

Integron Analysis and Genetic Mapping of Antimicrobial Resistance Genes in *Salmonella enterica* serotype Typhimurium

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1. Introduction

Antimicrobial resistance determinants may be transferred among bacteria via mobile genetic elements including plasmids, transposons, and the more recently explored integrons (1). Integrons are naturally occurring genetic elements found as part of the Tn21 transposon family or located on various broad host-range plasmids (2). The fundamental integron structure consists of a 5'-conserved segment (5'-CS) of 1.4-kbp and a 2-kbp 3'-CS (3). Between these conserved regions are DNA sequences of variable length and molecular complexity. These intervening sequences are known as *gene cassettes*, and several have now been characterized (Fig. 1). Acquisition and dissemination of these genes located within the integron structure, results in an increase in antimicrobial resistance (4).

Three classes of integron structure have been described. Class 1 integrons are of principal importance in clinical isolates. The 5'-CS of class 1 integrons includes an *intI* 1 gene of 1358 bp, which encodes a specific recombinase, a member of the DNA integrase family (5). This gene contains the *attI* recombination site, required for specifically integrating gene cassettes (Fig. 1). Classes 2 and 3 also contain integrase genes (*intI* 2 and *intI* 3), with the former showing 40% sequence identity to those of class 1, and the latter showing 61% (1). All three classes of integrons contain similar gene cassettes from the same families, which suggests the existence of a common pool of gene cassette with cross-specificity between the classes (1).

When the 3'-CS region is examined in detail, it contains several open reading frames (ORFs). These include *qacEΔ1*, which confers resistance to quaternary ammonium compounds, often associated with antiseptics, along with a *sulI* gene expressing resistance to sulphonamide antimicrobial agents. The *sulI* gene encodes the enzyme hydropteroate synthase. Transcription of the *sulI* gene begins at a promoter located in

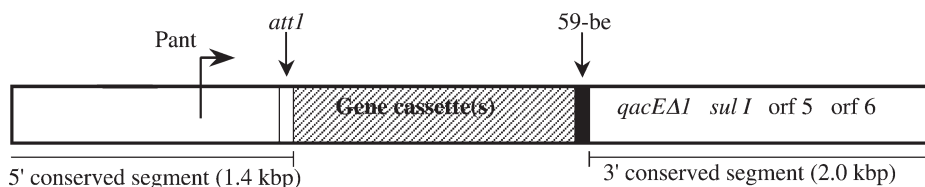


Fig.1. Schematic representation of a class 1 integron structure. Several gene cassettes (hatched box) have been found located between the 5'- and 3'-CS. Often, more than one open reading frame (ORF) gene cassette can be found within the same integron, in the same orientation, and transcribed from a common promoter located proximal to the 5'-site of insertion.

the 5'-CS. The latter is also responsible for the transcription of the inserted gene cassette(s) (6). Two additional ORFs, ORF-5 and -6, are located toward the distal end of the 3'-CS. The gene product of ORF 5 appears to share some sequence similarity with puromycin acetyltransferase, and this feature suggests a possible role in antimicrobial resistance (7). A biological function has yet to be ascribed to ORF-6.

Gene cassettes are discrete mobile DNA elements, which may exist as free, circular, nonreplicating DNA molecules (possibly) in transit from one genetic locus to another (8). They usually contain one gene along with a recognition sequence known as the 59-base element (59-be). The latter is a necessary component required for their integration into the larger mobile elements (i.e., integrons). Examples of gene cassettes commonly found in integrons include the aminoglycoside-modifying enzyme-encoding genes *ant* (3'')-1a, *ant* (2'')-1a, *aac* (3')-1, and *aac* (6')-1b, the β -lactamase-encoding genes, *bla*_{PSE-1}, *bla*_{OXA-1}, *bla*_{OXA-2}, *bla*_{OXA-5}, and *bla*_{CARB-3}, genes conferring resistance to chloramphenicol (*cat*, *cml*), and *dfr* genes resulting in resistance to trimethoprim. Gene cassettes lack a functional promoter (9) and therefore cannot be transcribed until they are correctly positioned within an integron (10). Pant, the integron promoter responsible for controlling gene expression is located toward the 5'-end of the *attI* site, 214 bases from the inner boundary of the 5'-CS (Fig. 1). Thus these unusual genetic elements act as natural expression vectors (1,11).

Salmonella enterica serotype Typhimurium is recognized as a significant human pathogen. It is currently estimated that, of the 40,000 *Salmonella* isolates reported to the Centers for Disease Control and Prevention (CDC), 8.5% are identified as serotype Typhimurium (12). These organisms are often simultaneously resistant to five or more common antimicrobial agents including ampicillin (A), chloramphenicol (C), streptomycin (S), sulphomamides (Su), and tetracycline (T). The increasing spectrum of resistance among *Salmonella* Typhimurium, definitive type (DT)104, has led several investigators to investigate the mechanism(s) of resistance acquisition. Antimicrobial resistance in *S. Typhimurium* DT204c and DT193 has been entirely attributed to the presence of plasmids; however, DT104 represents an unusual case in that some of the resistance determinants have become chromosomally integrated (13).

Resistance to sulfonamides when encountered is suggestive of the involvement of a class 1 integron. This is a useful phenotypic indicator, as it is a property attributed to class 1 integrons (as outlined above). Multiple drug-resistant (MDR)-DT104 of resis-

tance (R)-type ACSSuT are often associated with the presence of two class 1 integrons, containing gene cassettes of 1.0 and 1.2 kbp. The former gene cassette contains *ant(3'')-Ia*, conferring resistance to aminoglycoside antibiotics, streptomycin, and spectinomycin; the 1.2-kbp gene cassette contains a *bla*_{PSE-1} gene, encoding a β -lactamase enzyme conferring resistance to β -lactam antibiotics including ampicillin. Fine genetic mapping studies have located these structures to the chromosome of *S. Typhimurium* DT104 within a highly conserved 10-kbp *Xba*I DNA fragment (13-15). This chromosomal region has been described as a multiresistant gene cluster consisting of two integrons flanking an R-plasmid (16). Molecular analysis of antimicrobial resistance indicated that the same gene cassettes accounted for MDR-DT104 isolates from diverse geographical regions (14,17-20).

