A novel tRNA-associated locus (*trl*) from *Helicobacter pylori* is co-transcribed with tRNA^{Gly} and reveals genetic diversity

William G. Dundon,^{1†} David G. Marshall,¹ Colm A. Ó Moráin² and Cyril J. Smyth¹

Author for correspondence: William G. Dundon. Tel: +39 049 8276056. Fax: +39 049 8276049.
e-mail: dundon@civ.bio.unipd.it

¹ Department of Microbiology, Trinity College, Dublin 2, Ireland

² Department of Gastroenterology, The Adelaide and Meath Hospital, Tallaght, Dublin 24, Ireland

To date several genes have been identified in *Helicobacter pylori* that are expressed in only a proportion of strains, some of which are correlated with the pathogenicity of the bacterium. With this in mind, the present study was undertaken to identify other genes that are not expressed in all clinical isolates of *H. pylori*. Using arbitrarily primed PCR of RNA, a cDNA fragment of 187 bp (designated *trl* for transfer RNA-associated locus) was identified that was expressed in only one of two clinical isolates being tested. The fragment was purified, cloned and sequenced. A search of public databases prior to the release of the complete genome sequence of *H. pylori* strain 26695 showed no similarity with any other known genes or gene products. Inverse PCR was used to obtain further nucleotide sequence information surrounding the *trl* locus. A DNA probe derived from the *trl* locus hybridized with 32 (50%) of 64 clinical *H. pylori* isolates tested. Comparison of the nucleotide sequences of a *trl*-positive and *trl*-negative isolate showed that the locus is situated between two tRNA genes, tRNA^{Gly} and tRNA^{Leu}, in *H. pylori*. Primer extension analysis showed that the *trl* locus is co-transcribed with tRNA^{Gly}. Analysis of the region between tRNA^{Gly} and tRNA^{Leu} in *trl*-negative isolates revealed additional genetic diversity among these isolates.

Keywords: arbitrarily primed PCR, diversity, tRNA, horizontal transfer

INTRODUCTION

Helicobacter pylori, which colonizes the gastric mucosa of humans, is now recognized as the major aetiological agent of chronic gastritis and is strongly associated with both gastric and duodenal ulcers (for review see Dunn *et al.*, 1997). Infection with *H. pylori* has also been linked with an increased risk of adenocarcinoma and lymphoma (O'Connor *et al.*, 1996; Parsonnet *et al.*, 1997). To date several virulence factors have been identified and characterized from this Gram-negative bacterium (Fauchère & Blaser, 1990; Eaton *et al.*, 1991; Leying *et al.*, 1992; Covacci *et al.*, 1993; Tummuru *et al.*, 1993). Among those present in all *H. pylori* isolates

are the urease, the flagella and a number of putative adhesins that ensure tissue-specific colonization. In addition, a subset of strains produce a potent cytotoxin (VacA) and a surface-exposed immunodominant antigen (CagA), which is associated with cytotoxin expression and is part of the *cag* pathogenicity island (Xiang *et al.*, 1995; Censini *et al.*, 1996).

H. pylori has an extremely plastic and variable genome and extensive allelic diversity exists (for review see Marshall *et al.*, 1998). Correlations between strain variations and clinical outcomes of infection suggest that differences in virulence between isolates of *H. pylori* may be due to the presence or absence of particular genes or to variations in gene expression. For example, it has been reported that strains that possess the *cagA* gene are more likely to cause ulcer disease and gastric adenocarcinoma (Covacci *et al.*, 1993; Parsonnet *et al.*, 1997). Peek *et al.* (1998) have described the identification of a genetic locus (*iceA*) that was induced by contact of *H. pylori* with gastric epithelial cells. There are two distinct *iceA* sub-groups, *iceA1* and

† Present address: Centro CNR Biomembrane e Dipartimento di Scienze Biomediche, Università di Padova, Via G. Colombo 3, 35121, Padova, Italy.

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iceA2. *H. pylori* strains of the *iceA1* genotype have been shown to be strongly associated with duodenal ulcer disease, while *iceA2* strains are associated with gastritis (Peek *et al.*, 1998; van Doorn *et al.*, 1998). Recently, Akopyants *et al.* (1998) identified 18 DNA fragments specific to a monkey-colonizing strain of *H. pylori* by subtractive hybridization against the unrelated *H. pylori* strain 26695. A number of these strain-specific DNA fragments revealed nucleotide sequence identity with known genes from other bacteria. There are therefore probably several other genes in addition to *cagA*, *iceA* and those identified by Akopyants *et al.* (1998) in *H. pylori* that are not expressed in all strains and which may be important for the virulence of the bacterium. To investigate whether such genes could be identified in clinical isolates of *H. pylori*, a modification of a differential display technique was applied.

