signals. The advantages, compared with the previously used dye EtdBr in conjunction with a phosphor imager, are in the higher linearity over a broader range of total RNA loaded onto the gel. This range extends to 20 µg total RNA, which is an amount often used when hybridizing to low-abundance transcripts. The method is applicable for all laboratories that use a phosphor imager for quantifying hybridization signals derived from radioactively labeled probes. The method is also fast enough to use for checking inaccuracies of RNA quantitations done by UV photometry or pipetting measurements done before gel loading.

With the same instrument, signal normalization can be accomplished, eliminating hybridization steps involving less accurate "constitutively" expressed genes thus saving time and avoiding the generation of unnecessary radioactive waste.

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Procedure for the Investigation of Bacterial Genomes: Random Shot-Gun Cloning, Sample Sequencing and Mutagenesis of *Campylobacter jejuni*

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With the rapidly growing volume of bacterial sequencing information [20 prokaryote genomes have been sequenced, and the sequencing of 45 further genomes is in progress according to the Multipurpose Automated Genome Project Investigation (MAGPIE) data; **http://www-fp.mcs.anl.gov/~gaasterland/genomes.html**], the availability of experimental approaches validating gene function prediction is of prime importance. Unfortunately, molecular genetic approaches such as transposon mutagenesis and phage transduction are not available for many bacteria. In this report, we reveal how limited sequencing information resulted from random sequencing of a library, based on a suicide vector, can effectively be used in such bacteria. In addition, such libraries, if used for entire genome sequencing projects, can be applied for systematic mutational analysis of the genomes with limited genetic tools.

Campylobacter jejuni is a Gram-negative microaerophilic bacterium, which is a major cause of human enterocolitis (7). The development of disease prevention and infection control strategies are hindered by a poor understanding of the genetics and pathogenicity of the organism. Methods of genetic analysis used to characterize other enteric pathogens (e.g., transposon mutagenesis and phage transduction) are not available for *C. jejuni*. One of the most powerful tools for the identification of virulence determinants and characterizing pathogenic mechanisms is the construction of genetically defined mutants. On another hand, the procedure for construction of defined mutants by allelic replacement implies availability of the sequencing data. pUC18 has recently been used in different bacterial genome

sequencing projects (3,9). This vector does not replicate in *Campylobacter* and *Helicobacter* species and gives an important advantage as a suicide delivery system for allelic replacement in these bacteria. Selection of relatively small fragments (2 kb) for library construction increases stability of inserts.

C. jejuni NCTC 11168 was grown on blood-free selective agar base (Oxoid USA, Columbia, MD, USA) at 37°C in an atmosphere of 10% CO_2, 5% O_2 for 2 days. Chromosomal DNA was extracted by lysis with 1% sodium dodecyl sulfate (SDS) in the presence of 100 µg/mL of proteinase K, followed by phenol/chloroform-extraction and precipitation with isopropanol. DNA was resuspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) to a final concentration of 100 µg/mL and stored at 4°C. To ensure that only single inserts were present in the pUC18-based random library, a strategy similar to that described elsewhere (3) was used. Briefly, chromosomal DNA was sheared by ultrasonic disintegration, and DNA fragments of appropriate sizes (1.8, 2.0 and 2.3 kb) were gel-purified. DNA samples were treated with *Bal*31 and ligated with alkaline phosphatase (AP)-treated, *Sma*I-digested pUC18. The vector and insert (v+i) DNA band was gel-purified, blunt-end digested with T4 DNA Polymerase (Promega, Madison, WI, USA), religated and transformed into Epicurian Coli® XL2-Blue Competent Cells (Stratagene, La Jolla, CA, USA). Vector-derived primers (ak1, 5′-GTTTTC-CCAGTCACGACGTTG-3′ and v2, 5′-ATGTTGTGTGGAATTGTG-3′) were used to investigate the insert sizes in the pUC18 library. Crude cell lysates were used for PCR as described previously (6). The PCR products were analyzed by electrophoresis in 1% agarose gels and, after purification using MicroSpin S-300 HR Columns (Amersham Pharmacia Biotech, Uppsala, Sweden), were sequenced on a Model 373 DNA Analysis System with PRISM™ Ready Reaction Dye Terminator Cycle Sequencing Kit (PE Applied Biosystems, Foster City, CA, USA) using internal vector-derived primers (ak2, 5′-GAGCGGAT-AACAATTTCACACAGG-3′ and ak3, 5′-GTAAAACGACGGCCAGTG-3′).

The insert size of over 100 colonies was determined by vector-primer PCR and found to be in the expected range, indicating that concatamers had not formed during ligation. Thirty-five PCR products from individual clones were used for direct sequencing, generating 70 DNA sequences from both ends and

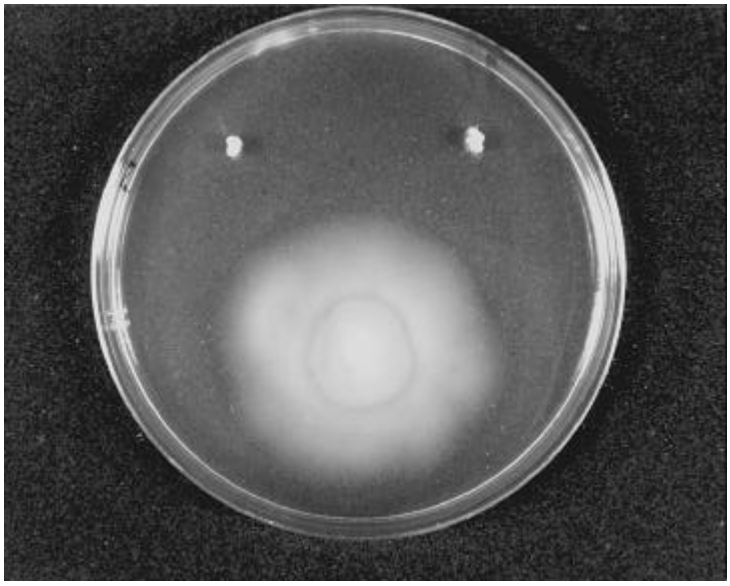

Figure 1. A swarm plate showing the motility of the *C. jejuni* wild type (large area) with that of two independent *fliN* recombinants. The diameter of the wild-type swarming colony is 44 mm, whereas the average diameter of the mutant colonies is 3.5 mm.

