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Ribosomal RNA gene patterns of *Helicobacter pylori* from surgical patients with healed and recurrent peptic ulcers

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SUMMARY

Fifty-two strains of *Helicobacter pylori* were examined by DNA restriction endonuclease digestion, ribosomal (r)RNA gene probe hybridization and biotyping. Most (49) strains originated from gastric (antral) biopsies of patients before or after elective surgery for duodenal ulcers. Chromosomal DNA *Hin*d III ribopatterns showed 9 strain clusters of which the largest contained 12 strains each with 3 common bands (1·50, 3·45, and 4·26 kb) but which were heterogeneous with respect to biotype and total digest pattern. Isolates from post-operative patients with either healed or recurrent ulcers showed ribopattern heterogeneity and exhibited a similar distribution of *H. pylori* ribopattern types; no single type predominated in any patient group or was more highly associated with recurrent ulcers than with healed ulcers. Multiple isolates from two surgical patients had only minor genomic variations in each set whereas isolates from two brothers had different ribopatterns. We conclude that *Hin*d III ribopatterns in conjunction with total digest patterns might provide the basis for future epidemiological typing studies.

INTRODUCTION

Helicobacter (*Campylobacter*) *pylori*, first isolated in 1982 [1], is accepted as a major cause of chronic gastritis in man and is believed to be a dominant factor in peptic ulcer formation [2]. Although a causal role in ulceration is not yet proven for *H. pylori*, there is increasing evidence that eradication of the organism by treatment with bismuth salts and antibiotics markedly alters the natural history of duodenal ulcer disease resulting in significantly lower 1-year ulcer relapse rates [3]. *H. pylori*-associated gastritis is also of possible importance as a risk factor in the development of gastric cancer [4].

Serologic studies have demonstrated that *H. pylori* is extremely common in the general population worldwide and in most individuals is not associated with any gastric disease, although prevalence of infection increases with age [5]. The possibility that some strains are potentially more virulent than others was

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suggested by toxigenicity and immunoblotting studies of isolates from different patient groups [6, 7] and by animal model studies [8].

The aim of the present study was to investigate genomic heterogeneity amongst strains of *H. pylori* from patients with peptic ulcers including a group that had undergone elective surgery for duodenal ulcers. The effect of surgical procedures on *H. pylori* colonization had not previously been investigated and the clinical aspects of the studies will be reported on separately. In earlier studies we demonstrated that restriction endonuclease digest patterns and ribosomal (r) RNA gene probe patterns are valuable molecular tools for precise characterizing of strains of *H. pylori* [9–12]. Results are presented here to show than *Hin*d III-ribopatterns can be used as a basis for high resolution identification (typing) of strains. The DNA patterns have been used to compare the diversity of isolates of *H. pylori* from different patients and to determine if the persistent post-operation isolates possessed particular genomic features that might distinguish them from isolates from patients with healed ulcers.

MATERIALS AND METHODS

Bacterial strains

Fifty-two strains of *H. pylori* were used. Forty-nine isolates were obtained from antral biopsies of 44 duodenal or gastric ulcer patients admitted to the Groote Schuur Hospital, Cape Town for highly selective vagotomy or truncal vagotomy with antrorectectomy as part of a study on ulcer recurrence after elective surgery. The isolates are listed in Table 1 according to patient or subject group as defined by diagnosis and treatment. Patients were aged between 24 and 54 years and most were male. These strains included multiple sets of four isolates each from two patients. Two further isolates were from brothers aged 40 and 43 years. All other Cape Town isolates were from unrelated patients. Two reference strains (NCTC 11637 and NCTC 11638) originating from Perth, Australia and one clinical strain (A673/90) from Dr C. Holcombe, University of Maidguri, Nigeria, were included for comparison.