Arbitrarily primed PCR of RNA has been used successfully in eukaryotes to identify genes that are differentially expressed under varying environmental conditions (Welsh *et al.*, 1992). The technique has been further developed for the identification of differentially expressed genes in prokaryotes (Wong & McClelland, 1994; Kwaik & Pederson, 1996; Thies *et al.*, 1998; Yuk *et al.*, 1998), including *H. pylori* (Peek *et al.*, 1998). Arbitrarily primed PCR of RNA involves the use of random primers in the synthesis of single-stranded cDNA from total RNA isolated from two different sources, i.e. from bacteria subjected to different environmental conditions, or, as in the case of this work, from two separate clinical isolates. Second strand synthesis is then performed on the single-stranded cDNA by PCR with radiolabelled dATP and an arbitrary primer. By using a selection of several different primers on reverse-transcribed mRNA isolated from the two different isolates, it is possible to obtain cDNA fingerprints for each isolate following electrophoresis on polyacrylamide sequencing gels. Comparison of the fingerprint profiles allows the pinpointing of DNA fragment differences which should represent genes that are expressed in one isolate and not the other. These fragments can be readily isolated and purified from the polyacrylamide gels and subsequently cloned for further characterization.

The present study reports the application of arbitrarily primed PCR of RNA in isolating a genetic locus that is only present in 50% of our clinical isolates. It describes the characterization of the locus (designated *trl* for transfer RNA-associated locus) at the nucleotide level. Additional genetic diversity in the corresponding region among *trl*-negative strains is also reported.

METHODS

Bacterial strains and culture conditions. Clinical isolates were obtained from antral biopsies of patients undergoing upper gastrointestinal endoscopy at the Meath, Adelaide and St James' hospitals in Dublin, Ireland. After primary culture the isolates were routinely grown under microaerophilic conditions for 3 d on Campylobacter agar base containing 7% (v/v) lysed horse blood (LHB), amphotericin (5 µg ml⁻¹) and

vancomycin (10 µg ml⁻¹). *Escherichia coli* strain ER2267 (New England Biolabs) [*ER1821 Δ(argF-lac)U169 recA1/F' proA⁺ proB⁺ lacI^a Δ(lacZ)M15 zxf::mini-Tn10(Kan^r)*] was used as a host for plasmid cloning experiments.

RNA preparation. Using a sterile swab, cells were harvested from LHB plates and washed in 1 ml TEN (10 mM Tris/HCl, pH 7.5, 1 mM EDTA, 0.1 M NaCl). Total RNA was subsequently isolated using an RNA isolator kit (Genosys Biotechnologies). The RNA concentration was determined by spectrophotometric analysis. One unit of RNase-free DNase I (Promega) was added (µg RNA)⁻¹ to remove any residual DNA contamination.

First strand synthesis of cDNA. First strand synthesis of cDNA was performed on total RNA by reverse transcription. Random hexamers (2 µg) were added to 4 µg total RNA in a final volume of 19 µl and incubated for 10 min at 70 °C. The mixture was allowed to cool to room temperature before adding 50 mM (each) dATP, dCTP, dGTP and dTTP, 10 mM DTT, 3 mM MgCl₂, 75 mM KCl, 50 mM Tris/HCl (pH 8.3), 1 U rRNasin (Promega) and 200 U M-MLV reverse transcriptase (Promega). The reaction was continued at 37 °C for 1 h and stopped by placing the mixture on ice. The volume was then made up to 100 µl with sterile distilled water. After phenol/chloroform extraction, the cDNA was precipitated with 1 vol. 4 M LiCl in 3 vols ethanol. The cDNA was resuspended in 20 µl sterile distilled water and stored at -70 °C.

Arbitrarily primed PCR. The PCR was carried out in a final volume of 25 µl containing 1 µl cDNA template, 3 mM MgCl₂, 40 pmol primer P1 (Table 1), 2.5 U *Taq* polymerase (Promega), 80 µM (each) dATP, dCTP, dGTP and dTTP, 1 µCi (3.7 × 10⁴ Bq) [α -³²P]dATP in 10 mM Tris/HCl (pH 8.3), 50 mM KCl and 0.001% (v/v) gelatin, and overlaid with 50 µl mineral oil. The thermal cycling parameters were as follows: five low-stringency cycles of 94 °C (1 min), 40 °C (1 min), 72 °C (2 min), then 30 high-stringency cycles of 94 °C (1 min), 55 °C (1 min), 72 °C (2 min), and finally 72 °C (10 min). Stop solution (10 µl; 80% formamide with 0.25% bromophenol blue and 0.25% xylene cyanole) was added to the completed PCR reaction. The samples were heated to 65 °C for 10 min and aliquots (4 µl) were loaded onto a 5% (w/v) polyacrylamide sequencing gel and electrophoresed at 35 V cm⁻¹ for 3 h. The gel was dried onto 3 MM paper (Whatman) and the separated PCR fragments were visualized following autoradiography.

