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Molecular typing of *Helicobacter pylori* isolates from asymptomatic, ulcer and gastritis patients by urease gene polymorphism

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SUMMARY

The gastric-adapted bacterium *Helicobacter pylori* plays an important role in gastritis and ulcer disease, but no phenotypic typing scheme presently exists for this organism. With a view to the development of genotypic typing, we have compared isolates of *H. pylori* from gastritis or ulcer patients with those from subjects exhibiting no disease. Variation was analysed at the urease genes, *ure*A and *ure*CD, by employing PCR-generated probes in genomic Southern blot hybridizations. Whilst *ure*A restriction fragments provided a fourfold subgrouping of strains, *ure*CD fragments were considerably more discriminatory. Twenty-four combined *ure*ACD profiles were generated with *Hin*d III, subdividing the 64 strains into 11 types and 13 single profiles. The most prevalent profile (*UI*) was found in 33% of strains, almost all from gastritis or ulcer patients. On the other hand strains isolated from asymptomatic individuals had the most diverse *ure*ACD profiles. A key finding from this set of isolates was that strains of *H. pylori* associated with general gastroduodenal disease were genetically more homogeneous than strains carried by people without disease symptoms.

INTRODUCTION

The Gram-negative bacterium *Helicobacter pylori* was first isolated in 1982 by Warren and Marshall [1]. Endoscopic and seroprevalence analyses indicate that this organism has a worldwide distribution, and is frequently isolated from asymptomatic persons [2]. By the age of 60 years, 40–60% of adult humans in developed countries show evidence of *H. pylori* infection without necessarily showing signs of disease [3]. The presence of the bacterium has however been causally associated with type B gastritis and is a contributing factor in peptic ulcer disease [4]. It has also been implicated as a contributory factor in certain gastric carcinomas [5, 6].

H. pylori is well adapted to the human stomach as its preferred ecological niche. Its microaerophilic physiology enables it to survive in the reduced oxygen tension of the mucous layer coating the gastric epithelium, whilst its spiral shape and polar flagella enable movement through such a viscous environment [7]. A key feature of *H. pylori* is its possession of a potent membrane-located urease. At

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Table 1. *Isolates of* H. pylori *used in this study*

