Characterization of an rRNA gene-specific cDNA probe: applications in bacterial identification

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Discontinuous DNA complementary to *Escherichia coli* 16S + 23S ribosomal RNA was synthesized by random oligonucleotide priming using reverse transcriptase. cDNA generated from native or denatured rRNA template was labelled by incorporation of either [α -³²P]dCTP or digoxigenin-labelled dUTP during synthesis, followed by template hydrolysis. The specific activity of the radiolabelled cDNA was 10^7 – 10^8 c.p.m. (μ g rRNA template)⁻¹ with 60–92% incorporation after 5 h. The length of the reverse transcript was between 20 and 1140 nucleotides and was unaffected by exclusion of primer. The cDNA probe could detect 3 pg rRNA by quantitative slot blot. In the non-radiolabelling digoxigenin system 3 μ g template gave 0.5–2.0 μ g cDNA after 24 h with a length of between 100 and 1225 bases. This probe could detect 50 pg rRNA. Probes were evaluated in the comparison of *Pasteurella haemolytica* biotypes by hybridization to Southern blots of restriction-endonuclease-digested total DNA. The digoxigenin-labelled probe was used to identify clinical isolates of *Campylobacter jejuni* to demonstrate its potential use in laboratories requiring high-sensitivity detection without the use of radioisotopes.

Introduction

Analytical techniques in biochemistry and molecular biology have revolutionized microbial classification and identification. Although DNA-based identification schemes are an improvement on phenotyping, there are problems associated with current techniques. In plasmid analysis the plasmids may be unstable or transferred; in restriction digests of chromosomal DNA too many fragments are generated to allow easy comparison of strains and cloned sequences are often too specific. Recent attention has focused on ribosomal RNA analysis (Woese, 1987) and rDNA restriction patterns have been used to compare bacterial strains (Grimont & Grimont, 1986). Moureau *et al.* (1989) used an oligonucleotide probe to demonstrate a high degree of variation in rDNA restriction patterns amongst *Campylobacter* spp. but did not correlate this with biotype.

In the present study, as an alternative to end-labelling, rRNA-specific cDNA sequences were synthesized by random oligonucleotide priming. A new method for non-radioisotopic labelling by incorporation of a digoxigenin-labelled nucleotide was evaluated. The plant steroid digoxigenin, found naturally only in *Digitalis* spp., is linked via a spacer arm to dUTP. Detection is by an anti-

digoxigenin alkaline phosphatase conjugate followed by an enzyme-catalysed colour reaction. The digoxigenin-labelled and radiolabelled cDNA probes were compared in rDNA restriction analysis of *Pasteurella haemolytica* and the digoxigenin-labelled probe was used to identify biotypes of *Campylobacter jejuni*.

Methods

Bacterial strains and media. The strains used are listed in the legends to Figs 1 and 2. Clinical isolates of *C. jejuni* were recovered by conventional methods and biotyped (Table 2) according to Lior (1984).

cDNA synthesis. (a) Radiolabelled cDNA was synthesized by reverse transcription of *Escherichia coli* MRE 600 16S + 23S rRNA (Boehringer Mannheim) after random oligonucleotide priming (Feinberg & Vogelstein, 1984). cDNA from 40 ng native or heat-denatured template was labelled during synthesis (26 U reverse transcriptase, Anglian Biotec) with 20 μ Ci [α -³²P]dCTP (3000 Ci mmol⁻¹, 111 TBq mmol⁻¹) at 37 °C for 18 h. The rRNA template was hydrolysed by alkali, neutralized, and non-incorporated radioactivity removed by spun column chromatography. Incorporation was followed by adsorption onto DE-81 Whatman filter paper (Sambrook *et al.*, 1989).

(b) Digoxigenin-labelled cDNA from 1–3 μ g template was labelled as above but with 0.7 nM-digoxigenin-11-dUTP (Boehringer Mannheim kit), ethanol precipitated, and redissolved in TE buffer (10 mM-Tris/HCl, 1 mM-EDTA, pH 8.0). The rRNA portion of the

cDNA:rRNA heteroduplex was degraded using ribonuclease H (Pharmacia). cDNA synthesis was followed by [^3H]dATP incorporation and by comparison to digoxigenin-labelled linearized pBR328 ($20\ \mu\text{g ml}^{-1}$, Boehringer Mannheim).