Isolation and cloning of arbitrarily primed PCR products. The autoradiogram was aligned with the gel using Glogos (Stratagene) autoradiogram markers and bands were cut from the gel using a razor blade. The gel slice was placed into a dialysis bag and 100 µl 0.1 × TBE (594 mM Trizma, 592 mM boric acid, 16 mM EDTA, pH 8.3) buffer was added. The dialysis bag was placed in a horizontal electrophoresis tank containing 0.1 × TBE. The DNA was electroeluted from the polyacrylamide gel for 2 h at 9.0 V cm⁻¹. The DNA was then precipitated with 0.1 vol. 4 M LiCl and 3 vols ethanol and resuspended in 10 µl distilled water. The DNA sample (5 µl) was amplified by PCR in a final volume of 25 µl using the same primer and reaction conditions as above. The thermal cycling parameters were as follows: 35 cycles of 94 °C (1 min), 50 °C (1 min), 72 °C (2 min), and then 72 °C (10 min). The PCR fragment was purified from a 2% (w/v) agarose gel in 1 × TAE (40 mM Tris/acetate, 1 mM EDTA, pH 8.0) using the GeneClean II kit (Bio 101), and cloned into the appropriate restriction site of the vector plasmid pUC18.

Confirmation of differential expression by Northern blotting. Northern blot analysis was carried out using the Digoxigenin

Table 1. Oligonucleotides and plasmids used in this study

Primer/plasmid	Sequence or relevant information	Strand	Position (bp)*/ size (bp)
Primers			
P1	5'-AGTGAATTCGCGGTGAGATGCCA-3'	+	412-434
		-	602-624
P2	5'-AACACTCCAACAACCTCG-3'	-	475-491
P3	5'-ATGGTGAGCCGTGAGAC-3'	+	564-580
P4	5'-GCCTCCATTTATTGTAG-3'	+	934-950
P5	5'-GCACGACCTTGCCAAGGT-3'	+	252-269
P6	5'-CACGGTATTGCTACCGAG-3'	-	742-759
P8	5'-ACGATAAGGCTTAACGC-3'	-	1215-1232
P9	5'-ATTATTGAGTTTAGATAC-3'	+	183-201
Plasmids			
pWD1	<i>trl</i> -I cloned into <i>Eco</i> RI site of pUC18	n/a	187
pWD2	PCR fragment using P8 and P9 cloned into <i>Hind</i> III and <i>Bam</i> HI site of pUC18	n/a	1049

*Base pair position of oligonucleotide with reference to the *trl* locus (Fig. 3b).

(DIG) Nucleic Acid Detection kit (Boehringer Mannheim) following standard protocols (Sambrook *et al.*, 1989). Briefly, 10 µg total RNA was electrophoresed through a 1% (w/v) agarose/2.2 M formaldehyde gel and then transferred to a nylon membrane. The distances migrated by the 23S, 16S and 5S rRNA of *H. pylori* were recorded for use as molecular mass markers. Hybridization was performed using the appropriate DIG-labelled DNA probe at 50 °C for 16 h.

Amplification of the *trl* locus by inverse-PCR. Genomic DNA (10 µg) from isolate MI 212 was digested with restriction endonuclease *Hind*III. After two phenol/chloroform extractions the DNA was precipitated, washed and dried. Approximately 3 µg DNA was then self-ligated in a total volume of 200 µl with 9 U T4 DNA ligase. The ligation was performed at room temperature for 16 h. The ligation mixture was extracted twice with phenol/chloroform, precipitated, dried and resuspended in 20 µl distilled water. The DNA was then used in a PCR amplification. The amplification was carried out using the Expand Long Template PCR system (Boehringer Mannheim) in a final volume of 50 µl containing 1 µl DNA template, 1.75 mM MgCl₂, 350 µM (each) dATP, dCTP, dGTP and dTTP, 300 nM (each) primer P2 and P3 (Table 1) and 2.6 U Expand polymerase in 20 mM Tris/HCl (pH 7.5), 100 mM KCl, 1 mM DTT, 0.1 mM EDTA, 0.5% (v/v) Tween 20 and 0.5% (v/v) Nonidet P40. The thermal cycling parameters were as follows: 1 cycle of 94 °C (2 min), followed by 25 cycles of 94 °C (10 s), 65 °C (30 s) and 68 °C (45 s), and a final elongation step of 68 °C (7 min).

Primer extension analysis. Primer (10 pmol) was end-labelled with [γ -³²P]dATP using T4 polynucleotide kinase. The end-labelled primer (2 pmol) was then added to 50 µg total RNA, heated to 90 °C and allowed to anneal for 15 min on ice. The primers were then extended using 200 U SUPERSCRIPT reverse transcriptase (Gibco-BRL) at 42 °C for 90 min. RNase (10 µg µl⁻¹; 5 µl) was added and the mixture was incubated for a further 30 min at 42 °C. After extraction with phenol and chloroform, precipitation and resuspension in 4 µl stop solution (see above), 3 µl was run on a 5% (w/v) polyacrylamide sequencing gel. Sequencing reactions were performed with primers P2 and P3 and plasmid pWD2 as a template (Table 1).