The inserted gene cassettes described above may be recovered by DNA amplification using the previously characterized Int1 F and Int1 R primer set (2) (Table 1). In a standard polymerase chain reaction (PCR), the complete amplification of any inserted gene cassette(s) within a class 1 integron structure can be successfully achieved. A typical result, for a DT104 isolate of R-type ACSSuT, is shown in Fig. 2 (lane 1). Two intensely ethidium bromide-stained DNA fragments corresponding to the 1.0- and 1.2-kbp amplicons outlined previously are detected after conventional agarose gel electrophoresis. These structures accounted for three (ASSu) of the five resistance traits normally associated with MDR-DT104 isolates (see below). Larger amplicons (approx 1.6 kbp [Fig. 2, lane 2]) have also been identified in *S. Typhimurium* isolates including phage types DT170a and -193. The amplicon shown in Fig. 2 (lane 2) contained two resistance genes, *dfr*I and *aad*A, fused in a classical head-to-tail fashion (15).

Chloramphenicol acetyl transferases (CATs) are the enzymes most frequently responsible for resistance to chloramphenicol. However nonenzymatic mechanisms have also been described including efflux pumps, membrane transporter impermeability, and ribosomal modifications. A *cml*A gene found in MDR-DT104 and recently described by Briggs and Fratamico (14) encodes an exporter enzyme and not the more common acetyltransferase. The *flo*R gene, which belongs to the MDR efflux pump family of proteins, was described (16) and is responsible for cross-resistance to chloramphenicol and florfenicol. The *flo*R gene is located downstream of the integron containing the *ant* (3'')-Ia gene cassette. Distal to the *flo*R gene are the *tet*RA genes (class G tetracyclines). Tetracycline resistance arises as a result of the production of an efflux pump. These resistance determinants are located on the integrated R-plasmid, outlined above.

The R-type pattern ACSSuT can therefore be accounted for by the presence of this conserved MDR locus on a 10-kbp *Xba*I digested DNA fragment. However, additional resistances to trimethoprim and fluoroquinolones are now being encountered in MDR-DT104 isolates (15). Trimethoprim resistance can arise owing to the presence of a nonconjugative but mobilizable 4.6-MDa plasmid, which can also encode resistance to sulphonamides (SuTp). Genes associated with trimethoprim resistance (including those of the *dfr* gene family), expressing a dihydrofolate reductase enzyme, have also been found in integrons (15). Considering the nature of integrons, this presents an opportunity for trimethoprim resistance determinants to become chromosomally inte-

Table 1
Sequence and Characteristics of Oligonucleotide Primers Used for PCR

Primer	Sequence 5' → 3'	Conc. per reaction (pmol)	% GC	PCR T-annealing (°C)
IntI1 F	GGC ATC CAA GCA GCA AGC	25	61	55
IntI1 R	AAG CAG ACT TGA CCT GAT	25	44	55
<i>sulI</i> F	CTT CGA TGA GAG CCG GCG GC	25	70	65
<i>sulI</i> R	GCA AGG CGG AAA CCC GCG CC	25	75	65
<i>qac</i> EA1 F	ATC G2CA ATA GTT GGC GAA GT	25	45	55
<i>qac</i> EA1 R	CAA GCT TTT GCC CAT GAA GC	25	50	55
<i>ant</i> (3'')-1a F	GTG GAT GGC GGC CTG AAG CC	25	70	65
<i>ant</i> (3'')-1a R	ATT GCC CAG TCG GCA GCG	25	67	65
<i>pse-1</i> F	CGC TTC CCG TTA ACA ACT AC	25	55	55
<i>pse-1</i> R	CTG GTT CAT TTC AGA TAG CG	25	45	55
<i>dfpI</i> F	GTG AAA CTA TCA CTA ATG GTA GCT	25	37	65
<i>dfpI</i> R	ACC CTT TTG CCA GAT TTG GTA ACT	25	42	65
<i>flo</i> F	ACC CGC CCT CTG GAT CAA GTC AAG	25	58	70
<i>flo</i> R	CAA ATC ACG GGC CAC GCT GTA TC	25	54	70

grated. A larger second plasmid of 60-MDa is also found in DT104s of R-type ACSSuT; however, this plasmid has not been linked with antimicrobial resistance but rather contains virulence determinants (13,21). *Salmonella* plasmid virulence (*spv*) genes are carried on this plasmid.

The presence of putative resistance islands can be investigated using a combination of molecular approaches including pulsed-field gel electrophoresis (PFGE), Southern blotting and probe hybridization experiments. PFGE is a useful molecular typing protocol, wherein the complete bacterial chromosome is investigated with a rare cutting restriction endonuclease (22). The macrodigested DNA fragments produced must be resolved by PFGE. These can then be denatured to yield single-stranded DNA fragments and transferred by Southern blotting to nylon membranes. Selected molecular probes for hybridization can be conveniently prepared by PCR, with the specific primer sets outlined in **Tables 2** and **3**.

For example, when making the *bla*_{PSE-1} probe, the primer set given was used to amplify a segment of the *pse* gene from the gene cassette. In this case the *int1* primer set is not used for probe generation purposes, as the flanking sequences are common among many gene cassettes and may lead to false-positive hybridization signals. Furthermore, the PFGE profiles may also provide epidemiological data and can be analyzed to indicate the genomic relationships of isolates (**Fig. 3A**) prior to Southern blotting. Suitably labeled molecular weight markers must be included for size determination, and these must be chosen depending on the type of hybridization and detection system used. When a digoxigenin (DIG)-labeling and detection system is used, prelabeled molecular weight markers can be included in two or three gel lanes. Once

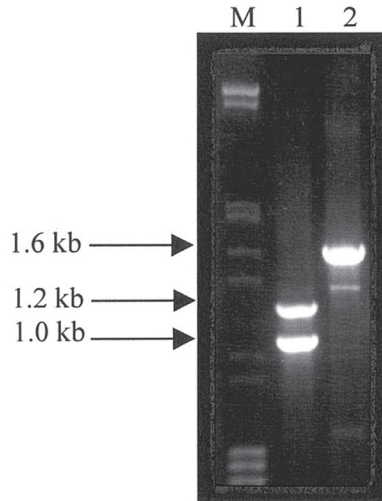


Fig.2. Amplified gene cassettes of two representative strains of *Salmonella enterica* sero-type Typhimurium. After PCR, 10 μ L of the amplified reaction mixture was loaded into a 1% (w/v) agarose gel in 1X Tris-EDTA-acetate (TAE) buffer containing 0.1 μ g of ethidium bromide per mL. Samples were horizontally electrophoresed at 80 V for 60 min. The lanes marked M contain an equal mixture of molecular weight markers grade III (ranging in size from 0.56 to 21.2 kb) and molecular weight markers grade V (ranging in size from 8 to 597 bp; Roche Diagnostics). Lane 1, CIT-F45, DT 104 isolate, containing two integrons with gene cassettes of 1.0 and 1.2 kbp; lane 2, CIT-F41, DT 193 isolate, containing one integron carrying a gene cassette with two ORFs, producing a PCR amplicon of 1.6 kbp.