Length of cDNA synthesized. (a) The oligonucleotide primers were excluded from the labelling mixture to measure the effect of self-primed reverse transcription on the length of cDNA synthesized. The radiolabelled mixture was passed through a Sephadex G-50 column (Pharmacia) with $0.5\ \text{mg tRNA ml}^{-1}$ (Sigma) and TE buffer, the radioactivity in each eluted fraction counted and the rRNA template hydrolysed. Samples were electrophoresed through 6% (w/v) polyacrylamide gels containing 42% (w/v) urea for 3 h at 20 W and the gels washed and dried (Sambrook *et al.*, 1989). Autoradiography using pre-flashed Hyperfilm MP (Amersham) was for 16 h at -70°C .

(b) The digoxigenin-labelled mixture was passed through a Sephadex G-50 column, eluted in 20 mM-Tris/HCl, pH 8.0, 100 mM-NaCl, 1 mM-EDTA, pH 8.0, and fractions assayed for digoxigenin incorporation. Samples were then treated with ribonuclease H and S_1 nuclease (Pharmacia), glyoxalated and electrophoresed in 1.4% (w/v) agarose gels for 3 h ($5\ \text{V cm}^{-1}$) using 0.01 M-sodium phosphate (pH 7.0) buffer. Nucleic acid was transferred by blotting to nylon membranes (Amersham) and baked at 80°C for 2 h. Digoxigenin incorporation was detected according to the manufacturer's instructions (colour development for 16 h).

Sensitivity of rRNA detection. *E. coli* 16S + 23S rRNA was glyoxalated, diluted in $2 \times \text{SSC}$ ($1 \times \text{SSC}$ is $0.15\ \text{M-NaCl}/0.015\ \text{M-trisodium citrate}$, pH 7.0) and slot-blotted onto nylon membranes using a Schleicher & Schuell manifold. These were probed using either single-stranded or double-stranded radiolabelled or digoxigenin-labelled cDNA.

rDNA restriction patterns. Purified total DNA was prepared as described by Ausubel *et al.* (1987), digested with restriction endonucleases and fragments separated by electrophoresis in 1% (w/v) agarose gels ($5\ \text{V cm}^{-1}$ for 3 h) in E buffer ($0.04\ \text{M-Tris/acetate}$, $0.002\ \text{M-EDTA}$), followed by transfer to nylon membranes (Southern blot). λ DNA digested with *Hind*III was used as a marker (end-filled with either [α - ^{32}P]dCTP or digoxigenin-11-dUTP; digoxigenin-labelled DNA fragments were blotted directly without alkali denaturation).

(a) **Hybridization using radiolabelled probes.** Membranes probed with cDNA synthesized from 40 ng template were prehybridized and hybridized as previously described (Hayes *et al.*, 1988), washed for 30 min at 65°C in $6 \times \text{SSC} + 0.1\%$ (w/v) SDS decreasing to $0.1 \times \text{SSC} + 0.1\%$ (w/v) SDS and autoradiographed at -70°C .

(b) **Hybridization using digoxigenin-labelled probes.** Membranes were probed with cDNA synthesized from $3\ \mu\text{g}$ template as above except that 0.5% (w/v) blocking agent (Boehringer Mannheim) was used instead of salmon sperm DNA. Membranes were washed in $2 \times \text{SSC} + 0.1\%$ (w/v) SDS for 30 min at 65°C , hybrids detected and membranes photocopied or photographed and dried for long-term storage.