Amplification of the region between tRNA^{Gly} and tRNA^{Leu} in *trl*-negative isolates. PCR with primers P5 and P6 (Table 1) was performed in order to amplify the region between the tRNA^{Gly} and tRNA^{Leu} in *trl*-negative isolates. The cycling parameters were as follows: 1 cycle of 94 °C (2 min), followed by 25 cycles of 94 °C (1 min), 55 °C (1 min) and 72 °C (2 min), with a final elongation step of 72 °C (10 min).

Southern blotting and dot blot analysis. Southern blot and dot blot analyses were carried out using standard methods (Sambrook *et al.*, 1989) and the DIG Nucleic Acid Detection kit. Briefly, 10 µg genomic DNA was cleaved with restriction endonuclease *Hind*III, electrophoresed on a 1% (w/v) agarose gel in 1× TAE buffer and transferred to a nylon membrane. The DNA was hybridized with the appropriate DIG-labelled probe for 16 h at 68 °C. Detection of the hybridized probes was carried out according to the manufacturer's instructions. For dot blots, identical conditions were used except that 1 µg genomic DNA was spotted onto nylon membranes prior to hybridization.

DNA sequencing and analysis. The nucleotide sequence of the *trl*-I DNA fragment was determined by dideoxynucleotide chain termination using the Sequenase kit (USB/Amersham). The nucleotide sequence of the inverse-PCR product, *trl*-II, was determined by the sequencing service at King's College, London, UK, with primers P2, P3 and P4 (Table 1). The nucleotide sequence of the *trl*-negative PCR products was determined by the sequencing service at MWG-Biotech, Milton Keynes, UK. Nucleotide sequences were analysed using the BLAST program (version 2.0) from the National Centre for Biotechnology Information (NCBI) internet site covering the GenBank, EMBL, DDBJ and PDB databases. Searches for amino acid sequence motifs were carried out using the MOTIF World Wide Web server.

RESULTS

Arbitrarily primed PCR identifies a tRNA-associated locus (*trl*)

Total RNA was isolated from two clinical isolates, namely isolate MI 212 (recovered from an asymptomatic

Fig. 1. Arbitrarily primed PCR of RNA. Polyacrylamide gel electrophoresis of [α - 32 P]dATP-labelled, arbitrarily primed PCR products showing the cDNA fingerprints of two *H. pylori* isolates. Lanes: 1, isolate MI 299; 2, isolate MI 212. The arrow identifies the differentially expressed cDNA fragment *trl*-I.

individual) and isolate MI 299 (recovered from a patient with a duodenal ulcer). Isolate MI 212 was *cagA*-positive while isolate MI 299 was *cagA*-negative. Both isolates were plasmid-free. Single-stranded cDNA was prepared from the RNA of both isolates, followed by

second strand synthesis by PCR using radiolabelled dATP and a selection of arbitrary primers. Following polyacrylamide electrophoresis the cDNA fingerprints were visualized by autoradiography. Of the arbitrary primers used, primer P1, which had an internal *Eco*RI site, gave different fingerprints for the two isolates (Fig. 1). The isolates differed with respect to a 187 bp fragment (designated *trl*-I) that was present in isolate MI 212 but absent in isolate MI 299. This fragment was purified as described in Methods. It was subsequently digested with restriction endonuclease *Eco*RI and cloned into the unique *Eco*RI site of pUC18 to give plasmid pWD1. To confirm the differential expression of the *trl* locus, Northern blot analysis of total RNA from isolates MI 212 and MI 299 was performed using DIG-labelled *trl*-I DNA as the probe. A hybridization band of approximately 400 bp was present in isolate MI 212. No hybridization signal was observed for isolate MI 299, confirming that the *trl* locus was only expressed in isolate MI 212 (Fig. 2b). The 187 bp of fragment *trl*-I was sequenced. A search of public databases was carried out [prior to the release of the genome sequence of *H. pylori* strain 26695 (Tomb *et al.*, 1997)] but no significant similarities with known genes or gene products were found.

To further characterize the *trl* locus, Southern blot analysis of *Hind*III digests of genomic DNA from isolates MI 212 and MI 299 was carried out using DIG-labelled fragment *trl*-I DNA as the probe. This analysis located the *trl* locus on a *Hind*III-digested genomic DNA fragment of approximately 3 kbp for isolate MI 212 (Fig. 2d). No hybridization signal was observed for isolate MI 299, suggesting that not only was the *trl* locus not expressed in isolate MI 299, but also that homologous DNA was not present in the genome of isolate MI 299.