No.*	Strain designation	Geographical origin	Urease gene bands†		*ure*ACD‡ profile
			*ure*A	*ure*CD	
1	NCTC 11637	Australia	4·5	0·4, 0·5, 0·9, 1·0	—
Isolates from asymptomatic subjects					
2	A816·90	SA	4·5	0·4, 0·9, 1·3	*U*III
3	A822·90	SA	6·0	0·4, 0·9, 5·8	*U*V
4	A824·90	SA	4·5	0·4, 0·9, 1·5	*U*I
5	A827·90	SA	6·0	0·4, 0·9, 5·8	*U*V
6	A834·90	SA	4·5	1·4, 1·5	*U*VIII
7	A838·90	SA	1·3	0·2, 0·4, 0·9	—
8	A840·90	SA	3·4	0·4, 0·9	*U*VI
9	A842·90	SA	4·5	0·4, 0·9, 1·5	*U*I
10	A844·90	SA	4·5	1·4, 1·5	*U*VIII
11	A846·90	SA	6·0	0·2, 1·3, 1·4	—
12	A710·91	SA	3·4	0·4, 0·9	*U*VI
13	A856·90	SA	6·0	0·4, 0·9, 5·8	*U*V
14	A911·90	SA	3·4	0·4, 0·9	*U*VI
15	A711·91	SA	4·5	0·2, 1·2, 1·4	*U*VII
16	A715·91	SA	4·5	0·2, 1·2, 1·4	*U*VII
17	A718·91	SA	4·5	1·2, 1·4	—
18	A720·91	SA	4·5	0·2, 1·2, 1·4	*U*VII
19	A828·90	SA	6·0	0·4, 0·9, 2·0, 5·8	—
20	A830·90	SA	4·5	1·1, 1·4, 2·5	*U*IX
21	A839·90	SA	4·5	1·1, 1·4, 2·5	*U*IX
22	A721·91	SA	4·5	0·4, 0·9, 1·5, 2·2	—
Isolates from ulcer patients					
23	A725·90	SA	4·5	1·3, 1·4	—
24	A683·90	SA	6·0	1·3, 1·4	—
25	A850·90	SA	4·5	1·5, 2·5	—
26	A851·90	SA	4·5	0·4, 0·9, 1·5	*U*I
27	A854·90	SA	4·3	0·2, 1·3, 1·4	—
28	A686·90	SA	4·5	0·4, 0·9, 1·5	*U*I
29	A675·90	SA	4·5	0·2, 0·4, 0·9, 1·2	*U*IV
30	A637·91	UKL	1·3	0·4, 0·9, 1·5	—
31	A654·91	UKL	4·5	0·4, 0·9, 1·5	*U*I
32	A656·91	UKL	4·5	0·2, 0·4, 0·9, 1·2	*U*IV
33	A658·91	UKL	4·5	0·4, 0·9, 1·5	*U*I
34	A660·91	UKL	4·5	0·2, 0·4, 0·9, 1·3	*U*II
35	A662·91	UKL	4·5	0·2, 0·4, 0·9, 1·3	*U*II
36	A681·91	UKL	4·5	0·2, 0·4, 0·9, 1·3	*U*II
37	A689·91	UKL	4·5	0·4, 0·9, 1·5	*U*I
38	A600·92	UKL	4·5	0·4, 0·9, 1·5	*U*I
39	A712·90	SA	4·5	0·2, 0·4, 0·9, 1·3	*U*II
40	A693·90	SA	6·0	0·4, 0·9, 1·5	—
41	A710·90	SA	4·5	0·4, 0·9, 1·3	*U*III
42	A648·91	UKL	4·5	0·2, 0·4, 0·9, 1·2	*U*IV
43	A651·91	UKL	4·5	0·9, 1·5	—
Isolates from gastritis patients					
44	214	UKM	4·5	0·4, 0·9, 1·5	*U*I
45	217	UKM	4·5	0·4, 0·9, 1·5	*U*I
46	222	UKM	4·5	0·4, 0·9, 1·5	*U*I
47	229	UKM	4·5	0·4, 0·9, 1·5	*U*I
48	231	UKM	4·5	0·4, 0·9, 1·5	*U*I
49	232	UKM	1·3	0·2, 0·4, 0·9, 1·3	*U*XI
50	238	UKM	1·3	0·2, 0·4, 0·9, 1·3	*U*XI

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Table 1. (*cont.*)

No.*	Strain designation	Geographical origin	Urease gene bands† *ure*A	Urease gene bands† *ure*CD	*ure*ACD‡ profile
51	A641·90	Italy	4·5	0·9, 2·0	*U*X
52	A645·90	Italy	4·5	0·4, 0·9, 1·5	*U*I
53	A650·90	Italy	4·5	0·2, 0·4, 0·9, 1·2	*U*IV
54	A653·90	Italy	4·5	0·2, 0·4, 0·9, 1·3	*U*II
55	A736·90	Italy	4·5	0·4, 0·9, 1·5	*U*I
56	A707·89	Italy	4·5	0·4, 0·9, 1·3	*U*III
57	A708·89	Italy	4·5	0·4, 0·9, 1·5	*U*I
58	A709·89	Italy	4·5	0·4, 0·9, 1·5	*U*I
59	A714·89	Italy	4·5	0·4, 0·9, 1·5	*U*I
60	A717·89	Italy	4·5	0·4, 0·9, 1·5	*U*I
61	A719·89	Italy	4·5	0·4, 0·9, 1·5	*U*I
62	236	UKM	4·5	0·4, 0·9, 1·3	*U*III
63	A656·90	Italy	4·5	0·4, 0·9, 1·5	*U*I
64	A711·89	Italy	4·5	0·9, 2·0	*U*X

* Corresponds to track numbers in Figures 1 and 2.
† Sizes of homologous *Hin*d III bands (kbp).
‡ Profiles designated as described in text.
SA, South Africa; UKL, UK (London); UKM, UK (Manchester).

physiological concentrations of urea the enzyme is responsible for neutralization of stomach acid in the local environment of the bacterium [8]. Urease-deficient (*ure*) mutants of *H. pylori* are sensitive to the effects of low pH *in vitro*, and do not exhibit cytotoxicity towards eucaryotic cells. Human gastric cells appear to be especially susceptible to the cytotoxic activity of *H. pylori* urease [9].

