# 2. Materials and Methods

## 2.1 Materials

### 2.1.1 Bacterial Strains and plasmids

**Table 2.1:** *C. jejuni* strains and plasmids used in this study

| Strain | characteristics | Penner serotype | Reference/Source |
|---|---|---|---|
| NCTC 11168 | Sequenced strain, human isolate (Worcester 1977), source: unknown | 0:2 | NCTC 11168 [A] [3;8] |
| 81-176 | Human isolate (Minnesota 1981), source: raw milk | 0:23/36 | Black *et al.* 1988 [B] [36] |
| 81-176 plasmids | pTet and pVir | - | Bacon *et al.* 2000 [55] |
| M1 | Human isolate (UK 2000), source: poultry | 0:9 | Unpublished/ VLA, Weybridge[C] |
| 40671 | Human outbreak isolate (UK 2000), source: water source suspected, unproven by epidemiology | 0:50, phage type 6 | Champion [D] [93] |
| 52472 | Blood invasive human isolate, source: unknown | untypable, phage type 1 | Unpublished/ PHLS, Colindale[D] |

[A] National Collection of Type Cultures, Colindale, London, UK

[B] Vanderbilt University, Nashville, Tennessee, USA

[C] Veterinary Laboratories Agency (VLA), Weybridge, UK

[D] Public Health Laboratory Service (PHLS), Colindale, London, UK

Purified genomic DNA from all strains and plasmids was provided by Brendan Wren's group at the London School of Hygiene and Tropical Medicine (LSHTM, London, UK).

### 2.1.2 Reagents

Reagents used in this study were purchased from Fisher Scientific (Loughborough, Leicestershire, UK), BDH Laboratory supplies (VWR International, Dorset, UK) or Sigma (Dorset, UK) unless otherwise stated. Restriction endonucleases were purchased from New England Biolabs (NEB) (Hitchin, Hertfordshire, U.K.).

## 2.2 General Methods

### 2.2.1 Growth of *Escherichia coli* clones

*Escherichia coli* clones were grown for 18-22 hrs at 37°C, agitating at 320 rpm in 2x Luria-Bertani broth (2LB; 20 mg/ml tryptone, 10 mg/ml yeast extract and 10 mg/ml NaCl) containing 0.1 mg/ml Ampicillin for pUC clones, 12.5 μg/ml Chloramphenicol for pBAC clones or other antibiotics as appropriate.

### 2.2.2 Preparation of DNA

#### 2.2.2.1 Isopropanol preparation for isolation of pUC plasmid DNA

pUC clones were grown (section 2.2.1) in a volume of 1 ml of 2LB per well in 96-well boxes (Beckman). Boxes were spun in a centrifuge (Eppendorf 5810R) at 4000 rpm to pellet the cells. Culture supernatant was decanted and cells were resuspended in 120 μl Glucose-Tris-EDTA (GTE; 20% glucose, 1 M Tris-HCl, pH8.0, 0.1 M EDTA) plus 60 μg/ml RNase A (Q-Biogene, Cambridge, UK). After resuspension 120 μl 0.2 N NaOH/ 1% SDS was added followed by 120 μl 3 M potassium acetate. In order to remove cell debris and precipitate plasmid DNA 140 μl of cell lysate was pipetted into a filter plate (Costar 3504) which was placed above a storage plate (Costar 3365 serocluster) containing 140 μl of isopropanol, and both plates were spun together for 15 mins at 4000 rpm and 4˚C in a centrifuge. The supernatant was discarded and pellets washed with 100 μl 70% ethanol by spinning for 5

mins at 4000 rpm and 4°C. Pellets were dried then resuspended in 60 µl of autoclaved double distilled water (DDW).