Table 2
Thermocycler Programs, Step Design, Cycle Number, and Corresponding Citation Used With Oligonucleotide Primers^a

PCR program	Step 1 Predenaturation	Step 2 Denaturation	Step 3 Annealing	Step 4 Extension	No. of cycles	Ref.
IntI1	95°C, 5 min	95°C, 1 min	55°C, 1 min	72°C, 2 min	30	2
<i>sull</i>	95°C, 5 min	95°C, 1 min	65°C, 1 min	72°C, 2 min	30	24
<i>qac</i> Δ 1	95°C, 5 min	95°C, 1 min	55°C, 1 min	72°C, 2 min	30	6
<i>ant(3'')-1a</i>	95°C, 5 min	95°C, 1 min	65°C, 1 min	72°C, 2 min	30	25
<i>pse-1</i>	95°C, 5 min	95°C, 1 min	55°C, 1 min	72°C, 2 min	30	26
<i>dfrI</i>	95°C, 5 min	95°C, 1 min	68°C, 1 min	72°C, 1 min	30	15
<i>flo</i>	95°C, 5 min	95°C, 1 min	70°C, 1 min	72°C, 1 min	30	16

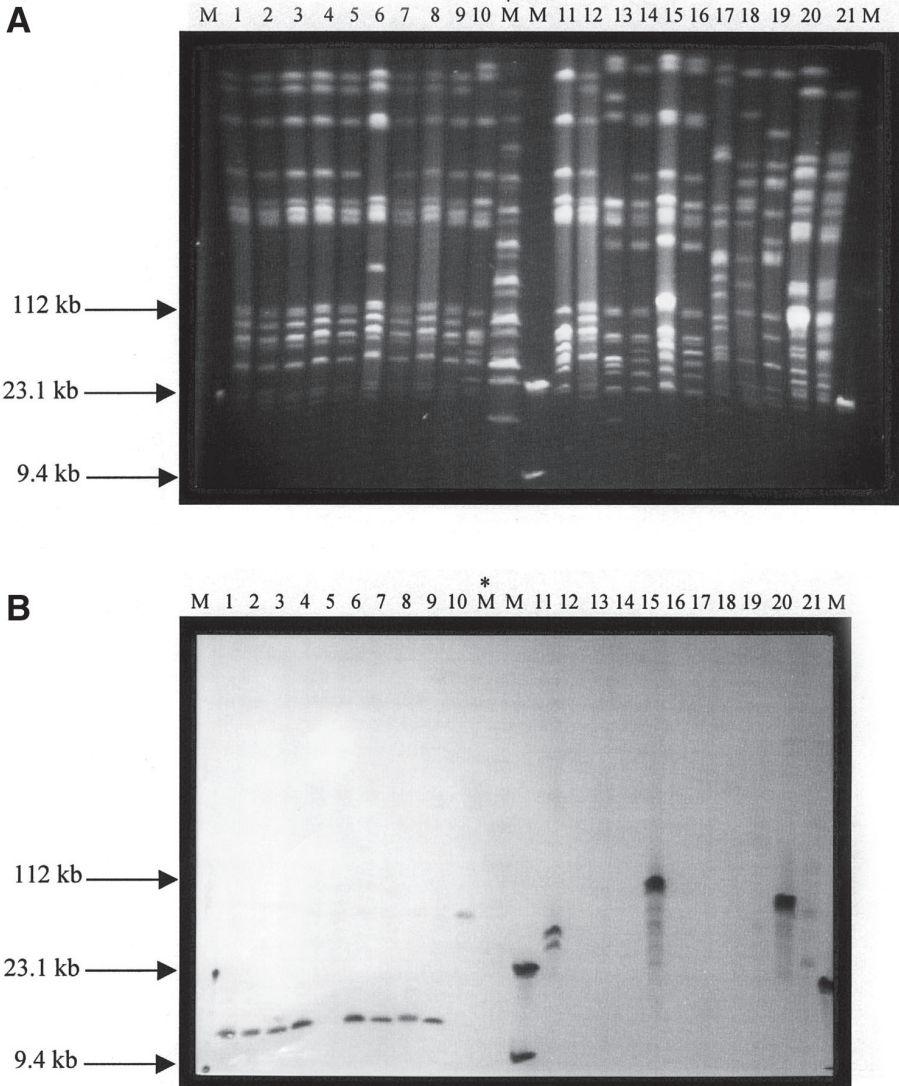
^aA final extension step of 72°C for 5 min was incorporated into each program; the samples are then maintained at 4°C until analysis.

Table 3
Southern Blotting of *Salmonella* Typhimurium Isolates and Probe Hybridization Using Selected Gene Cassettes

Phage type	R-type	10-kb <i>Xba</i> I fragment	Probe hybridization		
			<i>ant</i> (3'')- <i>Ia</i>	<i>pseI</i>	<i>dfrI</i>
DT104					
CIT-V38	ACSSuT	+	+	+	−
CIT-H164	ACSSuT	+	+	+	−
CIT-H176	ACSSuTTp	+	+	+	−
CIT-H183	SSu	−	−	−	−
CIT-V115	ACSSuT	+	+	+	−
CIT-F44	ASu	−	−	−	−
DT104 related					
CIT-F107 (104b)	ACSSuTK	+	+	+	−
CIT-H144 (104b)	ACSSuTTp	+	+	+	+
CIT-V37 (U302)	ACSSuTTp	+	+	+	−
DT193					
CIT-F34	ASSuTN	−	+	+	+
CIT-F41	ASSuTTp	−	+	+	+
DT195					
CIT-V60	SuTTp	−	−	−	−
DT208					
CIT-V75	T	−	−	−	−
DT170a					
CIT-V127	ASSuTTp	−	+	+	+
CIT-V129	SuTTp	−	−	−	−
Nontypable					
CIT-F40	Sensitive	−	−	−	−
CIT-F105	SuTp	−	−	−	−
CIT-H195	SuTp	−	+	+	−
Controls					
<i>E. coli</i> R100.1		−	+	+	+
<i>E. coli</i> R751		−	+	+	−

ant (3'')-*Ia*, aminoglycoside modifying enzyme coding gene; *pseI*, β -lactamase gene; *dfrI*, dihydrofolate reductase gene; R-type, antimicrobial resistance pattern; A, ampicillin; C, chloroamphenicol; S, streptomycin; Su, sulphonamides; T, tetracycline; N, nalidixic acid; K, kanamycin; V, veterinary; H, human; F, food.