Culture and conventional tests

All isolates were grown on Oxoid brain–heart infusion agar (BHI) containing 5% horse blood and supplemented with 1% Isovitalex (BBL Microbiology Systems, Becton Dickinson, Cowley, Oxford, UK). Cultures were incubated for 48 h at 37 °C under microaerobic conditions (5% O_2; 5% CO_2; 2% H_2; 88% N_2) in a Variable Atmosphere Incubator (Don Whitley Scientific Ltd, Shipley, Yorks). All strains were transported on blood agar plates in a microaerobic atmosphere. Stocks of strains were preserved in 10% v/v glycerol in Nutrient Broth No. 2 (Oxoid; CM 67) over liquid nitrogen.

The clinical strains were examined by several conventional bacteriological tests (Gram-stain, catalase, oxidase and urease) by methods described previously [13, 14]. To test for motility, strains were grown on slopes of BHI blood agar with added Isovitalex as described above. The liquid phase culture at the base of the agar slope was removed at 2 days and bacterial motility was determined by a standard method [13]. Cultures that were weakly motile or non-motile were

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Table 1. *Sources of* Helicobacter pylori *studied*

Patient group	Clinical details	Number of strains (patients)
A	Ulcer, pre-surgical biopsy	9 (8)
B	Healed ulcer, post-surgical biopsy	26 (22)
C	Recurrent ulcer, post-surgical biopsy	14* (11)
D	Other (ulcer healed spontaneously/treatment unknown)	3* (3)

* One gastric ulcer, all others were duodenal ulcers.

retested. Biotype was determined on the basis of preformed enzyme production in API Zym kits as described previously [15].

Chromosomal DNA extraction

Chromosomal DNA was extracted and rapidly purified using the guanidium thiocyanate method [16]. The concentration and purity of the DNA sample were determined by absorbance readings at 230, 260 and 280 nm.

DNA digestion and electrophoresis

The DNA (5 μg) was digested with the restriction endonucleases *Hae* III and *Hin*d III (*c*. 1 unit per μg DNA) for 4 h at 37 °C in the buffer recommended by the manufacturers (Northumbria Biologicals Ltd, Cramlington, Northumbria). The digested DNA was electrophoresed at 30 V for 16 h in horizontal 0·8% (wt/vol) agarose (Gibco-BRL Ltd, Paisley, UK: ultrapure, electrophoresis grade) gel in a buffer containing 89 mM Tris, 89 mM boric acid, 2 mM disodium EDTA (pH 8·3). After electrophoresis, the gels were stained in ethidium bromide (1 μg per ml) and photographed for a permanent record.

DNA transfer

After photography, the DNA in the gel was transferred to Hybond-N membrane (0·45 μm pore size: Amersham International) by vacuum-assisted transfer (Vacu-Gene XL; Pharmacia-LKB), involving depurination by treatment with 0·25 N-HCl for 20 min, denaturation in 0·5 M-NaOH–1·5 M-NaCl for 20 min and neutralization in 0·5 M Tris–HCl–1·5 M-NaCl–1 mM disodium EDTA (pH 7·2) for 1 h. The membranes were washed once in 2 × SSC, air dried and baked at 80 °C for 2 h.

Preparation of biotinylated probe cDNA

The biotinylated copy (c) DNA probe was prepared from 1 μg 16+23S rRNA from *H. pylori* NCTC 11638 using Moloney mouse leukaemia virus reverse transcriptase (Gibco-BRL Ltd), and was biotinylated by the incorporation of biotin-16-dUTP (Gibco-BRL Ltd) according to previously described methods [17].

Hybridization

Prehybridization (42 °C for 3–4 h) and hybridization (42 °C for 18 h) were carried out exactly as described previously [18]. The hybridization reactions were visualized colorimetrically with the BluGene (Gibco-BRL Ltd) non-radioactive

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Table 2. *DNA band size windows used for numerical analysis of* Hind *III ribopatterns*

Band window (locus)	Size range (kb)	Mean ± SD (kb)	N*	Frequency
1	1·4–1·6	1·50 ± 0·04	54	1·00
2	1·8–2·0	1·99 ± 0·03	3	0·05
3	2·8–3·1	2·85	1	0·02
4	3·2–3·6	3·45 ± 0·11	45	0·83
5	3·7–3·9	3·78 ± 0·08	18	0·33
6	4·0–4·5	4·29 ± 0·18	21	0·39
7	4·6–5·0	4·80 ± 0·13	10	0·19
8	5·1–6·0	5·39 ± 0·16	12	0·22
9	> 6·1	7·14 ± 1·44	4	0·07

* N, number of strains with ribopatterns containing the band.

nucleic acid detection system, which contained streptavidin–alkaline phosphatase conjugate and dyes, as recommended by the manufacturer.

