* could be explained by a low level of expression of the wild-type GFP. This could be because in *E. coli* the wild-type *gfp* gene is present on a multicopy vector, whereas in *C. jejuni* it is present at only one copy per cell due to integration into the chromosome. In order to circumvent the problem, we tried expressing an enhanced protein (EGFP) in *C. jejuni* cells.

Expression of the *egfp-SD* gene. The commercially available plasmid pEGFP was used as a source of the *egfp* gene (Table 1). Although both *E. coli*/pEGFP clones and cells were highly fluorescent, the expression vector had to be modified for expression in *Campylobacter*. The *egfp* gene was PCR amplified using a primer containing an SD sequence optimal for expression in *Campylobacter*, which resulted in generation of the vector pRED. The *E. coli* cells and clones containing pRED (Table 1) revealed bright fluorescence. After transformation into *C. jejuni*, the integration of the Cam^r–*egfp-SD* cluster into an rRNA cluster was confirmed by PCR as described above. Compared with wild-type *gfp* derivatives, the *egfp-SD* derivatives of *C. jejuni* revealed enhanced fluorescence under the confocal microscope. However, no fluorescence could be detected using a conventional fluorescence microscope. A relatively low fluorescence level in this case could be explained by a suboptimal codon usage in the *egfp* gene. Indeed, pEGFP was designed for expression in eukaryotic cells, particularly in human cell lines. Due to a low AT content in the *Campylobacter* genome, its codon preference is dramatically different from that in *E. coli* and human cells. Therefore, we decided to use the same technique for expression of the *egfp* gene from plasmid pMEK91, which as a codon content more optimal for *C. jejuni*.

Expression of EGFP from pMEK91. The source of this type of *egfp* gene was plasmid pMEK91 (24). The plasmid contains a version of the *egfp* gene (*egfp-pMEK*) under the control of the *Campylobacter ompE* promoter (P_{ompE}) (Fig. 1). The EcoRI fragment containing the *egfp-pMEK* gene was cloned into pRRC, and fluorescence of *E. coli* transformants was tested. Two types of colonies with different levels of fluorescence were detected. The highest level of fluorescence corresponded to both the Cam^r and *egfp-pMEK* genes transcribed in the same direction. A plasmid from one such clone was used in transformation into *C. jejuni*, and integration of the Cam^r–*egfp-pMEK* cassette into an rRNA cluster was confirmed as described above. The colonies of the *Campylobacter* derivatives were fluorescent but were not as bright as *E. coli* colonies containing the delivery plasmid. However, very strong fluorescence of the *C. jejuni* cells could be detected using a confocal microscope. Therefore, a better codon composition and the use of an additional promoter allowed efficient expression of GFP in *C. jejuni* even when the gene was present as just a single copy per cell.

Use of the GFP derivative of *C. jejuni* for visualization of bacterial forms in situ. The GFP derivative of *C. jejuni* strain 11168H (11168H/*egfp-pMEK*) was used for investigation of sessile bacteria attached to a coverslip in a stationary liquid culture. The sessile bacteria grew on glass coverslips immersed in flasks with stationary liquid cultures as indicated in Materials and Methods. Figure 6 demonstrates intense fluorescence of *E. coli*/pREM donor cells (Fig. 6A, panel 1) and 11168H/*egfp-pMEK* derivatives (Fig. 6A, panel 2). Similarly to the recipient 11168H cells (16), prolonged incubation (e.g., 4 days) of 11168H/*egfp-pMEK* on the solid medium resulted in conversion of spiral cells to coccoid forms (Fig. 6A, panel 3). Such a conversion was significantly reduced in bacteria attached to a coverslip in the stationary liquid culture (Fig. 6B, panels 1 to 3). The aggregates of sessile bacteria were found to contain extensive channels with highly motile darting rod- and spiral-shaped cells (Fig. 6B, panel 3).

DISCUSSION

In this study we describe the design and application of an efficient procedure for the introduction and expression of selected genes in *Campylobacter* cells. The procedure is based on insertion of a gene expression cassette into a noncoding conserved spacer region of one of several rRNA gene clusters present in the bacterial genome. The insertion occurs via a double recombination event that is common for *C. jejuni* (31). This is in contrast to the case for many other bacteria, including *E. coli* and *Yersinia*, in which insertions via single recombination events are more common and allelic replacement is achieved by either using an additional selection step, e.g., with the help of vectors containing sucrose sensitivity genes (5), or via introduction of exogenous genes required for allelic replacement (7).