thus yielding over 30 kb of DNA sequence information. Pairwise computer analyses of the nucleotide sequences showed the sequences in the subset to be unique. The DNA sequences were used in Basic Local Alignment Search Tool (BLAST) (1) searches against a nonredundant combined protein database, which includes translations of European Molecular Biology Laboratory (EMBL) DNA databases in all possible reading frames. Of these, 54% showed significant matches with known genes (including 10% that showed identity with published *C. jejuni* sequences), 22% showed similarity to hypothetical open reading frames (ORF) and 24% revealed no matches. Several genes encoded potential virulence determinants. Genes similar to those involved in flagella biogenesis, chemotaxis, polysaccharide biosynthesis and transport, gene regulation, extracellular protein secretion and host-determined restriction have been identified. The *Campylobacter* origin of these genes was confirmed by hybridization analysis, and some of them were mapped to the *C. jejuni* NCTC 81116 genome (5).

A gene encoding a protein similar to FliN, which is known to be a component of a motor-switch complex in *E. coli* (4), was identified on a recombinant plasmid a12 by sample sequencing. Flagellae play an important role in chemotactic motility in different bacterial pathogens (4). The structure, function and genetic organization of flagellae in *C. jejuni* remains to be elucidated. The product of *fliN* gene was found to be the smallest [103 amino acid (aa) residues] among similar proteins; the C-terminal region in the proteins being the most conserved. In contrast to other bacteria (2,8), no *fliM*-like gene was detected upstream from *fliN*.

Because *fliN* mutants in other bacteria are known to be nonmotile, this phenotypic marker was chosen in a pilot study to construct *C. jejuni* mutants from the random library by allelic replacement. Two primers (ak31, 5′-GTAAAAAGATCTGCCCATGATGA-ATGTTAAACTC-3′ and ak32, 5′-TT-TTGGAGATCTCCAAGGCGAGGTT-ATGGTTTATG-3′) were used to introduce a 172-bp deletion in the gene and for insertion of the kanamycin-resistance cassette (10). Inverse polymerase chain reaction mutagenesis (IPCRM) (12) conditions: 94°C for 1 min, followed by 45 cycles at 94°C for 1 min, 45°C for 1 min and 70°C for 3 min. The generated *Bgl*II site was used for insertion of a 1.5-kb *Bam*HI fragment containing kanamycin-resistant cassette to create a12K. Kanamycin-resistant colonies of *C. jejuni* obtained by natural transformation (11) with a12K were found to contain the mutated *fliN* gene, as revealed by PCR analysis and Southern blotting. In the PCR experiments, a

fliN-specific primer f1 (5′-ATGAGCGATGATATAGAG-3′) and a primer, corresponding to a downstream gene (*orf1*), ak18 (5′-GTTCTTTTAATTCATCTTTAGAATACG-3′) were used. Southern blot analysis was carried out using Gene Images Labelling and Detection System (Amersham Pharmacia Biotech) and was performed according to the manufacturer's instructions with a fluorescein-labeled PCR probe generated by primers ak18 and f1. Chromosomal DNA was extracted from *C. jejuni* NCTC 11168 and the *fliN* mutant and digested with *Cla*I. DNA was separated by agarose gel electrophoresis and transferred to positively charged nylon membranes (Boehringer Mannheim GmbH, Mannheim, Germany). Both PCR and Southern hybridization tests revealed a single band with increased mobility due to insertion of the kanamycin cassette, indicating a double recombination in the *fliN* gene. Thus, flanking DNA as small as 160 bp was sufficient for generation of double recombinants for the construction of *fliN* mutants. Subsequent analysis on 0.3% Mueller-Hinton agar (Oxoid) swarmer plates confirmed that the mutants were nonmotile (Figure 1). Electron microscopy studies showed that the mutants were non-flagellated, suggesting that FliN, being a component of the *C. jejuni* flagellar-switch complex, might also be involved in flagella assembly.

We report the construction of a random 2-kb *C. jejuni* library, which we partially sequenced, and a sample gene *fliN* was chosen for functional analysis. Cloning small inserts has distinct advantages for the genetic analysis of *Campylobacter* species, as the 2-kb library should avoid problems encountered with the cloning and analysis of large AT-rich fragments of *Campylobacter* DNA in an *E. coli* host (7). Rapidly expanding databases of annotated nucleotide sequences with experimentally found or predicted functions ensure increase in the proportion of the genes that could be identified by partial sequencing. We have demonstrated that following the identification of a target sequence, a defined mutant can be constructed rapidly using PCR-based technology. This procedure could be adapted to a 96-well microplate format for the large-scale functional analysis of *C. jejuni* and other bacterial genomes in which the library vector cannot replicate in the host strain. We also found that for those strains where natural transformation is less efficient, electroporation (11) can be routinely used instead. We have exploited this approach in construction of a number of other mutants, e.g., *capM*, *neuB* and *hsdR*, in different strains of *C. jejuni* (unpublished). As in the case of *fliN*, all mutants were found to be double recombinants. Apparently, in other bacteria, single recombinants would be more common. The approach described is particularly useful for genomes that have not been sequenced to rapidly derive functional information on novel genes.

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