Results

Characterization of labelled cDNA

The amount of [α - ^{32}P]dCTP incorporation depended on the nature of the rRNA template. cDNA synthesized from the native and denatured templates after 5 h showed 60% and 92% incorporation (compared to incorporation at 24 h), respectively. By 24 h, five times more incorporation was obtained using the native template [$1 \times 10^8\ \text{c.p.m.} (\mu\text{g template})^{-1}$] than for the denatured template [$2 \times 10^7\ \text{c.p.m.} (\mu\text{g template})^{-1}$]. The length of the cDNA synthesized varied from between 20–620 and 20–1140 nucleotides for the denatured and native templates, respectively. Subsequent degradation of template with ribonuclease H or sodium hydroxide, or exclusion of primer, did not alter the length of the reverse transcript. However, in the absence of primer a fivefold decrease in incorporation was observed with the native template and a 20-fold decrease with the denatured template when compared to the standard reaction after 24 h using native template (data not shown). Incorporation of digoxigenin-dUTP into cDNA after 5 h was 82% and 100% for the native and denatured templates, respectively. Twice as much incorporation was seen at 24 h with the native template. The amount of labelled cDNA synthesized from $3\ \mu\text{g}$ native or denatured template was $2\ \mu\text{g}$ and $0.5\ \mu\text{g}$, respectively. The length of the reverse transcript was 100–800 nucleotides for the denatured and 100–1225 for the native template.

Table 1. Sensitivity of detection after hybridization of radiolabelled cDNA probes (8 ng per ml hybridization solution) and digoxigenin-labelled cDNA probes ($20\ \text{ng ml}^{-1}$) to rRNA slot blots

Membranes were washed for 30 min at 65°C in $2 \times \text{SSC} + 0.1\%$ (w/v) SDS.

Probe	Sensitivity of detection (pg)							Digoxigenin-labelled cDNA Colour development time (h) 16
	Radiolabelled cDNA Exposure time (h)							
	0.67	1.25	2.5	4	6	8	16	
Native template								
Double-stranded	96	48	24	12	12	12	3	390
Single-stranded	48	24	12	6	6	6	3	3100
Denatured template								
Double-stranded	192	96	48	24	24	24	6	390
Single-stranded	192	96	48	24	24	12	6	6300

Fig. 1. Southern blot analysis of restriction-endonuclease-digested *P. haemolytica* DNA. Hybridization probes (prepared from native rRNA template) were (a) radiolabelled single-stranded cDNA (autoradiography for 6 h) and (b) digoxigenin-labelled double-stranded probe (detection for 4 h). Lanes 1-3, *Hind*III-cleaved DNA; lanes 4-6, *Ava*I-cleaved DNA. Lanes: 1 and 4, biotype A serotype 1 NCTC 10609; 2 and 5, biotype T, serotype 4 NCTC 10369; 3 and 6, type strain, NCTC 9380.

Ribonuclease H degradation of the rRNA template followed by S_1 nuclease degradation of the single-stranded cDNA reduced the size of the labelled cDNA to <100 bases for both templates (data not shown).

The level of sensitivity of detection by the radiolabelled cDNA probes decreased from the native template single-stranded probe to the denatured template double-stranded probe (Table 1). The nature of the template did not significantly affect the sensitivity of the digoxigenin-labelled probes, although ribonuclease H treatment reduced the sensitivity.

rDNA restriction patterns

As with sensitivity of rRNA detection, the nature of the template or probe used was reflected in the intensity of

the signal obtained after hybridization to DNA restriction digests. The biotypes and serotypes of *P. haemolytica* were readily differentiated (Fig. 1). The 0.1 kb *Hind*III fragment of biotype T was found to be characteristic of all biotype T serotypes (data not shown), whereas the 0.7 kb and 1.5 kb *Hind*III fragments were common to all types.

*Cla*I-cleaved *Campylobacter* DNA produced a simple rDNA restriction pattern (Fig. 2b). Isolates identified as *C. jejuni* biotype 3 by biochemical analysis gave a similar profile to the NCTC biotype 3. Four out of five strains identified as biotype 2 gave exactly the same profile and all five were more closely related to the NCTC biotype 2 (with a unique band at 3.4 kb). Four out of six isolates identified as biotype 1 gave the same profile as the four biotype 2 isolates with a further isolate similar to biotype

Fig. 2. Southern blot analysis of (a) *Hind*III and (b) *Cla*I digested *Campylobacter* DNA hybridized with digoxigenin-labelled cDNA probe (60 ng double-stranded probe per ml hybridization solution). Colour development was for 4 h. Lanes: A, λ *Hind*III-digested DNA end-filled with digoxigenin-labelled dUTP; B, *C. jejuni* biotype 1 NCTC 11351; C, *C. jejuni* biotype 2 NCTC 12106; D, *C. jejuni* biotype 3 NCTC 12108; E, *C. coli* biotype 1 NCTC 12110; F, *C. coli* biotype 2 NCTC 12143; 1–13, *C. jejuni* clinical isolates.