### 2.2.2.2 Vacuum preparation for isolation of BAC DNA

Clones were grown (section 2.2.1) in a volume of 1.5 ml per well in 96-well boxes (Beckman). The boxes were spun in a centrifuge (Eppendorf 5810R) for 3 mins at 4000 rpm to pellet the cells and the culture supernatant was discarded. Cells were resuspended in 100 µl GTE containing 0.1 mg/ml RNaseA (Q-Biogene) on a box vortexer (Luckham) set on speed 8 for 3 mins. After resuspension, 100 µl 0.2 N NaOH/ 1% SDS was added and cell suspensions were mixed again on a box vortexer for 1 min, incubated at room temperature for 2 mins then 100 µl 3 M potassium acetate was added and the cell suspensions mixed using a box vortexer for 2 mins. The cell lysate was then transferred into a filter plate with pore size 0.65 µM (MADVN6550 Millipore) on a vacuum manifold with a second filter plate (MANUBAC50 Millipore) underneath and a vacuum of 10-15 mmHg was applied until the cell lysate had passed through the top filter plate. The bottom filter plate (MANUBAC50) was then transferred to the top of the vacuum manifold and a vacuum of 20-25 mmHg was applied until the contents had passed through the filter plate into a waste receptacle. To wash the DNA, 200 µl of DDW was added to the filter plate and the vacuum of 20-25 mmHg reapplied until the plate was dry. The filter plate was then removed from the vacuum manifold and 35 µl 10 mM Tris-HCl pH8 was added to neutralize and resuspend the DNA. Filter plates were vortexed on box vortexer (Luckham) speed 5 for 10 mins to aid resuspension. The DNA was then pipetted from the filter plate into a 96-well plate for storage (costar serocluster).

#### 2.2.2.3 Low-throughput preparation of DNA

Clones were grown (section 2.2.1) in a volume of 6 ml. Overnight cultures were spun in a centrifuge (Eppendorf 5810R) in 50 ml tubes (Falcon) for 15 mins at 3000 rpm and 4°C. The culture supernatant was discarded and cells pellets resuspended in 200 µl GTE containing 0.1 mg/ml RNaseA (Q-Biogene). The cell mixture was then pipetted into 1.5 ml tubes (eppendorf) containing 400 µl 0.2 N NaOH/ 1% SDS and incubated at room temperature for 5 mins, 300 µl 3 M Potassium acetate was then added and the tubes were spun in a centrifuge (Eppendorf 5415D) for 15 mins at 13000 rpm. To precipitate the DNA, 750 µl of cell lysate was transferred into a fresh 1.5 ml tube (eppendorf) containing 450 µl of isopropanol and the tubes were spun for 15 mins at 13000 rpm in a centrifuge. The supernatant was removed and the pellets were washed with 1 ml 70% ethanol. The pellets were dried and then resuspended in 20-60 µl TE (10 mM Tris: 0.1 mM EDTA) as appropriate to the pellet size.

### 2.2.3 Gel electrophoresis

DNA fragments were separated by agarose gel electrophoresis using 0.5% or 0.8% w/v agarose or low melting point (LMP) agarose (Invitrogen, Paisley, UK) in either Tris-acetate-EDTA (TAE) or Tris-Borate-EDTA (TBE) buffer [94]. Samples were loaded in ficoll loading dye (1mg/ml Bromophenol blue, 0.1mg/ml Ficoll 400, 1xTBE v/v) diluted ¼ with running buffer .

DNA was visualized by adding 10 µg/ml ethidium bromide to the running buffer and leaving to stain for 10-30 mins before viewing under ultraviolet. Alternatively, DNA was visualized by adding VistraGreen (Amersham, Buckinghamshire, UK) according to the manufacturers instructions, in a volume of DDW sufficient to cover the gel, and staining for 30 mins before viewing on a Dark Reader (Clare Chemical research,

www.clarechemical.com). Fragment size was determined by comparison with appropriate DNA ladders including a λ *Hin*dIII digest (NEB) and pBR322 *Bst*NI digest (NEB) mix, 1 Kb ladder (Invitrogen), 100 bp ladder (Invitrogen) or Raoul™ (Q-Biogene).

### 2.2.4 Polymerase Chain Reaction

All Polymerase Chain Reaction (PCR) amplifications including sequencing were carried out on a Peltier Thermal Cycler (PTC-225 MJ Research, Bio-Rad, Hertfordshire, UK) except for reactions incorporating radio-labelled nucleotides (see section 2.3.5). Oligonucleotide primers were all synthesized in-house.

PCR amplification was performed using *Taq* DNA polymerase (Applied Biosystems, Foster City, CA, USA). Reactions contained 10-100 ng of DNA template, 50 pmoles of each primer (forward and reverse) and dNTPs (Amersham) at a final concentration of 0.25 mM each in a total reaction volume of 50 µl. PCR conditions consisted of 94°C for 30 s to denature the template, followed by 30 cycles of 92°C for 30 s, a variety of temperatures (based on individual primer Tm) for 30 s and 72°C for 1-4 min (depending on expected length of product), unless otherwise stated.