Fig. 2. Northern and Southern blot analysis. (a) Ethidium-bromide-stained agarose/formaldehyde gel with total RNA from isolates MI 212 and MI 299. Lanes: 1, isolate MI 212; 2, isolate MI 299. The positions of the 23S, 16S and 5S *H. pylori* rRNA are labelled. (b) Northern blot analysis of the same total RNA as in (a) probed with DIG-labelled *trl*-I DNA. Lanes: 1, isolate MI 212; 2, isolate MI 299. (c) A similar membrane was hybridized with a DIG-labelled probe complementary to the 23S rRNA of *H. pylori* as a control. (d) Southern blot analysis of *Hind*III digests of genomic DNA from isolates MI 212 (lane 1) and MI 299 (lane 2). The membrane was hybridized with DIG-labelled *trl*-I DNA.

(b)

ATTTTAGGCTTTGTTGCCATCAATTTTAAAGCTACCACTCGCTTGCAAGCCTTTTATT - 60
 ATGGCTTATTGTAATAATGCCTTTGAGCATTTTACAGACACTCATTGCCACTATAAAG - 120
 AGAGCAAAGCCACAATCTGTTTAAAGAGAATTGGAAATTGAACAAACCTTGACTT - 180
 TTTATTATTGAGTTTAGATACAATAACAATCGTCTTTTGAATAAAGAGCGCGGAATAGC - 240
 TCAGTGGTAGAGCACGACCTTGCCAAGGTGCGGGCCGCGGGTTCGATCCCCGTTTCCCGC - 300
 TCCATAACTTAATTAGTATTTAAGAGGATTTAAGATTGCGTTTGAAGTTAGCTAGCGTAT - 360
 GCTTgggcGTTTTGATGAGTGGTTGTGCGTCTTCTTCGCCAACTGGTGTCTTATCACCA - 420
 M S G C A S S S P T G A L I T
 TGGTAACGATGCCAGTTTCTGGGAATGATGTGCAATACTCCAAAGAAGGCGTGCGAGTT - 480
 M V T M P V S G N D V Q Y S K E G R A S
 GTTGGAGTGTTTTAGTCTTGTGGCTGCCGGTAATTGTTCCGGTAGAAAAAGCGGCTAAAA - 540
 C W S V F S L V A A G N C S V E K A A K
 GCGGCGGTGTTACCAAGATTAAAAATGGTGAGCCGTGAGACAAACAACCTTTTAGGTATTG - 600
 S G G V T K I K M V S R E T N N F L G I
 TTGGCAATACACCACGATCGTTCAAGGCGACTAGTTTAAATATTAGAGAGCGTAGTTG - 660
 V G K Y T T I V Q G D *
 AATCATCTTTCGTTCCACTCTAGGCTTAAAGCCTGAATTGCCAGGTGGTGAATTGGTA - 720
 AACACAAGGGACTTAAATCCCTCGGTAGCAATACCGTGCCGGTTCAGTCCGGCTTTGG - 780
 GCACCATCGTTGCAATTAACCAAATTAATTAACAATGGTTTGATTTGTTATCTCA - 840
 ATAGACGCACTATTGTTGAATTTAAGGCGACATAACCAAGTGGTAAGGCATGGGTCTGCA - 900
 AAACCTTGATTCCCCGGTTCGAATCCGGGTGTGCGCTCCATTTATTGTAGTCATTTAGGG - 960
 ACTTTTGTAGGACTTTTATGAAATAGCGGGAGATGGCTGAGTGGTTGAAAGCGGCGG - 1020
 TATTGAAAACCGTTGAGGGTCATACCTCCGGGGGTTCGAATCCCTCTCTCCCGGCCACTT - 1080
 GATTAATAATCTTTAAGCGATTTGTTTGGCAAATTCATCTCTCTTTTAAACAAAGAATT - 1140
 TTGTGAATATTGATTGTCTCTTTTAAATGAAATTTAAAGATTAGTTTAAAGGATTTATT - 1200
 CGGTGGGATTGTGACGCTTAAGCCTTATCGTTTCTTTAGCGTTATTTAATGGTGGTTT - 1260
 TCACTAAAAGAGTCGCTCTCTCGTTGTTGTAGGCATTTAGTGAGCGCTGTTTAAATA - 1320
 CATCGTTACACCTTTCTCACTTGTAGAATATTTATCATAAATCACTTCCGTTTTT - 1380
 TACATTTACGAGCCAGAAAAGGGCTTAATTTGAATCTTTCCAACCTCTATGTTTTCGGG - 1440
 GTTT -1445

Fig. 3. *trl* locus. (a) Schematic representation of the *trl* locus and contiguous tRNA genes. The base pair numbering is based on Tomb *et al.* (1997). The filled arrows represent the respective tRNA genes and their direction of transcription. The shaded arrows represent the two possible ORFs for the *trl* locus and their putative directions of transcription. (b) Nucleotide sequence of the *trl* locus and contiguous tRNA genes. The ORF (bp 375–635) of the *trl* locus is underlined in bold. The derived protein sequence is shown directly below the ORF. The transcription start site [G (position bp 230)] is doubly underlined. A putative ribosome-binding site is shown in bold lower-case letters starting 6 bp upstream of the start codon for *trl*. The tRNA genes are shown in bold italics.