The biosynthesis of the structural subunits of urease is encoded by two chromosomal genes, *ure*A and *ure*B whose nucleotide sequence has been reported [10]. They are among four genes which are located in a 4·2 kb DNA region, which confers urease activity when genetically transferred to *Campylobacter jejuni*, a related species which lacks the enzyme. These four genes have been sequenced from *H. pylori* strain 85P [11] and their order in the chromosome is *ure* CDAB. This study examined strains isolated from patients with one of two major disease syndromes (gastroduodenal ulcer and gastritis), and asymptomatic subjects infected by *H. pylori*. Its secondary objective was to characterize restriction fragment length polymorphisms (RFLPs) at the *ure*A and *ure*CD loci, with respect to the development of molecular typing for *H. pylori*.

METHODS

Bacteria and culture conditions

Sixty-three clinical isolates of *H. pylori* from different geographical origins were selected and grouped in three sets on the basis of associated clinical symptomologies: asymptomatic, gastric or duodenal ulcer and gastritis. *H. pylori* NCTC 11637^{T} (T = type strain) was included as a control in all the three sets. The details of the isolates are listed in Table 1. All strains were grown on Brain–Heart Infusion agar (BHI, Oxoid) containing 5% (v/v) horse blood and supplemented with 1% Isovitalex (BBL Microbiology Systems, Becton Dickinson, Cowley,

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Oxford, UK). Cultures were incubated for 48 h at 37 °C under microaerobic gaseous conditions (5% O_2, 5% CO_2, 2% H_2, 88% N_2) in a Variable Atmosphere Incubator (VAIN; Don Whitley Scientific Ltd., Shipley, Yorks, UK). Stocks of all cultures were preserved in 10% v/v glycerol in nutrient broth (Oxoid) over liquid nitrogen.

Extraction of genomic DNA

H. pylori cultures were harvested from a single Petri-dish after 48 h incubation. Cells were washed in 0·5 ml of TE buffer (Tris-EDTA buffer, pH 8·0). Genomic DNA was extracted by the protocol of Wilson [12]. DNA concentrations were quantified by u.v. spectrophotometry.

Preparation of DNA probes by polymerase chain reaction

A 411 bp internal fragment of the *ure*A gene (hereafter termed '*ure*A-411') corresponding to nucleotides [nt] 304 to 714, of the sequence of Clayton and colleagues [10] was amplified by the polymerase chain reaction (PCR) using the primers (5′-GCCAATGGTAAATTAGTT-3′ and 5′-CTCCTTAATTGTTTTTAC-3′) described by Clayton and colleagues [13]. Similarly, a 1·7 kb fragment spanning the *ure*C and *ure*D genes (hereafter termed '*ure*CD-1·7'), and corresponding to nt 521 to 2240 of the sequence of Labigne and co-workers [11], was amplified using the primers 5′-TGGGACTGATGGCGTGAGGG-3′ and 5′-ATCATGACATCAG-CGAAGTTAAAAATGG-3′ [14]. Amplification was carried out from 50 ng of genomic DNA of *H. pylori* NCTC 11637^T. The final reaction mixture of 100 μl contained 10 μl of standard PCR buffer (Boehringer Mannheim), 200 μmol/l deoxynucleotide triphosphate (dNTPs), 0·2 μmol/l of each primer and 2·5 units of *Thermus aquaticus* (Taq) DNA polymerase (Boehringer Mannheim). Samples were overlaid with 100 μl of mineral oil and subjected to one of two thermal cycling conditions. For the *ure*A fragment, denaturation was for 30 s at 94 °C; annealing for 1 min at 48 °C and extension for 1·5 min at 72 °C, and 30 cycles were made. For the *ure*CD fragment, denaturation was for 1 min at 94 °C; annealing for 1 min at 60 °C and extension for 2 min at 72 °C, and 20 cycles were made. For both fragments, the initial step was denaturation for 5 min at 95 °C, and the final step was extension for 5 min at 72 °C. The products were analysed by electrophoresis in 1% NuSieve Agarose (FMC BioProducts), purified by electro-elution, and labelled by random-priming for use as probes [15].