Fig. 3. (opposite page) The lanes marked M contain DIG-labeled DNA molecular weight marker grade II (Roche Diagnostics). Unlabeled mid-range PFG markers (New England BioLabs) in lane M* were included for fragment sizing before Southern transfer. Lane 1, CIT-V38; lane 2, CIT-F45; lane 3, CIT-H164; lane 4, CIT-H176; lane 5, CIT-H183; lane 6, CIT-V115; lane 7, CIT-F107; lane 8, CIT-H144; lane 9, CIT-V37; lane 10, CIT-F34; lane 11, CIT-F41; lane 12, CIT-F44; lane 13, CIT-V60; lane 14, CIT-V75; lane 15, CIT-V127; lane 16, CIT-V129; lane 17, CIT-F40; lane 18, CIT-F105; lane 19, CIT-H195; lane 20, *E. coli* R100.1 (control isolate); and lane 21, *E. coli* R751 (control isolate). (A) Pulsed-field gel electrophoresis



(Fig. 3. continued) profiles of *Salmonella* Typhimurium isolates digested with *Xba*I following electrophoresis through 1% (w/v) SeaKem Gold agarose in 0.5X TBE for 18 h at 10.5°C. Electrophoresis was performed at 200 V using a Gene Navigator system (Pharmacia) in the interpolation mode pulsing from 1 through 40 s. (B) Following pulsed-field gel electrophoresis the *Xba*I macrorestricted DNA fragments were transferred to nylon membranes. Individual gene cassettes were then used to probe the membranes. Southern blot using the *bla*_{PSE-1} gene as a probe.

the PFGE patterns are analyzed, DNA is transferred as an exact pattern replica onto a nylon membrane or other suitable solid phase. The membrane can then be hybridized with a specific antimicrobial resistance gene probe and the location of any hybridizing signal(s) generated detected visually (**Fig. 3B**). This entire procedure must be repeated for each new probe and the position of the hybridizing signal recorded each time. One possible limitation to this approach is the time required, as several replica sets of PFGE gels must be produced, transferred to nylon membranes, and subsequently probed. However, it is an advantage, if several agarose slices are digested simultaneously for PFGE (*see Note 1*).

Figure 3B shows the results of a hybridization experiment using the *bla*_{PSE-1} gene probe with the isolates listed in **Table 3**. The corresponding PFGE patterns are shown in **Fig. 3A**. A signal was detected for the DT104 isolates of R-type ACSSuT at approx 10 kbp. A similar result was obtained when using the *ant* (3'')-1a and the *flo* gene probes sequentially. This indicated that these genes are located within the same region on the *S. Typhimurium* genome. Larger fragments in non-DT104s can also be seen, the largest of which is 112 kbp (**Fig. 3B**, lane 15). This isolate (CIT-V127, **Table 3**) also had a hybridization signal at this position when probed with *ant* (3'')-1a and *dfpI* (**Table 1**). Analysis of hybridization signals from the various gene probes can therefore identify multiresistant gene clusters in bacterial isolates.

2. Materials

All reagents marked with an asterisk (*) are autoclaved at 121°C for 15 min prior to use.

2.1. DNA Extraction

This following protocol is a general purification method for Gram-negative organisms (**23**), yielding good-quality DNA templates (*see Note 2*).

1. All *S. Typhimurium* isolates used in this study are listed in **Table 3**.
2. Tryptone soya broth (TSB*; Oxoid, Hampshire, UK): dispensed in 5-mL volumes into sterile universal containers.
3. 1 M NaCl*.
4. TE solution*: 50 mM Tris-HCl, pH 8.0, 50 mM EDTA.
5. Lysozyme: obtained lyophilized from Sigma (Poole, UK), dissolved in sterile distilled water to a final concentration of 2 mg/mL, aliquoted, and stored at -20°C.
6. 20% (w/v) Sodium dodecyl sulfate (SDS).
7. Proteinase K: obtained lyophilized from Sigma, reconstituted in sterile distilled water to a final concentration of 10 mg/mL, aliquoted, and stored at -20°C.
8. Phenol/chloroform: isoamylalcohol (25:24:1; Sigma), saturated with 10 mM Tris-HCl, pH 8.0, 1 mM EDTA.
9. 3 M Ammonium acetate, filter-sterilized, and stored at room temperature.
10. 70 and 100% (v/v) ethanol stored at -20°C.
11. 1X TE solution*: 10 mM Tris-HCl, pH 8.0, 1 mM EDTA.

2.2. Polymerase Chain Reaction

1. Deoxyribonucleoside triphosphates (dNTPs; Promega, Madison, WI), stock concentration of 100 mM. Prepare a working concentration of 1.25 mM dNTPs by mixing 2.5 µL of

each (dATP, dCTP, dGTP, and dTTP) with 190 μ L of sterile distilled water (final volume 200 μ L). Store at -20°C until required.

2. 2.5 U *Taq* DNA polymerase (Promega). The enzyme is supplied with 25 mM MgCl_2 and 10X reaction buffer (100 mM Tris-HCl, pH 9.0, 500 mM KCl, 1% Triton X-100).
3. Sterile distilled water.
4. Oligonucleotide primers: all primers used for integron and gene cassette analysis were synthesized by Oswel (Southampton, UK) and purified by high-performance liquid chromatography (HPLC). These primer sequences together with their relevant characteristics are listed in **Table 1**.