### 2.2.5 Restriction enzyme digests

Restriction endonuclease digestion was performed over a period of 3-4 hrs at the specified optimum temperature (usually 37°C) using 1 unit of enzyme per µg of DNA unless otherwise specified. Double digests were performed using buffers compatible with both restriction enzymes according to the manufacturer's protocol. Complete digestion was verified using agarose gel electrophoresis (section 2.2.3).

## 2.2.6 DNA manipulation

### 2.2.6.1 Gel extraction

#### 2.2.6.1.1 Spin column kit

Fragments were excised from the agarose gel, and purified using MinElute™ gel extraction kit (Qiagen, Sussex, UK) according to the manufacturer's instructions.

#### 2.2.6.1.2 LMP agarace digestion

LMP agarose gel slices were melted in 1.5 ml tubes (eppendorf) in a waterbath at 65°C for 5 mins. The tubes were then transferred to a waterbath at 42°C and allowed to equilibrate before 5 µl of AgarAce® (Promega, Southampton, UK) was added for every 200 µl gel volume. Samples were incubated at 42°C for at least 20 mins. DNA was then extracted by phenol extraction (section 2.2.6.2) and purified by ethanol precipitation (2.2.6.3).

### 2.2.6.2 Phenol extraction

An equal volume of TE-buffered phenol (Sigma) was added to samples and agitated using a vortex genie (Scientific Industries, New York, USA) for 1 min. Samples were incubated on ice for 5 mins then spun in a centrifuge (Eppendorf 5415D) for 3 mins at 13000 rpm. The aqueous layer was pipetted into a 0.5 ml tube (eppendorf) and incubated on ice for 5 mins before being spun in a centrifuge for 3 mins at 13000 rpm. The aqueous layer was then transferred into a 1.5 ml tube (eppendorf).

### 2.2.6.3 Ethanol precipitation

DNA was precipitated by adding $1/10^{th}$ the sample volume of 1 M NaCl, 2.5 volumes of 70% ethanol; 1 µl pellet paint (Novagen, Darmstadt, Germany) was used to aid pellet visualization. Samples were incubated either at -70°C for 1 hr or -20°C overnight before

being spun in a centrifuge (eppendorf 5417R) for 30 mins at 14000 rpm and 4°C. Pellets were washed with 1 ml 70% ethanol before centrifugation for 5 mins at 1400 rpm and 4°C, and then dried before resuspending as appropriate.

## 2.3 Differential hybridization methods

### 2.3.1 Construction of a pUC19 library of DNA fragments

#### 2.3.1.1 Preparation of DNA

Approximately 10 µg of chromosomal DNA was sheared using a sonicator (xl2020 sonicator, Heat systems Inc., New York, USA) in a final volume of 60 µl to create fragments between 12 kb and 500 bp. The ends of sonicated DNA were repaired using 0.3 µl mung bean nuclease (256 U/µl, Amersham Pharmacia Biotech, Piscataway, NJ, USA) incubated at 30˚C for 10 mins in the presence of mung bean buffer (15 mM Sodium acetate, 25 mM NaCl, 0.5 mM $ZnCl_2$, 2.5% glycerol). The DNA was then ethanol precipitated (section 2.2.6.3) and size fractionated by LMP agarose gel electrophoresis (section 2.2.3). Fragments of appropriate size were recovered and purified (section 2.2.6.1.2).

#### 2.3.1.2 Ligation

Purified DNA fragments were ligated to pUC19 vector in the following reaction mix: 3 µl DNA solution, 0.3 µl pUC19 *Sma*I-Bacterial Alkaline Phosphatase (BAP) (Q-Biogene 40 ng/µl), 0.4 µl ligase buffer, 0.3 µl T4 DNA ligase (5 U/µl Roche, Lewes, East Sussex, UK). The ligation mixture was incubated at 12-14˚C overnight. Ligation was terminated using 1 µl Proteinase K (Roche 14 mg/ml) in a final volume of 50 µl and incubated at 50˚C for 1 hr.