DNA hybridization experiments and data representation

Genomic Southern blots were prepared in a standard format. *H. pylori* NCTC 11637^T was included as a control in each of the three sets of 21 DNAs, from asymptomatic subjects, ulcer patients, and gastritis patients, as described in Table 1. Ten micrograms of DNA was digested with *Hin*d III, electrophoresed in 0·8% agarose (30 V, 16 h) and Southern blotted on to Hybond-N nylon membrane (Amersham International). The membranes were baked at 80 °C for 2 h, and prehybridized (4 h) and hybridized (16 h) at 42 °C. Hybridization solutions were those previously described [16]. Stringent filter washing conditions (two washes, 20 min each, at 60 °C in 0·16 × SSC/0·1% SDS) were employed. The hybridized probes were detected colorimetrically with the BluGENE (Gibco-BRL Ltd) non-radioactive nucleic acid detection system. Computed similarities among strains were estimated by means of the Dice coefficient (negative matches excluded) and

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clustering of strains was based on the unweighted pair group method (UPGMA) to facilitate the generation and the plotting of a dendrogram [17]. All computations were performed using the DNAGE program, a modification of previously described software [18]. The developed filters were also scanned directly by an Apple scanner into a Macintosh Quadra 950 computer and imaged with Adobe Illustrator software to generate composite figures for each set of strains.

RESULTS

Variation at the ure*A locus*

Genomic DNAs of all strains were digested with *Hin*d III, which was predicted by analysis of the published nucleotide sequences [10, 11] to lack a recognition site within the 411 bp internal fragment of the *ure*A gene of *H. pylori*. In genomic Southern blots, the *ure*A-411 probe hybridized to one *Hin*d III fragment in each of the 64 strains. These *ure*A-411 bands fell into four general groups (Fig. 1A and Table 1). In 77% of the strains, a single *Hin*d III fragment of approximately 4·5 kb was found. In 11% of strains, the single band was sized at 6·0 kb, in 6% it was sized at 1·3 kb, and in the remaining 5% it was sized at 3·4 kb. One strain contained a band sized at 4·3 kb (isolate 27, Fig. 2B). Differences were observed in *ure*A-411 bands, with respect to the origin of the strains. The band sizes are listed in Table 1. Two bands of relatively rare frequency were not found among the strains from gastritis patients – one of 3·4 kb (also not found in strains from ulcer patients) and one of 6·0 kb.

Variation at the ure*CD locus*

The nucleotide sequence of the *ure* gene cluster from *H. pylori* strain 85P [11] indicated that two *Hin*d III sites were found within the 1338 bp sequence of *ure*C, and none within the contiguous 398 bp sequence of the *ure*D gene. The PCR product *ure*CD-1·7 was used to probe genomic Southern blots prepared with *Hin*d III, and consistent with restriction site predictions, 2, 3 or 4 bands were found in all strains. The most common individual band (found in 80% of strains) was sized at 0·9 kb, the second most common band (found in 75% of strains) was sized at 0·4 kb, the third, 1·5 kb in 44% of strains, the fourth, 0·2 kb in 28% of strains and a band of 1·3 kb in 23% of strains (Fig. 1B).

Certain bands were found in combination occurring with frequencies characteristic of the strain sets. For example, a combination of two bands of 0·4 and 0·9 kb was a feature of many strains (Fig. 1B, tracks 2–5, 7–9), being found in 90% of those from gastritis patients and 76% of those from ulcer patients. It was found in only 57% of strains from asymptomatic subjects. In the strains where these bands were absent, higher molecular weight *ure*CD bands were found instead (Fig. 1B, tracks 6, 10, 17, 20).