Band size estimation

DNA band sizes in the Southern blot hybridization patterns were calculated from migration distances either by the DNA SIZE program as described previously [18], or by an automated gel reader and analysis system (IBI, New Haven, Conn., USA). Biotinylated lambda phage (Gibco-BRL Ltd) digested with *Hin*d III was used to provide the size markers.

Computation of strain similarities

The blot hybridization patterns were screened for bands within nine different size ranges (see Table 2) that were defined by visual inspection, and positive (presence) and negative (absence) results were coded as 1 and 0 respectively. Bands of faint intensity were excluded. Double bands falling within a given range were scored as a single band. Computed similarities among strains were estimated by means of the Dice coefficient (negative matches excluded) and clustering of strains was based on the unweighted pair group method [19]. *H. pylori* NCTC 11637 was included as a reference on each gel.

RESULTS

Bacteriological characteristics

The strains of *H. pylori* were all Gram-negative, microaerobic, rod-shaped bacteria that produced catalase, oxidase, urease, alkaline phosphatase and acid phosphatase. Most strains (see Table 3) were API Zym biotype II (produced leucine arylamidase and phosphohydrolase but not esterase) except for the nine strains that were biotype III (similar to biotype II but lacking phosphohydrolase activity). Cultures of most strains contained some highly curved cells and in eight strains as many as 80% of cells in the microscopic fields observed were highly curved. Marked differences between strains were observed in the degree of motility, with 41% of strains being non-motile. Only 14 of the motile strains had more than 50% of highly actively motile cells whereas most motile strains had only the occasional actively motile cell and these were recorded as weakly motile.

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The motile and non-motile biotypes were distributed randomly amongst the various DNA ribogroups and types (Table 3).

Chromosomal DNA total digest patterns

Chromosomal DNAs from all the strains of *H. pylori* were digested with *Hin*d III, which cut with a high frequency to give multiple electrophoretic band patterns (> 15 bands). The fragments with sizes of about 5 kb and larger were generally well separated but the smaller sized bands were less well resolved (Fig. 1*a*). Visual comparisons of the total *Hin*d III digest patterns revealed clear differences between strains with the exception of the following five pairs of strains which had identical patterns: A677/90 and A678/90 (same patient); A681/90 and A683/90 (same patient); A688/90 and A689/90 (different patients); A692/90 and A693/90 (different patients), and A722/90 and A723/90 (different patients). Generally these total digest patterns were too complex and bands insufficiently resolved for further detailed analysis.

Digestion with *Hae* III also yielded a multiple electrophoretic band pattern for DNA from 36 strains (Fig. 1b) but DNA from the remaining 17 strains (32%) were not digested (Table 3). The similarities and differences between strains observed in the *Hin*d III digests were also reflected in the *Hae* III digests. The *Hae* III digests were not used here for ribotyping because digests were not obtained for every strain and were therefore not suitable for constructing a complete strain database of ribopatterns.

Ribosomal RNA gene patterns

The 16+23S rRNA gene probe was hybridized with *Hin*d III-digested chromosomal DNA from all strains and the resultant *Hin*d III-ribopatterns are illustrated in Fig. 2. Each strain pattern comprised between one and five strongly stained bands with sizes between 1·4 and 9·7 kb, and in some patterns several intermediate or weakly stained bands were evident. Six strains (A681/82, A682/90, A690/90, A719/90, A720/90, and A721/90) had distinctive multiband patterns of five or more bands of different intensities, which were attributed to partial digestion by *Hin*d III. The commonest bands were 1·5 kb (present in all strains) and 3·5 kb (present in 45 strains) whereas all other bands were each present in 21 or fewer strains. A 2·85 kb band was present only in A715/90.