2.3. Conventional Agarose Gel Electrophoresis

1. *ultra* PURE agarose, electrophoresis grade from GibcoBRL Life Technologies (Paisley, Scotland). Gelling temperature $36\text{--}42^{\circ}\text{C}$.
2. 10 mg/mL Ethidium bromide in distilled water (stored in a dark bottle).
3. 10X TAE buffer: 400 mM Tris-HCl, pH 7.8, 400 mM glacial acetic acid, and 2 mM EDTA; working concentration 1X.
4. Loading dye: prepared by mixing 100 μ L of 10% (w/v) bromophenol blue with 6.6 mL glycerol and 3.3 mL 10X TAE.
5. Molecular weight markers: several preparations of molecular weight markers are available, and the choice of a suitable marker is made based on the expected size of the amplified DNA fragment(s). Usually grades III and V molecular weight markers from Roche Diagnostics (East Sussex, UK) are used, and these have the following known DNA fragments:
 - a. DNA molecular weight marker III: consists of 13 DNA fragments ranging in size from 0.12 to 21.2 kbp; prepared by cleaving λ DNA with *Eco*RI and *Hind*III (250 $\mu\text{g/mL}$).
 - b. DNA molecular weight marker V: consists of 22 DNA fragments ranging in size from 8 to 587 bp; prepared by cleaving pBR322 with *Hae*III (250 $\mu\text{g/mL}$).

2.4. Pulsed-Field Gel Electrophoresis

2.4.1. Preparation of Agarose Plugs

1. Nutrient agar plates (Oxoid).
2. 1-mL Sterile plastic syringes with the nozzle removed.
3. Saline 0.85% (w/v) NaCl.
4. TEN buffer: 100 mM Tris-HCl, pH 7.5 100 mM EDTA.
5. Incert[®] agarose (FMC BioProducts, Vallesbaek Strand, Denmark).
6. 20 mg/mL Proteinase K in distilled water. Store at -20°C .
7. Lysis buffer: 6 mM Tris-HCl, pH 7.6, 1 M NaCl, 100 mM EDTA, pH 7.6, 0.5% (w/v) Brij-58 (polyoxyethylene-20-cetyl-ether), 0.2% (w/v) sodium deoxycholate, 0.5% (w/v) sarkosyl. Filter-sterilize and store at 4°C .
8. 400 mM EDTA, pH 9.3, 1% (w/v) sarkosyl.
9. 1X TE*: 100 mM Tris-HCl, pH 7.6, 100 mM EDTA, pH 7.6, 150 mM NaCl.

2.4.2. Restriction Endonuclease Digestion of Agarose Slices

1. DNS buffer: 100 mM Tris-HCl, pH 8.0, 5 mM MgCl_2 .
2. RNase super stock: RNase obtained from Sigma, diluted to 10 mg/mL with sterile distilled water, and frozen at -20°C in 1-mL aliquots.

3. RNase A 1:100 dilution: prepared by mixing; 200 μ L RNase A super stock, 200 μ L Tris-HCl, pH 7.5, 75 μ L 4 M NaCl, and 19.5 mL sterile distilled water in a sterile glass universal. Boil this mixture for 15 min, allow to cool gradually to room temperature in a water bath, and freeze 1-mL aliquots at -20°C .
4. 1 M MgCl_2 (Sigma).
5. BNE (buffer with no restriction enzyme): mix 5 μ L 10X restriction enzyme buffer with 5 μ L 1:100 RNase A dilution and 40 μ L sterile distilled water, per sample.
6. BWE (buffer with restriction enzyme): same as for BNE (in **step 5** above) but with 2 μ L of the appropriate restriction enzyme and only 38 μ L of sterile distilled water.
7. 10 U/ μ L *Xba*I restriction endonuclease.

2.4.3. Electrophoresis of Pulsed-Field Gels

1. SeaKem[®] Gold agarose (FMC BioProducts).
2. 5X Tris-borate-EDTA (TBE): 450 mM Tris-HCl, pH 8.3; 450 mM borate, pH 8.3; 10 mM EDTA (working concentration of 0.5X TBE).
3. 10 mg/mL Ethidium bromide.
4. Molecular weight markers: low-range and mid-range PFG markers embedded in 1% (w/v) low melting point (LMP) agarose supplied by New England BioLabs (Hertfordshire, UK). In addition, a DIG-labeled marker is also used to facilitate size determination from the colored developed blots: DNA molecular weight marker grade II, DIG-labeled, consisting of eight fragments ranging in size from 125 to 23,130 bp (Roche Diagnostics).

2.4.4. Visualization of DNA Bands

Ethidium bromide solution at a final concentration of 0.5 $\mu\text{g/mL}$ in distilled water.

2.5. Southern Blotting of Pulsed-Field Gels

2.5.1. DNA Transfer From Pulsed-Field Gels to Nylon Membranes

1. Depurination buffer*: 0.5 M HCl.
2. Denaturing buffer*: 0.5 M NaOH, 1.5 M NaCl.
3. 20X SSC*: 3 M NaCl, 0.3 M sodium citrate, pH 7.0.
4. Neutralizing buffer*: 0.5 M Tris-HCl, pH 7.0, 1.5 M NaCl.

DNA transfer of pulsed-field gels was achieved using a Vacu-Aid Blot processing Pump (Hybaid, Middlesex, UK) onto positively charged nylon membranes (Roche Diagnostics) (see **Note 3**).

2.6. Preparation and Detection of Digoxigenin (DIG)-Labeled Probes

2.6.1. Preparation of DIG-Labeled Probes

1. DIG-labeled dUTP, at a concentration of 1 nmol/ μ L (DIG-11-dUTP; Roche Diagnostics).
2. 4 M Lithium chloride.
3. Cold 100% ethanol.
4. Cold 70% (v/v) ethanol.

2.6.2. Hybridization and Detection

A DIG-DNA labeling and detection kit obtained from Roche Diagnostics was used for probe hybridization and for color detection of hybridization events.