2. The remaining biotype 1 strain, without the 3.4 kb band, resembled the NCTC biotype 1. The comparison between strains is summarized in Table 2. In contrast to the *Cla*I rDNA restriction pattern, *Campylobacter* spp. could not be distinguished easily using the complex *Hind*III pattern obtained (Fig. 2a).

Discussion

The commonest method for labelling RNA is by 5'-end labelling with [γ - 32 P]ATP using T4 polynucleotide kinase following dephosphorylation or phosphate exchange. However, the specific activity of the end-product is dependent on the activity of the labelled nucleotide used. We prepared cDNA labelled to high specific activity by random oligonucleotide priming using a rRNA template and reverse transcriptase. With the single-stranded cDNA probe there is no self-annealing (as with double-stranded probes) and no problem of ribonuclease degradation (as with RNA probes). It was found that heat denaturation of the rRNA to remove secondary structure produced shorter length cDNA with less incorporation of labelled nucleotide and consequently a lower sensitivity of detection. The reverse transcriptase was not inhibited by base pairing in the rRNA species. cDNA synthesis continued at a reduced efficiency in the absence of primer due to self-primed reverse transcription (Qu *et al.*, 1983). The length of the transcript was similar for both labelling methods, with discrete cDNA intermediates present due to base methylations terminating transcription (Youvan & Hearst, 1979).

The radiolabelled single-stranded probe was more sensitive than the double-stranded probe. However, the opposite was true for the digoxigenin-labelled probe. This may be due to incomplete degradation of the template with ribonuclease H (alkali hydrolysis of template resulted in a loss of incorporated digoxigenin, possibly by hydrolysis of the spacer arm). Inclusion of 10% dextran sulphate in the hybridization solution increased the sensitivity approximately eightfold to 50 pg for the double-stranded digoxigenin-labelled probe, although this also increased the background (data not shown).

Serotypes and biotypes of *P. haemolytica* strains were distinguished readily by their rDNA restriction pattern. A greater similarity could be seen between serotypes of the same biotype than between serotypes of different biotypes. The complexity of the pattern will be dictated by the choice of restriction enzyme; by using a two-dimensional scanner it would be possible to automate the analysis (Tabaqchali *et al.*, 1987).

Table 2. Biotyping of *Campylobacter* spp. by biochemical and rDNA restriction analysis

All isolates were sensitive to nalidixic acid (30 µg per disc) and resistant to cephalothin (30 µg per disc).

Strain	Biochemical test			Biotype	
	Hippurate hydrolysis	H ₂ S production	DNAase production	Biochemical analysis	rDNA analysis
NCTC strain					
<i>C. jejuni</i>					
11351	+	—	—	1	1
12106	+	—	+	2	2
12108	+	+	—	3	3
<i>C. coli</i>					
12110	—	—	—	1	1
12143	—	—	+	2	2
Clinical isolate					
<i>C. jejuni</i>					
1	+	—	+	2	2
2	+	—	+	2	2
3	+	—	—	1	2
4	+	—	—	1	2
5	+	+	—	3	3
6	+	—	+	2	2
7	+	+	—	3	3
8	+	—	+	2	2
9	+	—	—	1	1
10	+	—	—	1	2
11	+	—	—	1	2
12	+	—	+	2	2
13	+	—	—	1	2

Biotyping of *Campylobacter* spp. has been used to characterize strains. Comparison of the biochemical and rDNA profiles of *C. jejuni* revealed disagreement between five strains. This may be a result of (i) a misclassification due to a wrong biochemical profile (phenotypic variation) or insufficient tests in the scheme to allow reliable identification, or (ii) rDNA restriction patterns differentiating between serotypes within a given biotype.

The digoxigenin-labelled cDNA probe will be more appropriate for laboratories which lack the facilities for radioisotope handling or find the half-life of ³²P (14.3 d) limiting. By using a large amount of template (up to 3 µg in a standard reaction) sufficient labelled cDNA is synthesized to allow up to 15 hybridizations; this number can be increased by re-using the probe, which is stable at -20 °C for at least a year.

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