Visual comparison of the *Hin*d III-ribopatterns revealed that a number of strains had identical patterns but most patterns were different. The results from these ribopatterns, yielding nine unique bands of strong or intermediate intensity (Table 2), were used as the basis of a numerical analysis to determine the similarities between all 52 strains, and the dendrogram obtained is illustrated in Fig. 3. At the 55% similarity level (selected to define broad clusters) in the dendrogram, four groups of strains, comprised as follows, could be delineated.

Group R1 contained 44 strains of which all except one (A696/90) possessed a *Hin*d III ribopattern containing the 1·5 and 3·5 kb doublet.

Group R2 contained four strains that possessed a triplet in which the first band was 1·5 kb, the second band was either 3·8, 4·3 or 4·8 kb, and the third band was either 5·3 or 6·6 kb.

Group R3 contained four strains that possessed 1·5 and 5·3 kb bands.

Group R4 contained one strain that possessed 1·5 and 2·9 kb bands.

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Table 3. *Clinical and bacteriological characteristics of* H. pylori *strains in the various DNA groups**

Ribopattern group-type† (*Hind* III)	Strain number	Patient's sex	Diagnosis group	Motility	API Zym biotype	DNA cut by *Hae* III
R1–1	A673/90	nd	nd	(+)	II	+
	A676/90	M(B1)	B	–	II	+
	A680/90	M(P2)	B	+	III	+
	A692/90	M	B	–	II	+
	A693/90	M	A	–	II	+
	A707/90	F	D	–	II	+
	A708/90	M	C	+	II	+
	A725/90	M	A	(+)	II	+
R1–2	A677/90	M(P2)	B	(+)	II	+
	A678/90	M(P2)	B	+	II	+
R1–3	A684/90	M(P1)	A	(+)	III	–
	A688/90	M	A	(+)	II	+
	A689/90	M	B	–	II	+
R1–4	A675/90	M(B2)	C	–	II	+
	A679/90	M(P2)	B	–	II	+
	A691/90	M	A	+	III	–
	A698/90	F	B	(+)	II	–
	A709/90	F	B	+	II	–
	A717/90	M	B	(+)	III	+
	A724/90	M	A	(+)	II	–
R1–5	A683/90	M(P1)	A	+	III	+
	A685/90	M	A	–	II	–
	A694/90	M	C	–	III	–
	A697/90	M	C	+	II	+
	A699/90	M	B	(+)	II	+
	A700/90	M	C	(+)	III	+
	A704/90	M	C	+	II	–
	A705/90	M	B	–	II	–
	A716/90	M	B	–	II	–
	A719/90	M	B	(+)	II	–
	A722/90	M	B	(+)	II	+
	A723/90	M	B	–	II	+
R1–6	A711/90	M	B	+	II	+
	A712/90	M	A	+	II	+
R1–7	NCTC 11637	nd	nd	+	II	+
	A703/90	M	C	–	II	+
	A706/90	F	B	–	II	+
R2–1	A686/90	F	D	(+)	II	+
	A721/90	M	B	+	II	+
R3–1	A687/90	M	C	+	III	–
	A713/90	M	B	(+)	II	+
Others (type not designated)						
R1	A720/90	F	B	(+)	II	+
	A714/90	M	D	–	II	–
	A696/90	M	C	(+)	II	–
	A710/90	F	C	+	II	+
	A682/90	M(P1)	B	–	II	–
	A681/90	M(P1)	B	(+)	II	+

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Table 3. (*cont.*)

Ribopattern group-type† (*Hind* III)	Strain number	Patient's sex	Diagnosis group	Motility	API Zym biotype	DNA cut by *Hae* III
	A718/90		B	−	II	+
R2	A202/90	F	B	−	II	−
	A695/90	M	C	(+)	II	+
R3	A201/90	M	C	−	II	−
	A690/90	M	B	−	II	+
R4	A715/90	F	E	(+)	II	+

* Symbols used: M, male; F, female; nd, no details; +, motile; (+), weak motility; −, non-motile. For definition of diagnosis groups, see Table 1. P1, multiple isolate set patient 1; P2, multiple isolate set patient 2; B1, B2 brothers.