The solutions required are as follows:

1. Hybridization buffer: 5X SSC (1:4 dilution of 20X SSC), 1% blocking solution (from kit), 0.1% (w/v) *N*-lauroyl-sarcosine, 0.02% (w/v) SDS.
2. Wash solution A: 2X SSC, 0.1% (w/v) SDS (for 500 mL combine 50 mL 20X SSC, 450 mL sterile distilled water, and 0.5 g SDS).
3. Wash solution B: 0.1% SSC, 0.1% (w/v) SDS (for 500 mL combine 2.5 mL 20X SSC, 497.5 mL sterile distilled water, and 0.5 g SDS).
4. Buffer 1: maleic acid buffer; 0.1 M maleic acid, 0.15 M NaCl; adjust pH with NaOH to pH 7.5 (see **Note 4**).
5. Buffer 2: blocking buffer; dilute 10X blocking solution in buffer 1 (maleic acid buffer) to 1X final concentration.
6. Buffer 3: detection buffer; 0.1 M Tris-HCl, pH 9.5, 0.1 M NaCl, 50 mM MgCl₂.

3. Methods

3.1. DNA Extraction

This extraction method is a modification of the procedure described by Versalovic et al. (23).

1. Grow bacterial cells overnight in 5 mL TSB at 37°C and then pellet by centrifugation at 15,000g for 2 min.
2. Wash the cell pellet with 1 mL of 1 M NaCl followed by 1 mL TE solution (50 mM Tris-HCl [pH 8.0], 50 mM EDTA) and then resuspend in 0.7 mL TE solution (see **Note 5**). Transfer the cells to 1.5-mL Eppendorf tubes.
3. Add 100 µL lysozyme (2 mg/mL) to the cells and incubate at 37°C for 30 min. To complete lysis, add 30 µL of 20% (w/v) SDS and incubate at 65°C for 10 min. This is followed by the addition of 60 µL proteinase K (10 mg/mL) and returning the cells to 37°C for 1 h (see **Note 6**).
4. The protein must then be extracted from this crude nucleic acid preparation by performing two phenol/chloroform extractions: Add 800 µL phenol/chloroform/isoamylalcohol (25:24:1) to the lysate mixture and invert the tubes vigorously for 5 min. Centrifuge the samples for 5 min at 20,000g and then transfer the *top* aqueous phase (containing the DNA) to a new 1.5-mL tube. Repeat the phenol/chloroform extraction on the aqueous phase.
5. After the second extraction, precipitate the DNA by adding a 1:10 volume of 3 M ammonium acetate and cold 100% ethanol to fill the 1.5-mL Eppendorf tube and place at -20°C to precipitate overnight (see **Note 7**).
6. After precipitation, centrifuge the DNA samples at 18,000g for 30 min at 4°C. Wash the DNA pellet with 70% ethanol, remove all ethanol with a pipet tip, and dry the DNA by leaving the tubes open on the bench for 5 min. Finally, resuspend in 200 µL 1X TE solution.

3.2. Polymerase Chain Reaction

3.2.1. Integron and Gene Cassette Amplification

1. Amplification of gene cassettes located between the 5' conserved segment and the 3' conserved segment of class I integrons is performed in a final volume of 50 µL.

2. For each reaction combine 5 μL 25 mM MgCl_2 , 5 μL 10X reaction buffer, 8 μL working stock dNTPs to give a final concentration of 0.2 mM each (dATP, dCTP, dGTP, and dTTP), 100 ng of template DNA, and 2.5 U *Taq* DNA polymerase (5 U/ μL).
3. All primers are used at a concentration of 25 pmol per reaction (**Table 1**), and reaction conditions are given in **Table 2**.

A wide range of thermocyclers are available for PCR; the MiniCycler™ (MJ Research, Watertown, MA) is compact (if laboratory space is limited) and has proved very reliable.

3.3. Agarose Gel Electrophoresis

1. Prepare a 1.5% (w/v) agarose gel by mixing 1.5 g agarose and 100 mL 1X TAE (*see Note 8*). Heat gently in a microwave oven until the agarose bubbles, mix briefly, reduce the heat, and simmer for 30 s. Allow the gel to cool to 60°C and then add 1 μL of ethidium bromide (10 mg/mL). Mix in carefully and pour into the gel mold with the well comb in place.
2. Allow the gel to solidify for 20–30 min and then pour 1X TAE buffer containing 0.5 $\mu\text{g/mL}$ Ethidium Bromide into the electrophoresis tank until it just covers the surface of the gel. Remove the comb and mold barriers (if present).
3. Prepare the samples by combining 10 μL each PCR with 3 μL loading dye and load into the preformed wells.
4. Electrophoresis times vary from approx 1 h at 80 V for smaller gels (6.5 \times 8.0 cm) to 1.5–2 h at 100 V for larger gels (11.5 \times 14 cm).
5. The ethidium bromide-stained fragments can be viewed and photographed with a UV transilluminator, e.g., from UVP (Ultra-Violet-Products, Cambridge, UK), using Phoretix software (Newcastle-upon-Tyne, UK).

3.4. Pulsed-Field Gel Electrophoresis

3.4.1. Preparation of Samples in Agarose Plugs

for Pulsed-Field Gel Electrophoresis

Day 1:

1. For each strain to be analyzed plate out a single colony onto a nutrient agar plate and incubate overnight at 37°C.
2. Take one 1.5-mL Eppendorf tube per isolate and weigh the tube to 2 decimal places. Record the weights and add 1 mL of saline to each tube. Leave on the bench overnight.

Day 2:

1. Using a disposable inoculating loop, remove a large inoculum of each isolate from the agar plates and place in the saline. Mix well and centrifuge at 8500g for 2 min.
2. Remove the saline by pipeting and repeat this step twice. After the last wash fully aspirate any remaining saline and reweigh each Eppendorf tube to get the precise weight of the pellet. Resuspend the pellet in an equal volume of saline, to the nearest 10 (i.e., if the pellet weighs 0.038 g, add 40 μL saline). These tubes now represent stock samples.
3. To prepare a working suspension, mix 5 μL of each stock sample with 10 μL of saline and then to each of these working suspensions add 225 μL of TEN and mix.
4. Prepare 250 μL of 2% (w/v) Inert agarose for each sample (plus 250 μL extra), by combining the required amount of agarose with sterile distilled water in a heat-proof capped tube. Boil the agarose for 15 min, mixing occasionally, and then allow to cool to approx 50°C.

