MICROBIAL CHARACTERISATION

Phenotypic and genotypic properties of the genus *Hafnia*

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The present study characterised 73 *Hafnia alvei* isolates and five *Escherichia* isolates (originally identified as *H. alvei*) isolated from cases of diarrhoeal disease by the International Centre for Diarrhoeal Disease Research Branch (ICDDR) in Bangladesh. Based upon the hydrolysis of arbutin and aesculin and the fermentation of salicin and D-arabinose, four distinct biotypes could be recognised among the 73 *H. alvei* isolates tested; biotype 1 (D-(-)-arabinose-positive only) accounted for 75% of all isolates analysed. Hydrolysis of aglycone compounds such as arbutin, salicin and aesculin appeared to be associated with expression of β -glucosidase activity. ICDDR isolates, when compared with type or reference strains of *H. alvei*, were shown not to belong to the genus *Hafnia* based upon resistance to *Hafnia*-specific bacteriophage 1672, possession of the *phoE* gene, expression of glutamate decarboxylase activity and significant 16S rDNA sequence divergence (~8%) from the type strain, ATCC 13337^T. True *H. alvei* strains, implicated in outbreaks of diarrhoeal disease in Canada, lacked the *eaeA* gene in contrast to ICDDR isolates. Twenty-two *H. alvei* isolates were selected for further study. Based upon partial 16S rDNA sequencing, these 22 isolates fell into two genomic groups (genomospecies), identical to DNA groups previously established by DNA hybridisation studies. Markers such as motility, biotype, or enzymic or carbohydrate fermentation patterns did not correlate totally with DNA grouping, although malonate utilisation appeared to be the single best discriminatory phenotype. The results indicate that the genus *Hafnia* is heterogeneous and there do not appear to be any laboratory data available specifically linking these organisms to gastro-enteritis.

Introduction

The genus *Hafnia* was first recognised by Møeller [1] almost 50 years ago while studying amino acid decarboxylase patterns in members of the family Enterobacteriaceae. Møeller named this new group the 'Hafnia group'. Distinguishing phenotypic features of this taxon included a positive Voges-Proskauer reaction, production of lysine and ornithine decarboxylases, and a negative arginine dihydrolase test. Over the intervening years, the correct taxonomic position of these bacteria has been the subject of considerable controversy and a proposal to include them within the genus *Enterobacter* as either *Ent. alvei*, *Ent. hafniae* or a subspecies of *Ent. aerogenes* has been made [2].

However, DNA hybridisation studies conducted by Steigerwalt and colleagues [3] found that *Ent. hafniae* was not closely related to other *Enterobacter* species (18–22% relatedness). Thus, the genus *Hafnia* as described by Møeller [1] is a legitimate taxon with a single recognised species, *H. alvei* [2]. A second conclusion drawn from the study of Steigerwalt *et al.* [3] was the apparent fact that multiple genomospecies existed within the genus. This observation led to subsequent DNA relatedness studies by Brenner [4] that confirmed the existence of at least two DNA groups within *H. alvei*. Unfortunately, these DNA groups cannot be clearly separated from one another by conventional biochemical tests. In addition to multiple genomic species, several rare biogroups of *H. alvei* have been described [4, 5].

The clinical importance of *H. alvei* is presently unclear, although it is unquestionably an opportunist pathogen in debilitated hosts [6]. Recently, Albert and others

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[7, 8] described a number of '*H. alvei*-like' isolates from the diarrhoeal stools of Bangladeshi children <5 years of age. This link between *H. alvei* and bacterial gastro-enteritis has fuelled a series of case reports documenting *H. alvei* as a gastro-intestinal pathogen [9–11]. One problem with this association is the continued accumulation of phylogenetic, molecular and phenotypic evidence that these '*H. alvei*-like' isolates are either extremely aberrant *H. alvei* isolates or possibly members of the genus *Escherichia* [12, 13].

There have been no large taxonomic studies of *H. alvei* since the report of Greipsson and Priest [14]. In light of the genomic heterogeneity within the nomenclature *H. alvei* and its suggested association with diarrhoeal disease, a large collection of hafniae was characterised on a phenotypic and genotypic basis. These investigations are the basis of this report.