† Group defined at 55% S level and type defined at 90% S level in Fig. 4.

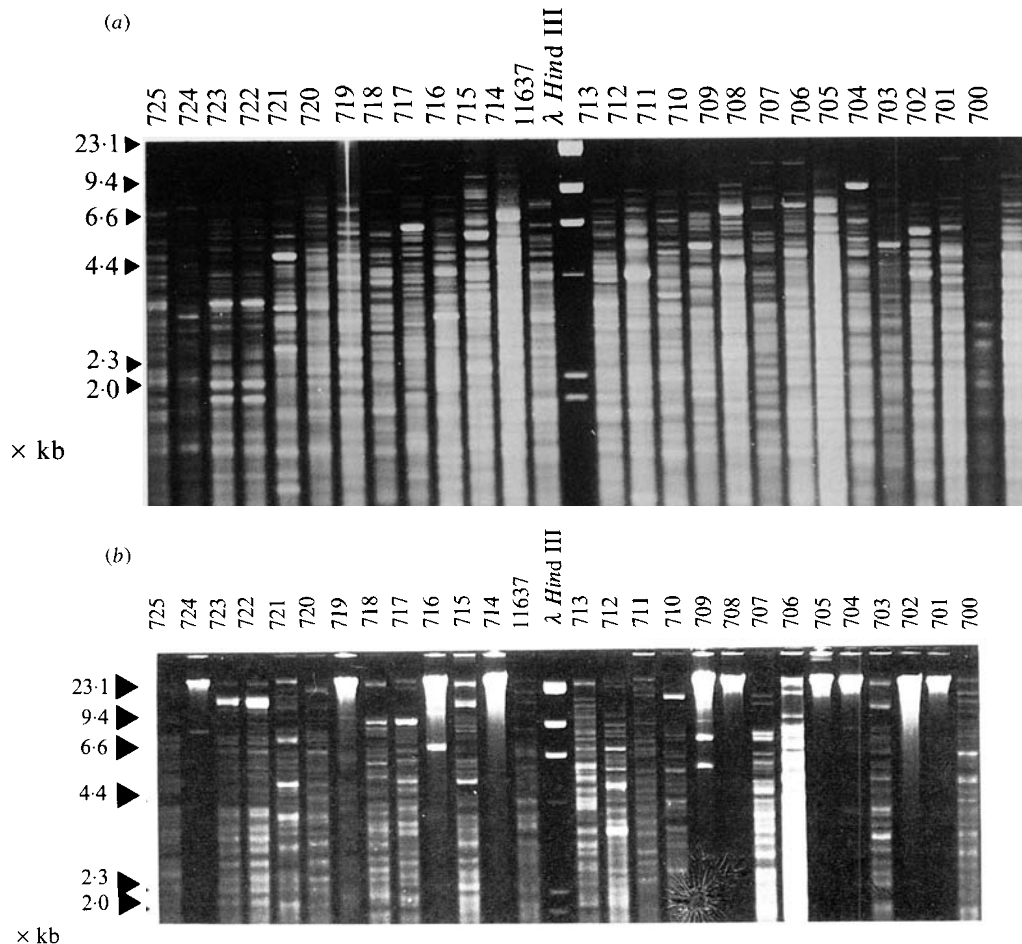

Fig. 1. Agarose gel electrophoresis of *Hind* III (a) and *Hae* III (b) digest fragments of chromosomal DNA from *H. pylori*. Sizes indicated are for bacteriophage λ-*Hind* III digests.