5. Working quickly, add 230 μ L of agarose to each sample, mix well using a 1-mL pipette tip (see **Note 9**) and aspirate into a syringe barrel. Seal the opening of the syringe with Parafilm and place on ice for 15–20 min.
6. When plugs have solidified, push them out into 10-mL tubes, add 2 mL of EC buffer to each tube, and put gently shaking at 37°C for 5 h.
7. After 5 h aspirate the EC buffer and add 3 mL TE buffer. Aspirate the TE buffer and add another 3 mL of TE buffer and then place the tubes on a gently shaking platform for 10 min. Aspirate the TE buffer, and then to each sample add 2 mL freshly prepared ES buffer and 100 μ L proteinase K (20 mg/mL). Place in a shaking waterbath at 50°C overnight.

Day 3:

1. Remove all samples from the waterbath and place them on ice for 10 min. Aspirate the ES buffer from each plug and wash the plugs in 3 mL TE buffer. Immediately replace the TE buffer with fresh TE and incubate with gentle shaking for 1 h.
2. Repeat this procedure for a total of four washes. The plugs can now be stored in fresh TE buffer at 4°C until ready for restriction digestion.

3.4.2. Restriction Endonuclease Digestion of Genomic DNA Embedded in Agarose Plugs

1. Aliquot 300 μ L of DNS buffer into the required number of Eppendorf tubes.
2. Chill the samples to be digested on ice for 10 min. Remove the surrounding TE buffer and aspirate the solid plug back into the syringe barrel originally used for preparing the plug.
3. Using a sterile scalpel, previously flamed with alcohol, slice 1-mm-thick pieces from the plug into the tubes containing the DNS buffer (see **Note 1**). Immediately replace the buffer with fresh DNS. Incubate the samples at room temperature for 1 h. Repeat this step for a total of four washes.
4. After aspirating of final wash, add 50 μ L BNE to all tubes and incubate for 1 h at 4°C. Replace the BNE with 50 μ L BWE, refrigerate for 2 h (at 4°C), and then place at 37°C for 12–20 h.
5. After incubation, add 250 μ L 0.5X TBE to all tubes to stop the enzyme digestion. Samples can now be stored at 4°C or run immediately.

3.4.3. Preparation and Loading of Pulsed-Field Gels

1. Prepare a 1% (w/v) agarose gel by mixing 1.1 g of SeaKem Gold agarose with 110 mL 0.5X TBE buffer in a conical flask. Record the weight of the flask along with its contents.
2. Heat the agarose on full power in a microwave oven until boiling. Reduce the heat until the agarose bubbles very gently for 3 min. Mix the agarose by gently swirling the flask (see **Note 10**) and reheat for a further 3 min. Reweigh the flask and replace the moisture loss with hot distilled water.
3. Allow the agarose to cool to 60°C before casting the gel mold. Leave to solidify for 30 min at room temperature.
4. Finally, pre-electrophorese the gel (prior to loading the samples) for 1.5 h under sample run conditions to improve resolution of DNA bands. Alternatively, place the gel in the electrophoresis tank and allow the 0.5X TBE buffer to wash the gel overnight.
5. Load the prechilled, digested agarose slices into the corresponding wells in the agarose gel with the aid of a glass coverslip and an inoculating loop.
6. Seal the wells with molten 1% (w/v) agarose before applying electric current. Samples are electrophoresed through the homogenous field using a Gene Navigator® System (Pharmacia Biotech, Uppsala, Sweden) or other similar PFGE system, with a hexagonal

(HEX) electrode array. The running buffer consists of 2.5 L of 0.5X TBE, and the following run conditions are applied: 200 V at 10.5°C for 18 h with pulse times ramped from 1 to 40 s.

3.4.4. Visualization of DNA Bands

1. Following electrophoresis, stain the gel for 30 min with an ethidium bromide solution (0.5 µg/mL). This may be followed by destaining in distilled water for 1–2 h.
2. All ethidium bromide-stained DNA fragments can then be directly viewed and photographed over a UV transilluminator (Ultra-Violet-Products). A permanent record is maintained either electronically by creating a TIFF file using suitable capture software such as Phoretix software, or a standard photograph can be taken.

3.5. Southern Blotting

3.5.1. Transfer of DNA From Agarose Gels to Nylon Membranes

DNA transfer from the PFGE gels to nylon membranes can be achieved efficiently using vacuum blotting with the Hybaid Vacu-aid apparatus (*see Note 3*).

1. Cut a piece of 3MM filter paper such that it is approx 2–4 cm larger than the gel to be blotted, and prewet the filter paper in 2X SSC (prepared by diluting 20X SSC 1:10 with sterile distilled water).
2. Being careful not to touch the membrane, cut it so that it is slightly (0.5 cm) larger than the gel. Again, prewet it in transfer buffer for 3–5 min.
3. Assemble the Vacu-aid (or similar device) as outlined in the manufacturer's instructions, and overlay the membrane with the gel.
4. The blotting process is greatly accelerated with the *in situ* pretreatment steps. First, apply the vacuum and add *depurination solution*. Allow this solution to penetrate the gel for 10 min and then replace it with *denaturation solution* for 10 min followed by the *neutralization solution*, also for 10 min. Start the transfer by overlaying the gel with 20X SSC and allow transfer to proceed for at least 1 h (*see Note 11*).
5. Once the transfer is complete, rinse the membrane in 2X SSC and place it on fresh filter paper to air-dry completely.
6. Fix the DNA to the membrane either by crosslinking in a UV crosslinker or by wrapping the membrane in aluminium foil and baking at 80°C for 1 h. The membrane is now ready for DNA probe hybridization.

3.6. Preparation and Detection of DIG-Labeled Probes

3.6.1. Preparation of DIG-DNA Gene Probes

1. Incorporation of a DIG label can be achieved using 1 µL of DIG-labeled dUTP (DIG-11-dUTP) in the integron PCR reaction mixture as previously outlined in **Subheading 3.2**.
2. After the labeling reaction, recover the probe by precipitating in a mixture containing 2 µL 4 M LiCl and 50 µL cold ethanol (100%).
3. Allow the probe to stand for 2 h at –20°C, followed by a centrifugation step at 18,000g for 15 min.
4. Decant the surrounding ethanol and wash the pellet (*see Note 12*) with 70% (v/v) cold ethanol. Centrifuge for 5 min at 18,000g, fully aspirate the ethanol, and allow the pellet to air dry for 5 min.
5. Resuspend the pellet in 50 µL 1X TE and solubilize the probe at 37°C for 30 min. Place the probe in 10 mL hybridization solution and store at –20°C until required.