A combination of three bands, 0·4, 0·9 and 1·5 kb (Fig. 1B, tracks 4, 9), was the most predominant (23/64 strains) of 19 different *ure*CD band profiles, of which 7 were unique to a single strain. The second most common profile, consisting of bands sized at 0·2, 0·4, 0·9 and 1·3 kb, was found in 7/64 strains overall, but was absent from asymptomatic subject strains. Twelve *ure*CD band profiles occurred in two or more strains.

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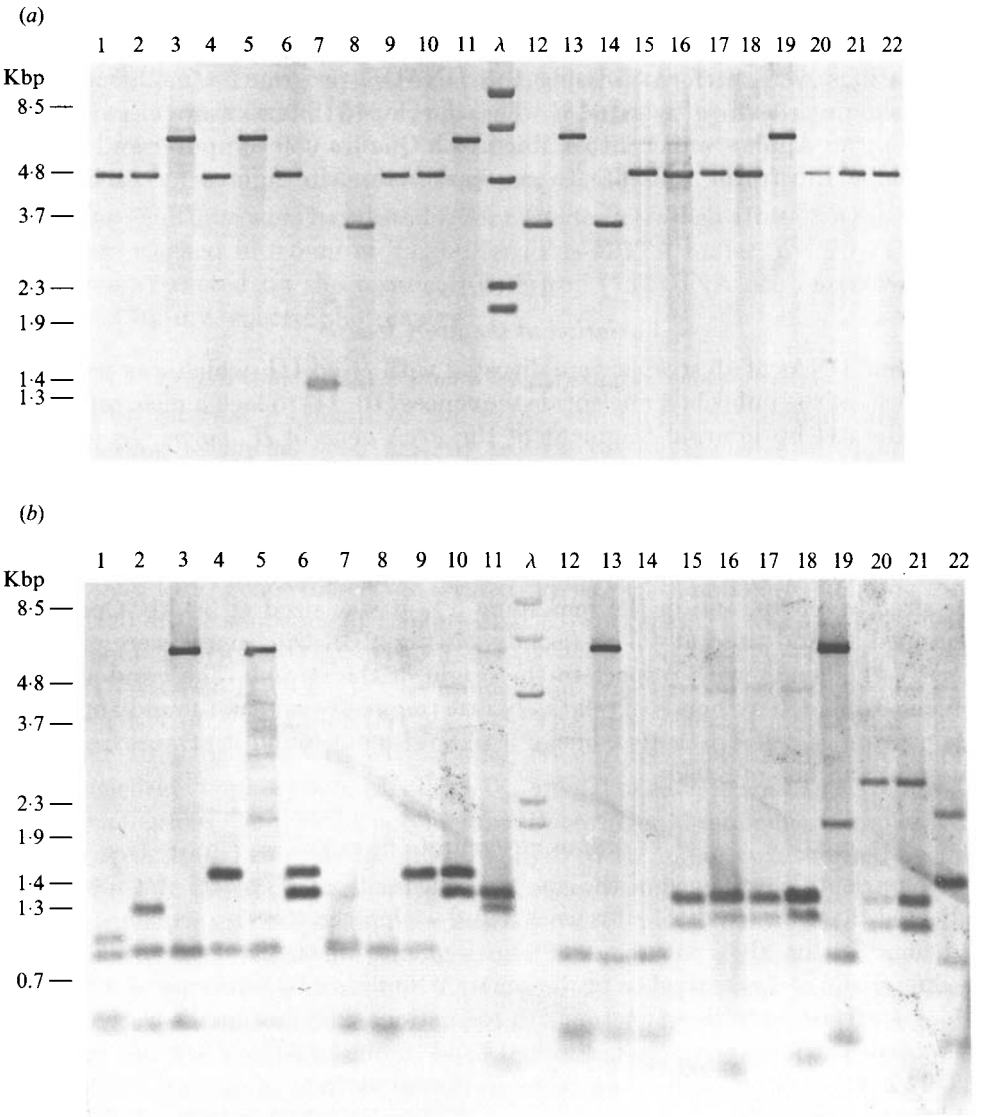

Fig. 1. A: *ure*A-411 bands of strains from asymptomatic subjects. Southern analysis of genomic DNA digested with *Hin*d III, and hybridized with *ure*A-411. Track 1 contained *H. pylori* NCTC 11637^{T}, and tracks 2–22 contained 21 strains from asymptomatic subjects as listed in Table 1. The central track contained *Hin*d III fragments of phage Lambda as molecular weight markers and the sizes of *Bst*E II fragments of phage Lambda are shown at left. B: *ure*CD-1·7 bands of strains from asymptomatic subjects. Southern analysis of genomic DNA digested with *Hin*d III (same format as A above), and hybridized with *ure*CD-1·7. Order and molecular weight markers as in Fig. 1A.