#### 2.3.1.3 Transformation

Transformation was performed using 0.5 µl of ligation mixture, 40 µl electrocompetent *Escherichia coli* DH10B (Invitrogen) and an electroporation device (BioRad, genepulser) set

at 1.7 Kv, 200 Ω, 25 μF. Cells were allowed to recover in 500 μl SOC [95] at 37°C for 1 hr and plated on Tryptone Yeast Extract (TYE) plates (15 mg/ml Agar; 8 mg/ml NaCl; 10 mg/ml Bacto Tryptone; 5 mg/ml yeast extract) containing 0.1 mg/ml ampicillin, with 2.5 mg 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) and 2 mg isopropylthio-β-D-galactoside (IPTG). White colonies were picked after overnight incubation at 37˚C.

## 2.3.2 Construction of a pBACe3.6 library of DNA fragments

### 2.3.2.1 Preparation of pBACe3.6 vector

The vector pBACe3.6 [96] was supplied in *E. coli* DH10B cells by Pieter de Jong from the Children's Hospital Oakland Research Institute, USA (http://bacpac.chori.org). The vector pBACe3.6 was prepared for cloning as described in 'Current Protocols in Human Genetics' [97].

Briefly, a caesium chloride gradient was used to remove and purify the vector DNA from the host cell DNA, after total DNA extraction. The pUC stuffer fragment was removed by restriction endonuclease digestion and the resultant "sticky ends" dephosphorylated to prevent vector recircularization. Control ligations with a 16 kb fragment of lambda DNA were performed to check the quality of the vector.

### 2.3.2.2 Preparation of DNA

DNA fragments of chromosomal DNA were prepared by limited digestion with an optimised dilution of *Sau*3A1 in 10x bovine serum albumin (BSA) for an optimized length of time (determined by previous trial digestions) at 37˚C in a 200 μl volume, to give DNA fragments of between 10-40 Kb as appropriate. The enzyme was inactivated and DNA extracted using phenol (section 2.2.6.2) followed by ethanol precipitation (section 2.2.6.3). DNA fragments were separated using a 0.4% low melting point (LMP) agarose gel (section 2.2.3). The appropriate size fractions were recovered from the gel and purified (section 2.2.6.1.2).

#### 2.3.2.3 Ligation

Approximately 20 ng of the purified DNA size fraction was used for ligation in a 50 µl reaction volume containing 10 ng pBACe3.6 pre-cut with *Bam*HI (section 2.3.2.1), 9 µl 30% polyethylene glycol (PEG) 8000, 1 µl 0.1 M $MgCl_2$, 5 µl ligation buffer and 1 µl 1/10 ligase (diluted in 10x BSA). The ligations were incubated overnight at 16˚C and terminated by adding 2.5 µl of 0.5 M EDTA and 1 µl of 14 mg/ml Proteinase K (Roche), incubated for 1 hr at 37˚C then 1 µl of 100 mM phenylmethylsulfonyl fluoride (PMSF) (sigma) was added. The ligation mixture was then dialysed on a 0.025 µm pore size microdialysis filter (MF-Millipore VSWP, Millipore UK Ltd, Watford, UK) floated on 0.5 x TE v/v in a petri dish and incubated at 4˚C for 3 hrs. The ligation mix was then pipetted from the filter.

#### 2.3.2.4 Transformation

Transformation was performed using 1 µl of each ligation, 20 µl electrocompetent *E. coli* DH10B cells (Invitrogen) and a CellPorator (Life Technologies, Paisley, UK) equipped with a voltage booster set at 4 kΩ, 330 µF, 13 kV/cm, fast charge. Cells were allowed to recover in 500 µl SOC [95] by incubation at 37°C for 1 hr before being plated onto TYE plates containing 20 µg/ml chloramphenicol and 5% sucrose, and incubated overnight at 37˚C. Colonies were picked the following day.

### 2.3.3 Propagation of library clones

Clones were picked either manually or using the Sanger Institute automated picking facility into media (section 2.2.1) plus 7.5% glycerol for storage. All libraries were routinely tested for phage and *Pseudomonas* contamination by spotting colonies onto agar plates seeded with a DH10B lawn or onto *Pseudomonas* selective agar with C-N supplement (Oxoid, Basingstoke, Hampshire, UK) according to manufacturer's instructions.

## 2.3.4 Preparation of colony arrays

### 2.3.4.1 Colony blotting

The clones were arrayed onto 78 x 119mm Nytran N membrane (Schleicher and Schuell, Dassel, Germany), supported on agar plates, using the Sanger Institute automated robotic arraying facilities. pUC plasmid clones were arrayed in duplicate in a 384-pin 4x4 gridding pattern resulting in 6144 clones per filter. BAC clones were arrayed in duplicate in a 96-pin 4x4 gridding pattern resulting in 1536 clones per filter. After colony blotting, the agar plates plus membranes were incubated for 16-18 hrs at 37°C.