3.6.2. Hybridization With DIG-DNA Probes

1. Prewarm the hybridization buffer to 58°C for Southern blots of PFGE gels. Place the membrane in a hybridization bottle with approx 50 mL prewarmed hybridization solution and incubate in a hybridization oven at 58°C for 1 h (*see Note 13*).
2. Boil the probe for 10 min to ensure that the DNA is completely denatured and cool rapidly in ice water for an additional 10 min. Remove the hybridization solution from the membrane, add the denatured probe, and allow to hybridize overnight at 58°C with constant agitation of the hybridization-probe solution. This is achieved in the hybridization bottle simply by rotating it. This step should continue for a period of 18 h.
3. Following probe-hybridization, remove the probe and freeze it at -20°C for later use (*see Note 14*).
4. Wash the membrane twice with *wash solution A* at room temperature for 5 min per wash, and wash twice with *wash solution B* at 68°C for 15 min per wash, with constant shaking.

3.6.3. Color Detection of the Hybridized DIG Label

1. Rinse the membrane briefly in *buffer 1* (maleic acid buffer), and then incubate the membrane for 30 min with 300 mL *buffer 2* (blocking buffer) at room temperature.
2. Dilute anti-DIG-AP conjugate to 150 mU/mL (1:5000 dilution) in *buffer 2* per the manufacturers instructions (8- μ L conjugate in 40 mL *buffer 2*) and incubate the membrane for a further 30 min with the antibody conjugate.
3. Remove unbound antibody conjugate by washing twice with 300 mL *buffer 1* for 15 min for each wash at room temperature. Then equilibrate the membrane in approx 60 mL detection buffer for 2–5 min followed by incubation with 30 mL freshly prepared color solution (600 μ L NBT/BCIP in 30 mL *buffer 3*), in a sealed plastic bag (*see Note 15*).
4. Once the color solution is added, place the membrane in the dark and do not shake. Allow the color to develop, which could take from several minutes to several hours.
5. After color development, wash the membrane in 1X TE or in sterile distilled water for a few minutes and then allow the membrane to dry before photographing and recording results.

4. Notes

1. When cutting the gel slices for restriction digests, it is useful to cut several slices together and digest them all at once in the same tube. This is particularly advantageous if the samples are to be electrophoresed more than once for hybridization with several probes.
2. The DNA extraction protocol outlined here can also be used for Gram-positive bacteria. However, note that lysozyme is not effective against Gram-positive bacterial cells and therefore must be substituted for by using mutanolysin (50–100 U per sample) or lyso-staphin (50–100 U per sample) for staphylococcal isolates.
3. The transfer of immobilized DNA fragments by Southern blotting can be greatly accelerated when using a vacuum-based blotting device. Denaturation, depurination, neutralization, and transfer of DNA to the solid-phase membrane can be achieved in under 2 h. Transfer can also be achieved using more traditional and slower methods including capillary action through multiple layers of filter paper.
4. When preparing the maleic acid buffer, to achieve the required pH 7.5, approx 7 g of NaOH per liter are required. Add the sodium hydroxide pellets carefully and allow them to dissolve, check the pH, and then add less concentrated NaOH until the desired pH is reached. **Caution:** *Be aware that granular NaOH can cause skin burns and must be handled carefully.*

5. It is very important to resuspend the bacterial pellet completely before adding the lysozyme. Repeated pipeting proves more efficient than vortexing in this respect.
6. After incubation with proteinase K for 1 h at 37°C, it is often difficult to manipulate the DNA in solution, owing to its “stickiness,” typically occurring following the release of chromosomal DNA. Incubating the samples for an additional hour at 65°C often improves this situation by reducing the “stickiness.”
7. As soon as the cold ethanol is added, strands of DNA should become visible as they precipitate. Allow the DNA to precipitate for at least 2 h or preferably overnight at –20°C, to increase the yield further.
8. Agarose gels can be made up in several different sizes; a gel of dimensions 11.5 × 14 cm requires 100 mL of agarose TAE buffer mix, whereas smaller gels approx 6.5 × 8.0 cm require only 30 mL.
9. When preparing plugs for PFGE, it is very important to prepare a homogenous mixture of bacterial cell culture and warm agarose. Maintain the solubilized agarose at a constant temperature of 50°C and dispense the 230-μL aliquot directly into each of the bacterial cultures. Mix by gently pipeting up and down, but avoid introducing air bubbles into the samples. When the samples are thoroughly mixed, immediately aspirate into the barrel of the syringe, again avoiding air bubble formation. If air bubbles form, gently flick the syringe barrel until the air bubbles escape by floating to the surface of the gel/culture mixture.
10. **Caution:** *a fully heated flask of molten agarose is very likely to boil over when disturbed, resulting in a severe skin burn. Care should be taken when gently swirling molten agarose to avoid injury. Make sure that gloves are worn.*
11. When performing the Southern blot with a vacuum blotter, allow the transfer to proceed for a minimum of 1 h, but as the macrorestricted DNA fragments are quite large, an additional 30 min often ensures complete transfer of the DNA. As the transfer proceeds, ensure that there is adequate 20X SSC buffer for the transfer. Generally the 20X SSC must be topped up every 20–30 min.
12. A pellet may not always be visible when the DIG-labeled DNA probe is precipitated; therefore it is reassuring if 5 μL of the PCR product for the probe is analyzed by agarose gel electrophoresis prior to the precipitation step.
13. If several Southern blotting and hybridization experiments are to be performed in the laboratory, it is wise to invest in a hybridization oven. The oven will maintain a constant temperature for hybridization, while continually rotating the membrane in a hybridization bottle to ensure even distribution of probe.
14. After hybridization, the probe can be frozen at –20°C and used for up to five hybridization experiments, depending on the strength of the original DIG-labeled PCR product. However, it is important to remember that probes must be denatured each time before use.
15. Using a sealed plastic bag has the advantage of increasing the membrane-solution contact by reducing the volume of the container.

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Public Health Microbiology

Methods and Protocols

Spencer, J.F.T.; Alicia L. Ragout de Spencer (Eds.)

2004, XVII, 548 p., Hardcover

ISBN: 978-1-58829-117-2

A product of Humana Press