Analysis of the combined ure*ACD* Hin*d III profiles*

Genomic Southern blots probed with *ure*A-411 and *ure*CD-1·7 were scanned directly, imaged by computer and electrophoretic molecular size marker scales were standardized. Data from both probes were superimposed to yield a schematic representation (Fig. 2A, B, C), where *ure*A bands are represented by thick lines

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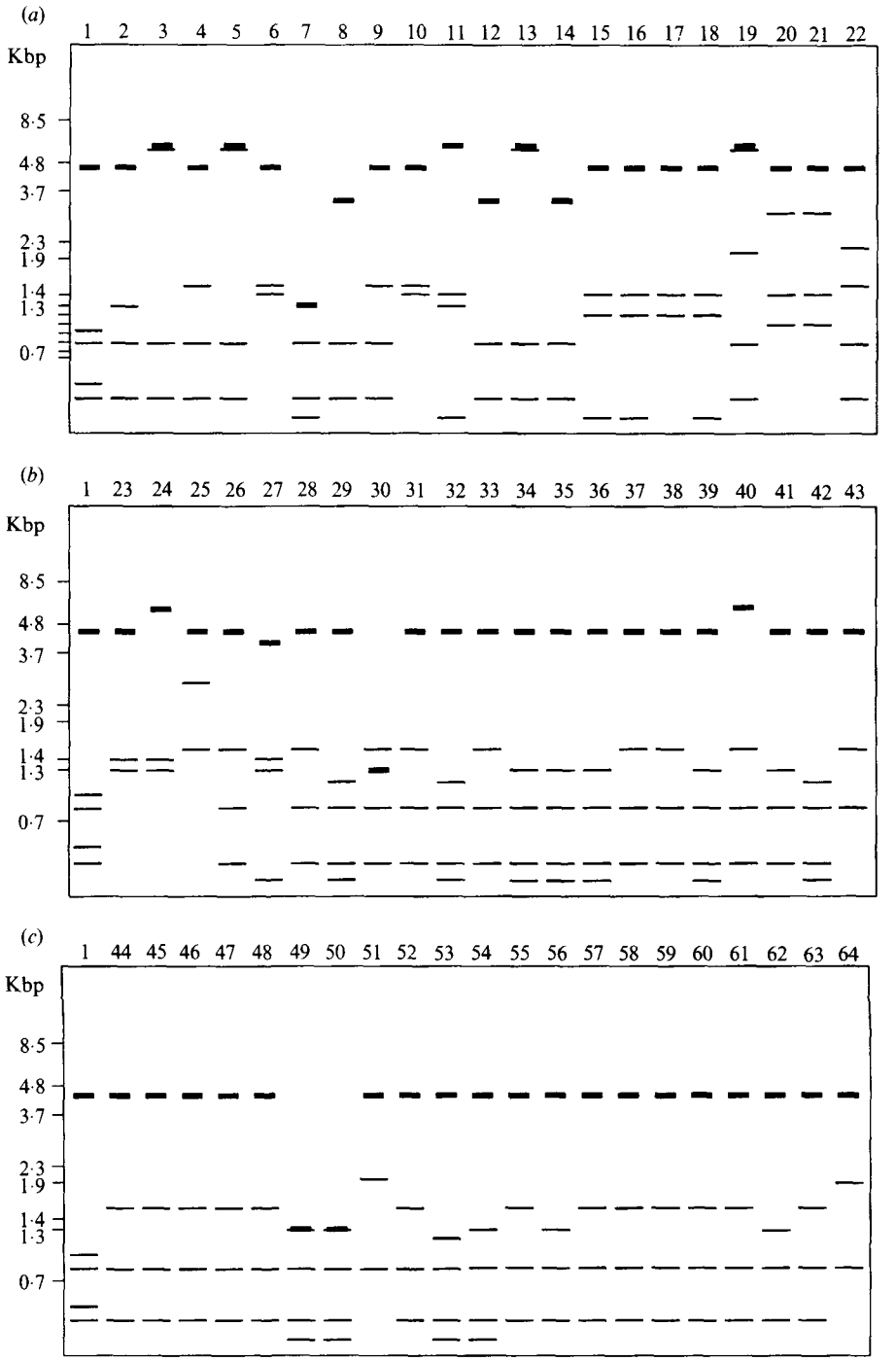