Materials and methods

Bacterial strains and bacteriophage

A total of 73 *H. alvei* isolates from the Microbial Diseases Laboratory was investigated. They were identified to genus and species level by conventional biochemical tests as described previously [15]. Included in these 73 isolates were reference strains ATCC 13337 (DNA group 1), ATCC 29926 (DNA group 1), ATCC 29927 (DNA group 2), all obtained from the American Type Culture Collection (Manassas, VA, USA) and CCUG 429 from the Culture Collection of the University of Göteborg, Sweden. Strains D46-NF and D67-NF, implicated in two outbreaks of diarrhoeal disease attributed to *H. alvei* [9, 16] were provided by A. Ismaili (Department of Microbiology, University of Toronto). Five other '*Hafnia*-like' strains (ICDDR strains 9194, 10457, 10790, 12502, 19982), previously obtained from and reported by Albert *et al.* [7, 8] were included for comparative purposes. The *Hafnia*-specific bacteriophage 1672, originally isolated by Guinée and Valkenburg [17], was obtained from ATCC (ATCC 51873-B1).

Phenotypic studies

H. alvei biotypes were determined on the basis of the hydrolysis of aesculin and arbutin (48 h) and the fermentation of D-arabinose, salicin, lactose, raffinose and adonitol (72 h). Fermentation reactions were determined in extract broth (Acumedia Manufacturers, Baltimore, MD, USA) containing Andrades' indicator. Some *Hafnia* isolates were also tested for glutamate decarboxylase activity by the method of Rice *et al.* [18]. Motility was assessed in motility deeps after 24 h and by determining the zone of migration (mm) in chemotactic agar plates [19]. The presence of pre-formed esterases, aminopeptidases, proteases, phosphatases and glycosidases was evaluated with API ZYM strips according to the manufacturer's instructions [20];

production of α -glucosidase and β -glucosidase was also determined with Wee-Tabs (Key Scientific, Round Rock, TX, USA). Alkylsulphatase, cellulase, chitinase, deoxyribonuclease, elastase, lecithinase, mucinase, protease, ribonuclease, siderophore, stapholysin and tyrosinase activities were determined in plate assays as described previously [20–22]. Susceptibility to *Hafnia*-specific bacteriophage 1672 was performed on nutrient agar quadrant plates with tryptone broth-grown suspensions of test organisms [17].

Genotypic studies, pathogenicity assays and animal studies

Pulsed-field gel electrophoresis (PFGE) of chromosomal DNA and detection of *eaeA*, *stx1*, *stx2* and *phoE* by PCR were performed as described previously [13]. Bacterial adhesins, invasins and cytotoxins were detected with HEp-2 cells [23, 24]. The relative pathogenicity of selected *Hafnia* and *Hafnia*-like isolates was assessed by determining the 50% lethal dose (LD50) for each isolate in Swiss-Webster mice by intraperitoneal injection [25]. Animal studies were conducted under the supervision and approval of the Institutional Animal Care and Use Committee, California Department of Health Services (protocol 00-14). Partial 16S rDNA sequencing of selected isolates was performed with an Applied Biosystems ABI 377 sequencer, and sequence assembly and analysis were accomplished with MicroSeq Analysis software [26]. *Hafnia* isolates were assigned to either DNA group 1 or 2 if there was <0.5% 16S rDNA sequence divergence between the test isolate and the reference strain for a given genomospecies (ATCC 13337, ATCC 29927).

Results

H. alvei biotypes

Barbe described two biotypes of *Hafnia* based upon aesculin and arbutin hydrolysis and the fermentation of D-arabinose and salicin [cited in 4]. Of the 73 *H. alvei* isolates tested, 75% fell into Barbe biotype 1 (Table 1). Biotype 2 isolates, characterised by the hydrolysis of aesculin and arbutin, and the fermentation of salicin, were less common. Two new biotypes, designated Barbe biotypes 3 and 4, were encountered in this survey. Biotype 3 isolates were positive for the hydrolysis of aesculin and arbutin and acid production from the fermentation of salicin and D-arabinose. Biotype 4 isolates lacked activity against these glycosides and failed to ferment salicin and D-arabinose. Together they comprised only 10% of the isolates studied. As arbutin, aesculin and salicin are the most common aglycone compounds (glycosides) that bacteria can utilise, Barbe biotype 2 and 3 isolates were investigated further with other aglycone compounds. All 13 aesculin-positive isolates failed to ferment two other aglycone compounds, amygdalin and α -methyl-D-glucoside, although they produced acid from the

Table 1. Barbe biotypes of *H. alvei*

Biotype	Biochemical reactions				Number (%) of isolates
	Aesculin	Arbutin	Salicin	D-(–)-Arabinose	
1	–	–	–	+	55 (75)
2	+	+	+	–	11 (15)
3*	+	+	+	+	2 (3)
4*	–	–	–	–	5 (7)

*As defined in this report.

fermentation of β -methyl-D-glucoside; eight aesculin-negative *H. alvei* isolates produced similar results. However, in rapid Wee-Tab enzymic assays, 11 of 13 aesculin-positive isolates produced both α - and β -glucosidases; in contrast, only 3 of 8 aesculin-negative *H. alvei* isolates produced β -glucosidase and none elaborated α -glucosidase. A second biotype or taxon of *H. alvei* has been described by Gavini *et al.* [27] on the basis of the rapid fermentation of lactose, raffinose and adonitol. Most isolates (95%) failed to ferment rapidly any of these three carbohydrates but four isolates fermented either lactose or raffinose or both rapidly; two of the latter isolates were from urine; the clinical source of the other two isolates was unknown. None of the 73 *H. alvei* isolates fermented adonitol.