### 2.3.4.2 Lysis of bacterial clones

The membranes were placed colony side up on chromatography paper soaked in 10% SDS for 5 mins. Membranes were then transferred to chromatography paper soaked in denaturing solution (0.5 N NaOH/ 1.5 M NaCl) for 10 mins then allowed to dry for 10-20 mins on dry chromatography paper. After drying the membranes were briefly submerged in neutralizing solution (0.5 M Tris-HCl pH 7.4/ 1.5 M NaCl), followed by a 5 min wash with fresh neutralizing solution on an orbital shaker. A further 5 min wash in neutralizing solution was performed, followed by a 5 min wash in 1/10 neutralizing solution. The membranes were then washed with agitation in 2X SSC/ 0.1% SDS for 5 mins, 2X SSC for 5 mins then washed twice with 50 mM Tris-Hcl pH7.4 for 5 mins. Membranes were air dried DNA side up on chromatography paper for a minimum of 6 hours before UV cross-linking for 2 mins on a transilluminator (254 nm).

## 2.3.5 Hybridization of membranes

### 2.3.5.1 Radiolabelled probe generation using random octamers

Random octamer primers were annealed to denatured DNA templates and extended by the Klenow fragment of DNA polymerase I, incorporating one radiolabelled nucleotide and three unlabelled nucleotides, to form a probe [98]. This labelling reaction was carried out using 100 ng of sonicated DNA and components of the BioPrime DNA labelling system (Invitrogen) according to manufacturer's instructions, except for using an in-house dNTP mix (0.6 mM of A, G, T) and 30 µCi [α-$^{32}$P]dCTP (Amersham). Labelling reactions were incubated at 37˚C for 1 hr then 5 µl of stop buffer (0.5 M EDTA) was added and the probes were purified using a microspin G25 column (Amersham). An equal volume of sonicated human placental DNA (Sigma) was added to each probe, then the mixture was denatured at 99˚C for 5 mins.

### 2.3.5.2 Generation of radiolabelled probe using PCR

Primer pairs complementary to pUC clone insert sequences were used to amplify regions of DNA from pUC clones (section 2.2.4). Products were checked for size using agarose gel electrophoresis (section 2.2.3) then purified through spin columns (microspin S-400HR, Amersham) according to the manufacturer's instructions. These purified templates were then added to 1 µl of 10x PCR buffer (50 mM KCl, 5 mM Tris pH8.5, 2.5 mM $MgCl_2$), 0.4 µl of 2.5 mM dATP, dTTP and dGTP, 0.5 units *Taq* polymerase and 4 µCi [α-$^{32}$P]dCTP in a 10 µl reaction volume. PCR amplification was performed on a thermocycler (Perkin and Elmer, Boston, MA, USA) at 96°C for 30 s to denature the template, followed by 30 cycles of 92°C for 30 s, 53°C for 30 s and 72°C for 2 mins. Probes were purified using a microspin G25 column (Amersham) then denatured at 99˚C for 5 mins.

#### 2.3.5.3 Hybridization

Membrane sets were soaked in 2x SSC (1x SSC is 15 mM sodium citrate and 0.15 M NaCl) prior to pre-hybridization. Membrane sets were pre-hybridized in glass tubes, in a hybridization oven at 65˚C in 15 ml Church buffer (1 mM EDTA, 0.5 M $NaHPO_4$ (pH7.2), 7% Sodium Dodecyl Sulphate (SDS) 1% BSA) for 1-3 hrs. Pre-hybridization buffer was decanted and probe was added to the membranes in a fresh volume of Church buffer and incubated at 65°C overnight.

#### 2.3.5.4 Washes

The day following hybridization (section 2.3.4.3) the membranes were washed twice at room temperature with 2x SSC/ 0.1% SDS for 20 mins, then twice at 65˚C with 0.1x SSC/ 0.1% SDS for 20 mins. The membranes were then sealed in Saran wrap, placed in an autoradiograph cassette and exposed to photographic film. It was found that a rinse step using 2x SSC/ 0.1% SDS conducted in the hybridization tubes improved the quality of results for differential hybridization reactions.