Fig. 2. Diagrammatic representation of *ure*ACD *Hin*d III profiles. *ure*A-411 profiles, represented by thick lines and *ure*CD-1·7 profiles represented by thin lines of all 64 strains listed in Table 1. Panel A: tracks 2–22 contained the strains from asymptomatic subjects; Panel B: tracks 23–43 contained the strains from ulcer patients; Panel C: tracks 44–64 contained the strains from gastritis patients. Track 1 in each panel contained *H. pylori* NCTC 11637^{T}. Molecular weight markers in kilobase pairs are shown on left.

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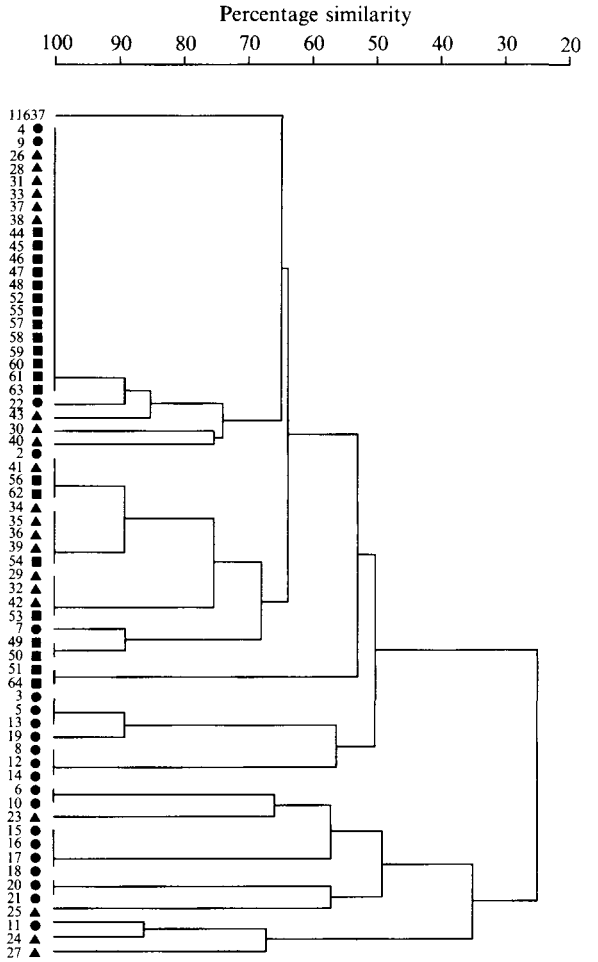

Fig. 3. Dendrogram of the UPGMA cluster analysis of *ure*ACD profiles. Strains (vertical axis) were numbered as presented in Table 1. Horizontal axis indicates percentage similarities of the *Hin*d III profiles, determined by the Dice coefficient. ● represents strains from asymptomatic individuals; ▲ from ulcer patients; ■ from gastritis patients.

and *ure*CD bands by thin lines. A series of 24 *ure*ACD profiles was generated. Eleven of these profiles were found in more than one strain, and the other 13 profiles were found only in single strains in this study. The conserved profiles were designated *U*I to *U*XI, in order to represent their relative prevalence among the 64 *H. pylori* strains. Those found in only one strain were not named (Table 1). The most prevalent profile, *U*I, was found in 21 of the 64 strains. Thirteen of these (62%) were from gastritis patients (Fig. 2C, tracks 44–48. 52), six (28%) from ulcer patients (Fig. 2B, tracks 26, 28, 31), and only two from asymptomatic subjects (Fig. 2A, tracks 4 and 9). The next most prevalent profile, *U*II, was found in 5/64 strains, of which 4 were from ulcer patients (Fig. 2B, tracks 34–36 and 39), and 1 was from a gastritis patient. In order to evaluate the degree of similarity between the different types and profiles, the *ure*ACD profiles were subjected to numerical (UPGMA cluster) analysis and the dendrogram obtained is illustrated in Figure 3.