Despite routine use of allelic replacement in *Campylobacter*, at present little is known about its mechanism and the genes involved. One of the candidate genes that could be responsible for allelic replacement was *xerC* (Cj*0863*). Indeed, the gene

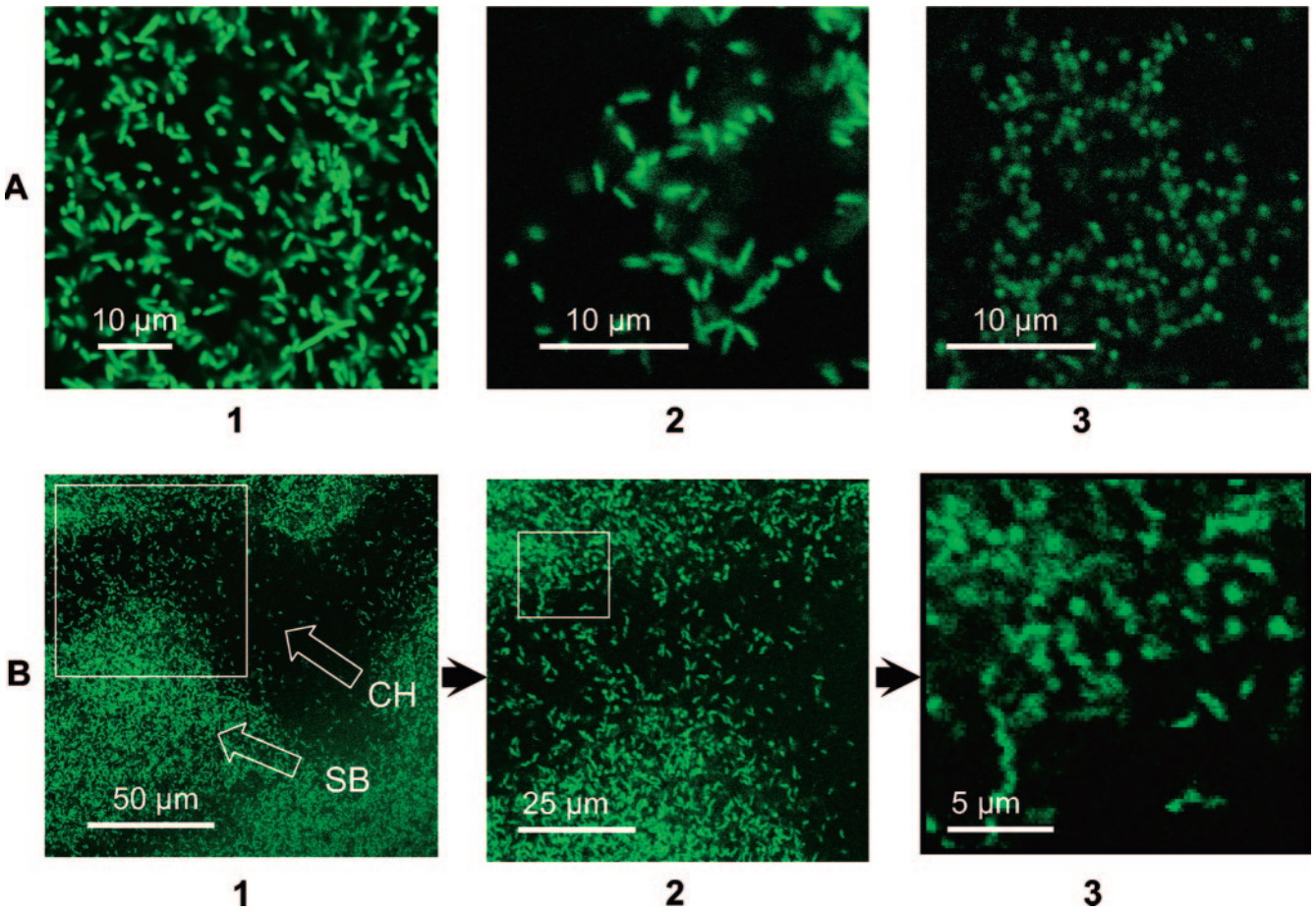

FIG. 6. Confocal microscope images of bacteria expressing EGFP. A, agar cultures. 1, *E. coli*/pREM5; 2, 11168H/*egfp-pMEK* 2-day culture; 3, 11168H/*egfp-pMEK* 4-day culture. B, *C. jejuni* biofilm formed on a glass coverslip placed at the bottom of a flask with stationary liquid culture (four day incubation, brucella broth). 1 to 3, images of the same area of the coverslip with increased magnification. Magnified areas are shown as squares. The open arrows point to the areas of sessile bacteria (SB) and channels (CH).