Diarrhoea-associated *H. alvei*

The ICDDR strains of Albert *et al.* [7, 8], previously linked to diarrhoeal disease, were compared to type or reference strains of *H. alvei* (Table 2). ICDDR strains possessed atypical biochemical properties for inclusion in the genus [13], were not susceptible to lysis by *Hafnia*-specific phage 1672 and were *E. coli* *phoE*-positive [13]. They also produced a ladder-like DNA pattern of high and low mol. wt bands by PFGE when cut with *Xba*I. All five ICDDR strains produced glutamate decarboxylase (GAD), a characteristic associated only with *E. coli* and some *Shigella* strains in the family Enterobacteriaceae [18]. In contrast, all three reference strains of *H. alvei* were susceptible to phage 1672, were *phoE*-negative and GAD-negative; they generated only low mol. wt bands on *Xba*I PFGE analysis. When the 16S rDNA of each ICDDR strain was sequenced and compared with the type strain of *H. alvei*, ATCC 13337^T, >7.85% sequence divergence

between each ICDDR strain and ATCC 13337^T was noted. The ICDDR strains shared greatest 16S rDNA sequence similarity to *Escherichia/Shigella* species following a search of the MicroSeq Database (Applied Biosystems, Foster City, CA, USA), further supporting the taxonomic relatedness of these organisms to *Escherichia* species. Strain 10790 was most closely related to *E. coli* and *S. boydii* (1.33% sequence divergence with each species). Several additional differences were observed (Table 3) when the pre-formed enzymic properties of these five ICDDR strains were compared to 13 typical *H. alvei* strains (including ATCC 13337^T, ATCC 29926 and ATCC 29927). *H. alvei* produced higher enzymic activities for both alkaline and acid phosphatase and leucine arylamidase than did ICDDR strains. The latter were enzymically more active against substrates used to detect β -galactosidase and naphthol-AS-BI-phosphohydrolase activities. No ICDDR or *H. alvei* isolate produced preformed C 4 or C 8 esterase, C 14 lipase, valine or cystine arylamidase, trypsin, chymotrypsin, α -galactosidase, β -glucuronidase, β -glucosidase, N-acetyl- β -glucosaminidase, α -mannosidase or α -fucosidase.

The pathogenic properties of ICDDR strains were also compared with two classic *H. alvei* strains that are representative isolates (D46-NF, D67-NF) from two previously identified outbreaks of gastro-enteritis in Newfoundland attributed to O3:H[–] hafniae [9, 16]. All seven strains were negative for Shiga toxin genes by PCR and failed to produce bacterial cytolysins or invade HEP-2 cells. The ICDDR strains produced a weak localised adherence pattern in initial HEP-2 adhesion studies, but this adherence pattern was not reproducible in subsequent trials; neither strain D46-NF

Table 2. Comparison of ICDDR strains to reference cultures of *H. alvei*

Strain	Biochemical test results	Lysis by phage 1672	<i>phoE</i>	GAD	PFGE (<i>Xba</i> I)	16S sequence divergence* (%)
ICDDR 12502	Atypical [†]	–	+	+	Ladder	7.86
ICDDR 10790	Atypical	–	+	+	Ladder	7.95
ICDDR 10457	Atypical	–	+	+	Ladder	7.86
ICDDR 9194	Atypical	–	+	+	Ladder	7.95
ICDDR 19982	Atypical	–	+	+	Ladder	7.95
<i>H. alvei</i> ATCC 13337 ^T	Typical	+	–	–	Low mol. wt bands	0
<i>H. alvei</i> ATCC 29926	Typical	+	–	–	Low mol. wt bands	0.09
<i>H. alvei</i> ATCC 29927	Typical	+	–	–	Low mol. wt bands	0.95

phoE, outer-membrane protein gene; GAD, glutamate decarboxylase; PFGE, pulsed-field gel electrophoresis.

*Sequence divergence relative to ATCC 13337^T.

[†]As previously defined [13].