Nucleotide sequencing locates the *trl* locus between the tRNA^{Gly} and tRNA^{Leu} genes and reveals two possible ORFs

To obtain further nucleotide sequence information surrounding *trl*-I, inverse-PCR was used as described in

Methods. A 3 kbp PCR product was obtained designated *trl*-II. A total of 1258 bp of nucleotide sequence was obtained from the PCR product *trl*-II. A public database search revealed strong nucleotide sequence identity between this new sequence and tRNA genes from several different organisms. By combining the

Fig. 4. Primer extension analysis. Determination of the transcriptional start site for the *trl* locus using total RNA from isolate MI 212 and primers P2 and P3. The transcriptional start site for the *trl* locus is indicated by the arrow. Sequence reactions (A, C, G, T) were performed with the same oligonucleotide as in the primer extension experiments. The nucleotide sequence surrounding the start site is shown.

original nucleotide sequence data of fragment *trl*-I and the extra 1258 bp from PCR product *trl*-II, the *trl* locus was shown to be located between two tRNA genes, tRNA^{Gly} and tRNA^{Leu} (Fig. 3). This was confirmed by the release of the complete genome sequence of *H. pylori* strain 26695 (Tomb *et al.*, 1997). Nucleotide sequence analysis revealed the possibility of two ORFs for the *trl* locus (Fig. 3a). The first of these ORFs (designated ORF-G; bp 616–322) begins with a GTG start codon and encodes a putative protein of 98 aa (10.8 kDa). The second ORF (designated ORF-A; bp 375–635) is in the opposite orientation to ORF-G, begins with an ATG start codon and encodes a putative protein of 86 aa (9.5 kDa). A search of the public databases revealed that ORF-G is the ORF that is predicted by Tomb *et al.* (1997) as its putative product shows 98% amino acid sequence identity with the hypothetical protein HP0945.

Nucleotide sequence analysis located the binding sites for the arbitrary primer P1 (bp 412–434 and 602–624; Fig. 3b). Each site showed 39% nucleotide sequence identity with P1. Analysis of the nucleotide sequence upstream of both putative ORFs did not reveal any obvious regions corresponding to Shine–Dalgarno consensus ribosome-binding sites (Shine & Dalgarno, 1974). However, one sequence (TGGGC) 6 bp upstream of the ORF-A start codon may be a possible ribosome-binding site due to a transversion mutation from an adenine to a cytosine. A search for motifs within the amino acid sequence of the two putative proteins revealed no identities with known amino acid motifs.

Primer extension reveals that the putative ORF for *trl* begins with an ATG start codon and that the locus is co-transcribed with tRNA^{Gly}

To determine the transcription initiation site of the *trl* locus, primer extension was performed. Two primers were used, P2 and P3, which would determine the

direction of transcription of the *trl* locus. A cDNA product of 261 bp was produced by extension with primer P2 (Fig. 4). There was no product from primer extension with primer P3. This result revealed that the *trl* locus is transcribed in the same direction as the transcript predicted by ORF-A and not ORF-G. Therefore, there is no transcript corresponding to hypothetical protein HP0945 in isolate MI 212. The result also shows that the transcription initiation site for the *trl* locus is located at base G (position bp 230; Fig. 4), the first base of the tRNA^{Gly} gene. The *trl* locus is therefore co-transcribed with tRNA^{Gly}.

Nucleotide sequence analysis of *trl*-negative isolates

In an attempt to identify a possible integration/excision site for the *trl* locus, the region between tRNA^{Gly} and tRNA^{Leu} in *trl*-negative isolates MI 270, MI 299, MI 339, MI 354 and MI 365 was amplified by PCR using primers P5 and P6. A product of 141 bp was obtained for three of the isolates (MI 299, MI 354 and MI 365), while a slightly larger product of 157 bp was obtained for the remaining two (MI 270 and MI 339) (Fig. 5a). The PCR products were sequenced directly using primers P5 and P6.

Analysis of the nucleotide sequences showed three main features. Firstly, the size difference between the PCR products was due to a 16 bp sequence present in isolates MI 270 and MI 339 but absent in the other three isolates (Fig. 5b). This 16 bp sequence is identical in both isolates and possesses a conserved 4 bp direct repeat (AGTT) at both its 5' and 3' ends. The other *trl*-negative isolates also have an AGTT sequence at the 5' boundary with the 16 bp sequence. With the exception of this 16 bp region, the sequences from all five isolates were identical. Secondly, a comparison of the sequences with that of isolate MI 212 confirmed that none of these isolates possessed the *trl* ORF. Thirdly, the sequence data