shows extensive similarity with other genes involved in resolution of cointegrants (data not shown). In order to check the possibility that this gene is also involved in allelic replacement in *C. jejuni*, we used our integration system as a model in the investigation of recombination in the 11168H/*xerC*::Kan^r mutant. Transformation of the pRRC delivery plasmid into this mutant resulted in insertion of the Cam^r gene cassette into different rRNA clusters. However, in all cases no vector-derived sequences could be detected, indicating double recombination and elimination of the vector sequences even in the *xerC*-negative strain. Therefore, genes other than *xerC* are likely to be responsible for allelic replacement.

The number of rRNA clusters in bacterial genomes varies significantly (18). The reason for such variation is not completely clear. There is no strong correlation between the rRNA gene copy number per genome and the bacterial growth rate. The reduction of the number of rRNA genes in *E. coli*, which is normally seven, has a moderate effect on the growth rate (2, 9). Overexpression of the rRNA gene may actually decrease the bacterial growth rate (28). A link between the rRNA gene copy number and ecological strategies of bacteria has been reported (17). Those authors showed that depending on environmental conditions, either a higher or a lower copy number of rRNA gene clusters may be preferable. It was suggested that the multiple copies of rRNA clusters may in some circumstances provide an advantage when a quick response to varying environmental conditions is required (9). However, for slowly growing bacteria, usually isolated from a low-nutrient environment, a low copy number of rRNA clusters is adequate (11). Our results indicated little or no effect of insertion of the Cam^r gene cassette on growth of *C. jejuni*. One should bear in mind, however, that our integration system was designed in such a way that only a minimal disruption (if any) of the rRNA cluster affected would occur.

Analysis of the insertion products revealed that the Cam^r gene cassette could be inserted randomly into any of the rRNA gene clusters. Interestingly, in some cases the PCR analysis indicated integration into two rRNA clusters simultaneously. This was not due to the presence of two different kinds of cells in the same sample, since subcloning resulted in colonies still producing PCR products with different primers. Simultaneous integration of the Cam^r gene into two rRNA clusters did not have a significant effect on the colony sizes or on the bacterial growth in a liquid culture. The additional minor PCR products produced by some derivatives suggest a possibility of migration of the Cam^r gene cassette between the rRNA clusters.

Various *Campylobacter* shuttle vectors have been described previously. However, these vectors have not been proven versatile. In our hands, none of the available shuttle vectors could be introduced into the sequenced strain *C. jejuni* strain NCTC 11168 or its derivative 11168H. In addition, even in a few cases of successful introduction of shuttle vectors into a *Campylobacter* strain as described by other authors, additional steps were required to overcome host-specific restriction. For example, in order to introduce pMW10-based vectors into *C. jejuni* RM1221 via electroporation, Miller and colleagues had to first transform these vectors into an Str^r derivative of this strain via conjugation (23). Only plasmids extracted from *C. jejuni* could then transform the same strain of *C. jejuni*. However, strains carrying other restriction-modification systems would be refractory to acquiring this DNA. Another limitation of the shuttle vectors may be a requirement for the presence of residential plasmids in the recipient strains, which may be required for plasmid rescue (30). An additional disadvantage of the shuttle vectors used in other studies is the requirement for using a complementing gene with its own promoter (19, 22, 27). However, this is not always feasible, since a promoter is often located at quite a significant distance from a gene. In our study we overcame the shuttle vector-related limitations by putting an exogenous gene under the control of a constitutive Cam^r promoter, followed by integration of this cassette into a spacer region within an rRNA gene cluster.