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DISCUSSION

There is at present no well-defined typing scheme for *H. pylori*, despite its documented role in gastric disease and its high prevalence rates in human populations worldwide [2, 3]. No serotyping or biochemical typing schemes have yet been described and it is clearly important to establish genotypic typing, upon which clinical and epidemiological studies may be based.

A single *Hin*d III site was found [10, 11] at the 5′ end of the *ure*A gene (nt 2743), outside the sequence which constitutes the probe (2961–3372). Therefore the single bands detected in all strains with *ure*A-411 are consistent with these published sequences, and it is likely that the differences in the size of the bands corresponds to variation in the position of a distal *Hin*d III site downstream of *ure*A. In *H. pylori* 85P [11], this lies beyond the *ure*B gene, as was the case for most strains in this study. In the 6% of strains where a 1·3 kb *Hin*d III band was detected, a *Hin*d III site must occur within *ure*B. The *ure*A probe was able to provide a fourfold grouping of all strains in the study. Furthermore, the differences observed between strain sets were notable. Strains from gastritis patients were the most homogeneous, 91% carrying a 4·5 kb *ure*A-411 band and the other 9% carrying a 1·3 kb band. The 4·5 kb band occurred in a high proportion (81%) of strains from ulcer patients, but was rarer in those from asymptomatic subjects, a group which was noticeably heterogeneous with *ure*A-411 (Fig. 1A).

The *ure*CD bands were considerably more discriminatory than the *ure*A bands. A *ure*A-defined group could be split into a number of *ure*CD band profiles – for example strains carrying the 4·5 kb *ure*A band had 13 different *ure*CD band combinations. There were also notable associations: 21/23 strains with the commonest *ure*CD band combination (0·4, 0·9 and 1·5 kb) also had the 4·5 kb *ure*A band. The most effective basis for subtyping was by the combined profiles of the *ure*ACD genes (Fig. 2A, B, C).

The *ure*ACD profile dendrogram (Fig. 3) reveals a significant branch point at 50% similarity. Here, a major group of isolates from asymptomatic subjects with divergent profiles was separated from the rest of the strains. The majority of isolates from ulcer and gastritis patients grouped together in a large subdivision at about a 64% level of similarity. One third of the total number of strains found in this subdivision shared an identical *ure*ACD profile which branched from the nearest neighbouring cluster at a 65% level of similarity.

We have elsewhere shown that strains of *H. pylori* may be individually fingerprinted by ribotyping with a general purpose probe [19] or by restriction site variation at the 16S rRNA (*rrn*) gene loci [16]. On the other hand, a genotypic subtyping scheme should be able to recognize specific types, as is found by *ure*ACD profiling which defined 11 types and 13 potential types among the 64 isolates of this study. This represents an improvement over 'ribotyping' and we conclude that a coherent approach to the molecular epidemiology of *H. pylori* would be to type strains by *ure*ACD profiles, and fingerprint individual isolates by 16S *rrn* profiling.

Lee [20] has postulated that there are no distinct ulcerogenic strains of *H. pylori*, and that comparative studies of isolates from ulcer and non-ulcer patients are unlikely to identify genotypic features of ulcerogenic strains. However, the present study does identify a relative homogeneity among disease-associated

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isolates in contrast to the heterogeneity of those from asymptomatic individuals. This relative homogeneity occurs in the physical map of genes encoding urease, whose activity is the characteristic feature of *H. pylori*, and which is necessary for its colonization of [8, 9] and survival in the host. This similarity suggests that further studies may indeed reveal common genotypic features of strains associated with gastroduodenal disease in general.

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