Fig. 5. Characterization of *trl*-negative isolates. (a) Agarose gel of the amplified products from a PCR using primers P5 and P6 complementary to the tRNA^{Gly} and tRNA^{Leu}. Lanes: 1, isolate MI 212 (*trl*-positive); 2, isolate MI 270; 3, isolate MI 354; 4, isolate MI 339; 5, isolate MI 365; M, 100 bp ladder (BRL). (b) Comparison of the nucleotide sequence from the PCR products obtained from the *trl*-negative isolates in (a) and the same region from isolate MI 299. The doubly underlined numbers above the nucleotide sequence are the base pair numbers with respect to the *trl* locus of MI 212 (Fig. 3b). Dashes indicate gaps that were introduced to allow optimal alignment between the five sequences. The repeat sequences (AGTT) are underlined.

revealed that the nucleotide sequence between tRNA^{Gly} and tRNA^{Leu} in both of the *trl*-negative isolate types (i.e. 37 bp for MI 299, MI 354 and MI 365 and 53 bp for MI 270 and MI 339) revealed no identity to the nucleotide sequence between tRNA^{Gly} and tRNA^{Leu} in isolate MI 212. This suggests that in the *trl*-negative isolates, the *trl* locus has been replaced by (or replaces) this 37 bp/53 bp segment due to a site-specific recombination event involving the nucleotide sequence of the tRNA^{Gly} and tRNA^{Leu} and not the sequences between the tRNAs. Therefore, the existence of *trl*-negative isolates is not due to a loss or gain of a DNA segment from the region between the tRNA^{Gly} and tRNA^{Leu} of *trl*-positive isolates but rather to the replacement of the whole of this tRNA^{Gly} and tRNA^{Leu} region.

The *trl* locus is only present in 50 % of clinical isolates tested

A collection of *H. pylori* clinical isolates was screened for the *trl* locus in order to determine whether there was an association between the presence of the locus and *H.*

pylori-related disease type. Dot blot hybridization of 64 clinical isolates with DIG-labelled *trl*-I DNA gave positive signals for 32 (50%) isolates. No correlation between *trl* status and clinical origin was revealed (data not shown).

DISCUSSION

This study reports the use of the technique of arbitrarily primed PCR in the identification of a locus that is expressed in 50 % of clinical isolates of *H. pylori*. The *trl* locus described shows 98 % nucleotide sequence identity with an ORF encoding the hypothetical protein HP0945 from the recently sequenced genome of *H. pylori* strain 26695 (Tomb *et al.*, 1997). However, primer extension analysis has shown that there is no transcript corresponding to hypothetical protein HP0945 and that the transcript of the *trl* locus is in the opposite orientation to that predicted by Tomb *et al.* (1997). Since the release of the genome sequence of strain 26695, it has become apparent that predictions of putative ORFs followed by assignment of putative roles must be performed with caution. ORF assignment can vary depending on the system used for analysis of the genome sequence (Berg *et al.*, 1997; Saunders *et al.*, 1998). For example, the analysis of the genome sequence by Saunders *et al.* (1998) used a different strategy to that of Tomb *et al.* (1997) and revealed clearly identifiable differences in ORF assignment. The incorrect assignment of ORF-HP0945 confirms these findings.

No correlation was seen between the presence of the *trl* locus and the clinical origin of the isolates. Whether the *trl* transcript is translated into a functional protein remains unclear. There are no obvious amino acid motifs within the putative amino acid sequence. Preliminary investigations using hybrid *trl*-fusion protein constructs in *E. coli* suggest that *trl* is not translated (data not shown) and that therefore the *trl* locus may be an example of transcribed 'junk' DNA.

A number of gene products in other bacteria are associated with tRNAs (Rossi *et al.*, 1981; van Delft *et al.*, 1987; Bösl & Kersten, 1991; Bremaud *et al.*, 1995). For example, the *tufB* operon in *E. coli* and *Stigmatella aurantica* is co-transcribed with four tRNAs which are subsequently removed by processing. This allows for the translation of the *tufB* transcript into a functional protein. Primer extension analysis revealed only one tRNA-*trl* transcript, indicating that the transcript remains unprocessed. Whether this extended tRNA^{Gly}-*trl* transcript affects the functioning of the tRNA is unclear. Tomb *et al.* (1997) have identified two copies of the tRNA^{Gly} gene in *H. pylori* strain 26695 so that if the tRNA^{Gly} which is associated with the *trl* locus is unable to function correctly, such isolates should have a second copy of the tRNA^{Gly} gene to counteract this defect.

The nucleotide sequence characterization of the tRNA^{Gly} and tRNA^{Leu} region in isolate MI 299 confirmed that the *trl* locus was absent. It also revealed that the absence of the *trl* locus in isolate MI 299 is not the

result of an excision or integration event within the region between the tRNA^{Gly} and tRNA^{Leu} in a *trl*-positive isolate but rather due to the complete replacement of this DNA region by a 37 bp/53 bp DNA segment. These data would suggest that an extra-chromosomal source for either the *trl*-positive or *trl*-negative regions exists which can integrate into the bacterial genome via the conserved tRNA sequences. In addition, the identification of two types of *trl*-negative isolates revealed the possibility of another site-specific recombinational event occurring in the region between tRNA^{Gly} and tRNA^{Leu}. It would appear likely that the extra 16 bp sequence identified in isolates MI 339 and MI 270 has been lost in isolates MI 299, MI 354 and MI 365 by a recombinational event involving the direct repeat AGTT sequence. The genome sequence of *H. pylori* strain J99 has been released since the submission of this paper (Alm *et al.*, 1999). Strain J99 is *trl*-negative with a 37 bp region between the tRNA^{Gly} and tRNA^{Leu} identical to that described herein.