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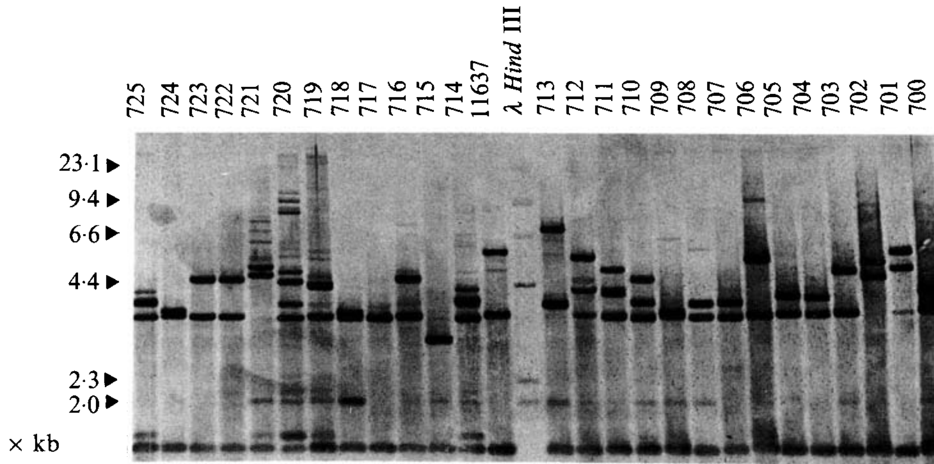

Fig. 2. The rRNA gene patterns (ribopatterns) for *Hind* III digests of chromosomal DNA (see Fig. 1) probed with biotinylated cDNA from 16+23S rRNA from *H. pylori* NCTC 11638.

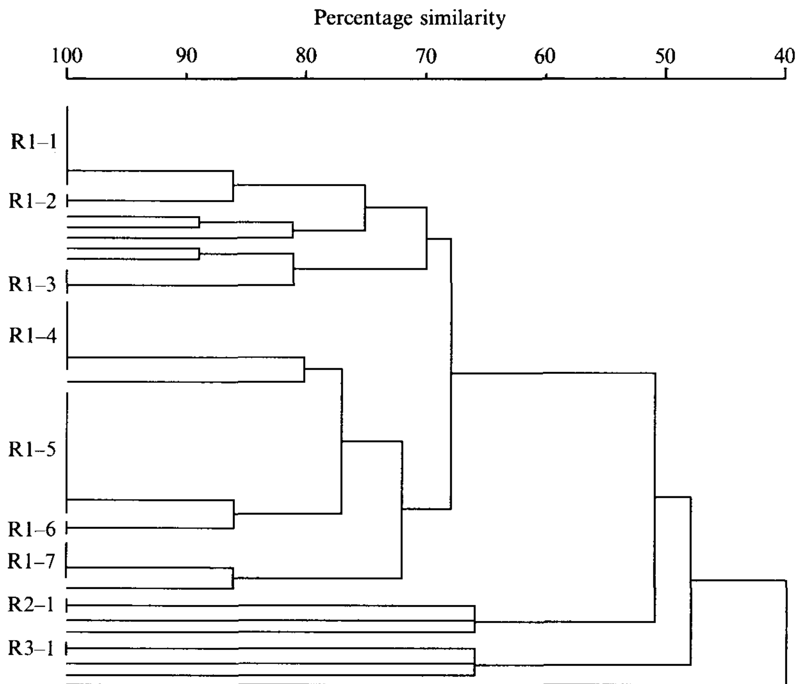

Fig. 3. Dendrogram of a cluster analysis based on the *Hind* III-ribopatterns of *H. pylori* isolates (vertical axis). The numbers on the horizontal axis indicate the percentage similarities as determined by the Dice coefficient.

At the 90% similarity level (selected to define types), nine clusters of two or more strains were formed (Table 3). Most strains were in Group R1 and they had the characteristic DNA bands listed in Table 4. A further 12 strains did not cluster at this level and were not given type designations. *H. pylori* NCTC 11637 was

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Table 4. *Sizes of DNA bands in* Hind *III-ribopatterns of* H. pylori *in strain clusters obtained in numerical analysis at 90% S level*