The use of the rRNA gene cluster as a gene insertion target has a number of other advantages. First, due to redundancy of the rRNA genes in *Campylobacter*, even if a certain rRNA gene cluster is affected by insertion, it would result in only a minor effect on cell functioning. Indeed, we could detect no significant difference in growth rates even after simultaneous insertion of the Cam^r cassette into two different rRNA clusters. Second, due to the very high conservation of rRNA genes between *Campylobacter* spp., a recombination vector designed for one strain can be effectively used for most other *Campylobacter* strains. The vector contains long conserved flanking regions of DNA, ensuring high efficiency of recombination. Using a total DNA preparation extracted from an already-constructed derivative, an inserted gene can be transferred to other *Campylobacter* strains with even higher transformation efficiency.

In this study we demonstrated the complementation of two knockout mutants. However, while the complementation completely restored the glycosylation phenotype in the *pglH* mutant, the complementation of the *maf5* mutation only partially restored motility. This could be due do tight requirements for the regulation, timing, and level of expression of the genes required for motility. However, even partial phenotype restoration confirms that the phenotype changes are really associated with a particular gene. Moreover, partial phenotype restoration after expressing a His tag fusion protein would provide a tool for isolating functional protein complexes, thus assisting functional analysis. We have demonstrated the same restoration of motility in a 11168H/*maf5*::Kan^r mutant complemented with a His-tagged Maf5 protein (data not shown).

We also investigated expression of three genes of non-*Campylobacter* origin (*gfp*) in *C. jejuni*. Despite extensive differences among the three *gfp* genes, the derived amino acid sequences are almost identical. Fluorescence properties of the EGFP-SD and EGFP-pMEK proteins are enhanced compared with those of wild-type GFP due to the presence of two mutations, F64L and S65T. The EGFP-pMEK protein also contains a Q80R replacement compared with EGFP. *E. coli* cells containing either pRED (EGFP-SD) or pREM (EGFP-pMEK) were highly fluorescent. However, the fluorescence level of *C. jejuni* cells expressing EGFP-pMEK was much higher than that of cells expressing EGFP-SD, which is due to the presence of two tandem promoters and more optimal codon usage (Table 3) in the latter construct.

TABLE 3. Examples of the difference in codon usage frequencies between two versions of *egfp* genes, with the highly expressed *C. jejuni* gene *ompE* shown for comparison

Codon (amino acid)	Frequency in:		
	egfp	*egfp-pMEK*	*ompE*
TTT (Phe)	0	8	9
TTC (Phe)	12	4	14
CTT (Leu)	0	11	17
CTC (Leu)	3	1	0
CTC (Leu)	0	3	4
CTG (Leu)	18	1	0
AAA (Lys)	1	15	22
AAG (Lys)	19	5	0

The construction of the fluorescent derivative of the sequenced strain of *C. jejuni* allows a wide variety of studies involving situ detection of bacterial cells in various forms both in vivo and in vitro. One advantage is the real-time observation of bacterial cells, which does not require any subsequent fixation or staining steps. This is particularly important when visualizing fragile communities of C. *jejuni* sessile cells attached to solid surfaces. Similarly to the recipient strain 11168H (16), the cells of its GFP derivative were mostly spiral or rod-like after 2 days incubation on blood agar plates (Fig. 6A, panel 2) but were mostly coccoid after 4 days of incubation (Fig. 6A, panel 3). The coccoid cells are considered to be a degenerate bacterial form accumulated in cultures during prolonged incubation or are induced under unfavorable environmental conditions (6). We were interested to investigate if attached aggregates (biofilms) of bacteria provide a mechanism of protection from such conversion. The results demonstrated much higher preservation of live rod-like or spiral forms, indicating that aggregation/immobilization is indeed advantageous for bacterial survival. Detailed analysis of the conglomerates of sessile bacteria under the confocal microscope revealed the presence of extensive internal liquid channels containing a large number of highly motile cells (Fig. 6B). It appears that the formation of the sessile form serves as a protective mechanism increasing bacterial survival under unfavorable conditions.

In summary, we demonstrated an efficient procedure of introduction and expression of various genes in *Campylobacter* cells. It is noteworthy that the method is applicable to the sequenced strain NCTC 111168, which is used by the majority of researchers. The fact that this strain was particularly difficult to complement was hindering functional genomics studies on this pathogen. This study therefore is a breakthrough for the research community. The method allows complementation of mutations and expression of other genes of non-*Campylobacter*

origin, assisting better understanding of the physiology of this microorganism, including the mechanisms of survival of bacteria in the environment, which may be important for the design of intervention strategies to reduce the presence of *Campylobacter* in the food chain.

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