### 2.3.6 Sequencing of library clones

#### 2.3.6.1 Sequencing primers

5` - 3`

M13F: TGTAAAACGACGGCCAGT

pUC18R: GCGGATAACAATTTCACACAGGA

T7: TAATACGACTCACTATAGGG

Sp6: ATTTAGGTGACACTATAG

### 2.3.6.2 Sequencing reactions

All sequencing was performed using ABI Prism® BigDye® terminator chemistry (Applied Biosystems) and loaded on ABI 3700 capillary sequencing machines at the Sanger Institute sequencing facility according to their protocols.

#### 2.3.6.2.1 pUC clone sequencing

Sequencing reactions were conducted using 3 µl of DNA (20 ng/ul), 0.25 µl BigDye v3.1, 2.5 µl BigDye buffer (400 mM Tris pH9, 10 mM $MgCl_2$), and primer (either M13F or pUC18R) to a final concentration of 3 pM in a total reaction volume of 9 µl. DNA was amplified by thermocycling with the following conditions:-

96˚C 30 s

92˚C 4 s
50˚C 4 s
60˚C 1 min 50 s
) 44 cycles

10˚C holding temperature

After thermocycling DNA was precipitated by adding 25 µl of precipitation mix (60 mM sodium acetate, 4 µM EDTA in 96% ethanol) and spinning in a centrifuge for 20 mins at 4000 rpm and 4°C. Pellets were then washed with 30 µl 70% ethanol before spinning in a centrifuge for 5 mins at 4000 rpm and 4°C and dried before being loaded on sequencing machines.

#### 2.3.6.2.2 Sequencing from primers internal to cloning vector

Sequencing reactions were carried out as in section 2.3.6.2.1 except primers designed from the insert sequence were used at a final concentration of 30 pM. Either plasmid or PCR products purified using spin columns (Microspin S-400HR, Amersham) were used as sequencing templates.

**2.3.6.2.3 BAC end sequencing**

Sequencing reactions were conducted using 10 µl DNA (650-800 ng), 3 µl of BigDye v3.1, 3 µl BigDye buffer (400 mM Tris pH9, 10 mM $MgCl_2$) and 30 pM of primer (either T7 or Sp6) in a reaction volume of 20 µl.

DNA was amplified by thermocycling with the following conditions:-

95˚C 5 mins

95˚C 30 s
51˚C 10 s
60˚C 4 mins
} 75 cycles

10˚C holding temperature

After thermocycling DNA was precipitated by adding 5 µl 3 M Sodium acetate and 125 µl 96% ethanol, the plates were spun in a centrifuge for 1 hr at 4000 rpm and 4°C. The pellets were washed with 100 µl 70% ethanol and spun in a centrifuge for 15 mins at 4000 rpm and 4°C and then dried before being loaded on sequencing machines.

## 2.3.7 Analysis of sequence

The trace files were processed using Asp (http://www.sanger.ac.uk/software/sequencing/docs/asp/) and basecalled using Phred [99]. The individual read sequences were compared to the query sequence using WUBLASTN from the Washington University Basic Local Alignment Sequence Tool algorithms (WU-BLAST; http://blast.wustl.edu [100]). MSPcrunch [101] was then used to map query sequences back to the relevant subject sequence. Reads to be further analysed were assembled using Phrap (Green, P., unpublished) into contiguous sequences. Coding sequences (CDSs) were predicted within Artemis [102] and the translated protein sequences were compared to a non-redundant protein database using WUBLASTP [100] and FASTA [103]. Predicted proteins were compared against the Pfam database of protein domain

Hidden Markov models (http://www.sanger.ac.uk/software/Pfam/). The protein sequences were also searched for signal peptides (http://www.cbs.dtu.dk/services/signalP-2.0/), transmembrane helices (http://www.cbs.dtu.dk/services/TMHMM-2.0) and prosite motifs (http://www.expasy.ch/prosite/). Clustal X was used for protein and DNA alignments [104]. Shading was supplied by Boxshade server (http://www.ch.embnet.org/software/BOX_form.html). NJplot [105] was used to visualize guide trees produced by clustal X. EMBOSS applications (http://emboss.sourceforge.net/apps/) "needle" for Needleman-Wunsch [106] global alignments and "water" for Smith-Waterman [107] local alignments were used.