The location of the *trl* locus within a tRNA cluster is noteworthy given that tRNA genes have been shown to be the sites of integration for transposons, plasmids and bacteriophages in various bacteria (Reiter *et al.*, 1989; Inouye *et al.*, 1991; Hayashi *et al.*, 1993; McShan *et al.*, 1997; Ratti *et al.*, 1997). Some bacteriophage carry tRNA genes in their genomes which can facilitate site-specific integration via conserved sequences in bacterial genomes (Cheetham & Katz, 1995). Plasmids (Hagblom *et al.*, 1986; Madon *et al.*, 1987; Reiter *et al.*, 1989; Richardson & Park, 1997) and pathogenicity islands (Ritter *et al.*, 1995; Hacker *et al.*, 1997; Hensel *et al.*, 1997; Sullivan & Ronson, 1998) have also been shown to be associated with tRNA genes. The highly conserved nucleotide sequences of the tRNA genes provide stable integration sites and the possibility of integrating into the genomes of a broad range of bacteria. Many of the proteins described in *H. pylori* show stronger amino acid sequence similarities to the corresponding proteins from Gram-positive bacteria and eukaryotes (Berg *et al.*, 1997; Tomb *et al.*, 1997). This would suggest that transfer of genetic information from different phylogenetic groups to *H. pylori* has occurred. Therefore, one possible explanation for the presence of the *trl* locus in only a proportion of *H. pylori* isolates is that the locus was acquired by horizontal transfer. The exact mechanism behind *trl* acquisition remains to be elucidated. Bacteriophage have been described in *H. pylori* but only at the morphological level (Schmid *et al.*, 1990; Heintschel von Heinegg *et al.*, 1993). Molecular studies of *H. pylori* bacteriophage might identify a possible mechanism of horizontal gene transfer in this organism. Plasmids have also been identified and well-characterized in *H. pylori* (Kleanthous *et al.*, 1991; Heuermann & Haas, 1995; Minnis *et al.*, 1995). They, too, could be important elements in the horizontal transfer of acquired genetic elements between strains of *H. pylori*.

In contrast to other reports on the use of arbitrarily primed PCR of RNA to identify differentially expressed genes in prokaryotes (Wong & McClelland, 1994;

Kwaik & Pederson, 1996; Peek *et al.*, 1998; Thies *et al.*, 1998; Yuk *et al.*, 1998), few arbitrarily primed PCR products were seen in the fingerprints produced in the present work. This is most likely due to the fact that total prokaryotic RNA contains large amounts of rRNA species which reduces the efficiency of obtaining arbitrarily primed PCR products (Wong & McClelland, 1994). Indeed, it would seem that the reason that the *trl* locus was identified using this technique is that, as it is co-transcribed with tRNA^{Gly}, it would be a well-represented transcript in a total RNA preparation from a *trl*-positive *H. pylori* isolate. Therefore, the technique as used in this work has not been as sensitive as previously described (Wong & McClelland, 1994; Kwaik & Pederson, 1996; Thies *et al.*, 1998; Yuk *et al.*, 1998).

This problem of abundant structural RNA species in a total RNA preparation could be overcome by using a method that would specifically remove the abundant 23S, 16S and 5S rRNA species. Complementary DNA to these specific rRNA species could be hybridized under suitable conditions giving DNA-rRNA hybrids that could then be removed by either hydroxylapatite chromatography or biotin-streptavidin binding (Li & Gruber, 1996).

The genetic variability of *H. pylori* has been well-documented, although the mechanisms responsible are as yet undefined (Marshall *et al.*, 1998). At the molecular level, the organism's genetic diversity has been shown by extensive point mutations and mosaic differences in conserved genes (Atherton *et al.*, 1995), the presence or absence of particular loci (Xiang *et al.*, 1995; Cao & Cover, 1997) and pathogenicity islands (Censini *et al.*, 1996), and by large-scale chromosomal rearrangements (Taylor *et al.*, 1992; Jiang *et al.*, 1996). The identification of the *trl* locus in only 50% of clinical isolates tested further confirms the genetic diversity in *H. pylori*, as do the differences seen in the nucleotide sequences of the region between the tRNA^{Gly} and tRNA^{Leu} genes in *trl*-negative isolates. Use of an optimized version of arbitrarily primed PCR of RNA in the identification of other such genetic variations may provide a better understanding of the genetic diversity and pathogenicity of *H. pylori*.

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