DNA type	No. strains*	Ribopattern band sizes (kb)
R1–1	8	1·50 ± 0·04, 3·43 ± 0·09, 3·79 ± 0·11
R1–2	2	1·50 ± 0·02, 2·01 ± 0·0, 3·63 ± 0·30, 3·79 ± 0·05
R1–3	3	1·54 ± 0·0, 3·53 ± 0·04, 4·84 ± 0·14
R1–4	7	1·52 ± 0·03, 3·41 ± 0·14
R1–5	12	1·50 ± 0·04, 3·45 ± 0·15, 4·26 ± 0·19
R1–6	2	1·54 ± 0·01, 3·53 ± 0·02, 4·23 ± 0·02, 5·26 ± 0·29
R1–7	3†	1·49 ± 0·04, 3·49 ± 0·08, 5·49 ± 0·22
R2–1	2	1·50 ± 0·04, 4·47 ± 0·04, 4·86 ± 0·16
R3–1	2	1·52 ± 0·01, 3·73 ± 0·01, 6·60 ± 0·51

* See Table 3 for strain numbers.

† Two field strains and NCTC 11637 (duplicate results of different analyses).

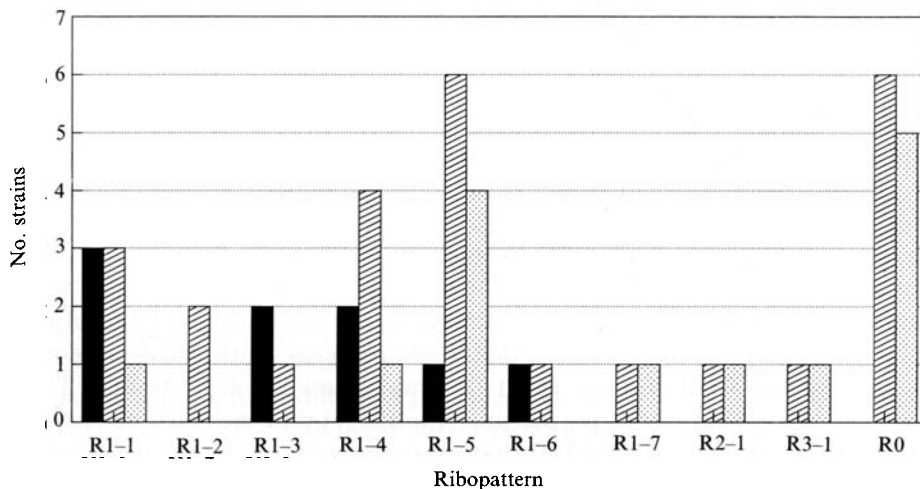

Fig. 4. Distribution of different *H. pylori Hin*d III ribopatterns (R-types) amongst ulcer pre-surgical patients (Group A) ■, post-surgical, ulcer healed patients (Group B) ▨ and post-surgical, recurrent ulcer patients (Group C) ▩. RO, indicates all other groups where types were not designated.

included as control on both gels used for the numerical analysis and the patterns of the two samples were identical (ribopattern R1–7).

Distribution of ribopattern types within patient groups

Fig. 4 illustrates the distribution of the different *H. pylori Hin*d III ribopattern types amongst the pre-operative ulcer patients (group A), whose final outcome was not known, amongst the post-operative healed ulcer patients (group B) and amongst the post-operative recurrent ulcer patients (group C). Three other post-operative strains (group D) were not included in the analysis as the final outcome was not known.

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DISCUSSION

DNA fingerprints based on restriction endonuclease digest patterns provide a sensitive and reproducible method of identifying strains of *H. pylori* [9–12, 20–24]. The band patterns are too complex and strains are too diverse to provide a convenient and reproducible general-purpose typing scheme for *H. pylori* although such patterns are useful for fingerprinting. In contrast, ribopatterns based on rRNA gene restriction patterns are comprised of fewer, more discrete bands and offer greater potential as a means of 'typing' isolates of *H. pylori* [10, 11]. The technique has been applied to an increasing number of bacterial species [25]. In *Campylobacter jejuni*, for example, discrimination with ribopatterns was comparable to that achieved by phage typing and was superior in some respects to standard serotyping methods [26].

In the present study, *Hin*d III-ribopatterns were used as the basis of the numerical analysis because they were relatively simple (fewer than five bands in most strains) and all the DNAs were cut by that endonuclease. In contrast, the disadvantages of using *Hae* III were that about 30% of strain DNAs were not cut at all, and previous studies [27, 28] showed that *Hae* III ribopatterns were more complex and more difficult to analyse although procedures based on the present study are currently being developed. With the exception of a few strains such as A681/90 and A682/90 that contained several weakly stained bands (suggestive of partial cutting), all strains had *Hin*d III ribopatterns that could be readily coded using the nine band loci described. Numerical taxonomic analysis enabled four broad groups of strains to be defined at the 55% S level and at least nine DNA types to be defined at the 90% S level. The majority (44/52) of strains contained a 1·5 and 3·5 kb doublet in common. A number of strains from different patients were observed to have similar *Hind*d III-ribopatterns with the commonest ribotypes being R1–5 (12 strains), R1–1 (8 strains) and R1–4 (7 strains). There were no obvious consistent biotypic features (motility, preformed enzyme activity and ease of DNA cutting with *Hae* III) that were common to strains with the same DNA *Hin*d III ribopattern. Likewise, no associations between *Hin*d III ribopattern and diagnosis group were observed in the series of patients investigated. A comparison of the results on the *Hin*d III ribopatterns (Fig. 4) showed furthermore that there were no marked differences in the distribution of different DNA pattern types between the post-operative healed ulcer patients and the post-operative recurrent ulcer patients. Although the numbers of strains in each ribogroup were small, persistent ulcers did not appear to be associated with any particular *Hin*d III ribopattern type of *H. pylori*. Thirty-seven of the strains (71%) in this study were isolated after surgical treatment for ulcers and in 11 (33%) of the patients, the ulcers were recurrent. The clinical significance of these investigations is outside the context of the present paper and will be reported elsewhere.

Our results confirm data reported previously [10] that some strains of *H. pylori* with the same DNA ribopattern can be present in different patients. Matches in *Hin*d III ribopatterns were observed in the present study between strains from several patients from widely separated geographical locations; e.g. South Africa and Nigeria (see R1–1) and South Africa and Australia (see R1–7) as well as between some South African strains from different patients although the strains

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had different total digest patterns (*Hae*III and *Hin*d III). No further clinical details were available to provide a possible explanation of those results. In contrast, consecutive isolates (in one case these included isolates before and after surgery) from two unrelated patients in South Africa had slightly different *Hin*d III ribopatterns between and within the isolate sets. Also single isolates from two family members (brothers) were different. These *Hin*d III ribopattern variations, albeit minor, were consistent with more obvious differences in the total DNA digest band patterns. Such DNA variations in total restriction digest patterns have been observed previously in isolates from patients in other countries [11, 12, 22, 23] but the present study is the first application of ribopatterns to such strain sets.

The 12 strains of *H. pylori* with ribopattern R1–5 were the largest single set of similar strains identified in this study. They were all from South African male patients but were heterogenous with respect to diagnosis group, total digest patterns, motility and enzyme biotype. The R1–5 ribopattern comprised a triplet of 1·50, 3·45 and 4·26 kb bands. As the typical bacterial rRNA cistron is about 5 kb, our data suggest the genomes of these *H. pylori* contain two copies of the rRNA gene set with a *Hin*d III cutting site within one of the sets. The ribopatterns of some strains of the R1–5 group also contained secondary, weaker intensity bands, which were excluded from the numerical analysis but might be useful for further subtyping. The existence of these secondary bands was unexplained but they may have resulted from partial digestion by *Hin*d III, and have been observed previously in ribopattern studies of strains from Canada [27] and Italy [28]. Our data indicate that such patterns are reproducible and can therefore validly be used for fingerprinting.

We conclude that *Hin*d III ribopatterns are a valuable means of characterizing *H. pylori*. The groups defined by numerical methods provide a broad basis for comparing strains from different patients and could be used in conjunction with *Hin*d III and *Hae* III total digest patterns, which are more strain-specific [11, 27], for use in future molecular epidemiological studies.

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