Table 3. Enzymic activities of ICDDR B and *H. alvei* strains*

Enzyme	Average relative activity for the following groups† (percent of strains)	
	ICDDR B strains (n = 5)	<i>H. alvei</i> (n = 13)
Alkaline phosphatase	1.8 (100)	3.15 (100)
Leucine arylamidase	1.4 (100)	2.85 (100)
Acid phosphatase	2.4 (100)	3.76 (100)
Naphthol-AS-BI-phosphohydrolase	1.2 (100)	0.38 (38)
β -Galactosidase	2.2 (100)	0.92 (62)
α -Glucosidase	0.4 (20)	0

*As determined with the API ZYM strip.

†As previously defined $\Sigma(n_1 \dots n_x)/x$ [26].

nor strain D67-NF adhered to HEp-2 cells in a recognisable pattern. As previously reported, all five ICDDR B strains were *eaeA*-positive [13] but neither strain D46-NF nor strain D67-NF contained this gene. There was no appreciable difference in the mean LD50 values between the ICDDR B (2.18×10^7) and Newfoundland (3.23×10^7) strains.

Enzymic studies and 16S rDNA sequencing

Twenty-two isolates representing different Barbe biotypes and carbohydrate fermentation patterns were selected for further analysis. All 22 isolates produced siderophores but lacked alkylsulphatase, cellulase, chitinase, deoxyribonuclease, elastase, lecithinase, mucinase, protease, ribonuclease, tyrosinase and stapholysin activities. Two DNA groups were demonstrated based upon the results of 16S rDNA sequencing (Table 4). DNA group 1, which included the type strain ATCC 13337^T, consisted of five isolates (<0.20% divergence). DNA group 2, represented by ATCC 29927, consisted of 17 isolates (<0.10% divergence). The two

O3:H⁻ strains associated with different outbreaks of diarrhoeal disease fell into different DNA groups based upon 16S rDNA sequencing. With the exception of Barbe biotype 1 and DNA group 2, Barbe or carbohydrate fermentation patterns were not linked specifically to either DNA group. Brenner [4] has previously suggested that motility at 24 h was the most useful phenotypic marker for recognising each DNA group. Only one (20%) of five DNA group 1 isolates was motile at 24 h both in motility deep and chemotactic plate assays; for DNA group 2, 12 (71%) of 17 isolates were motile. In this study malonate utilisation appeared to be a better marker as all DNA group 1 isolates were positive and only 2 (12%) of 17 DNA group 2 isolates were positive.

Discussion

The results of this study indicate that the genus *Hafnia* as presently defined consists of members with fairly homogeneous phenotypic profiles. Testing a selected

Table 4. Characteristics of selected *H. alvei* strains by DNA group

Strain	DNA group	16S divergence (%) from		Biotype*	Motility (24 h)		Malonate	Other tests
		ATCC 13337	ATCC 29927		tube	plate†		
ATCC 13337 ^T	1	0	0.95	2	+	54	+	Ure ⁺
D46-NF	1	0	0.95	1	–	12	+	
131-004	1	0.09	0.85	2	–	3	(+)	
ATCC 29926	1	0.09	0.85	1	–	11	+	
HA 2	1	0.19	0.85	4	–	5	+	
ATCC 29927	2	0.95	0	1	+	80	–	
CCUG 429	2	0.95	0	1	+	80	–	Raf ⁺
HA 11	2	0.95	0	1	+	80	–	
HA 5	2	0.95	0	1	–	5	–	
D67-NF	2	0.95	0	1	+	68	–	
131-009	2	0.95	0	1	+	80	–	Lac ⁺ , Raf ⁺
HA 1	2	0.95	0	1	–	5	–	
HA 18	2	0.95	0	1	+	80	–	
HA 6	2	0.95	0	1	+	80	+	
HA 8	2	0.95	0	1	–	5	–	
HA 3	2	0.85	0.09	1	–	5	–	
131-017	2	0.85	0.09	1	+	40	–	Lac ⁺ , Raf ⁺
HA 10	2	0.85	0.09	1	–	4	–	
HA 4	2	1.04	0.09	1	+	34	+	
HA 17	2	1.04	0.09	1	+	80	–	Cel ⁺
HA 9	2	1.04	0.09	1	+	80	–	
HA 7	2	1.04	0.09	1	+	78	–	

Ure, urease; Raf, raffinose; Lac, lactose; Cel, cellobiose.

*Barbe biotypes (see Table 1).

†Zone of chemotactic migration (mm).

group of *H. alvei* isolates for the production of extracellular enzymes by plate assays revealed uniformly negative results with the singular exception of siderophore production. Uniform production of a siderophore that is not related to enterobactin or aerobactin has recently been reported by Podschun *et al.* [28] in extra-intestinal isolates of *H. alvei*. Barbe, in 1969, described two biotypes of *H. alvei* based upon arbutin and aesculin hydrolysis and fermentation of salicin and D-arabinose [4]. Results from the present investigation indicate that at least four distinct biotypes can be detected based upon these four tests. Biotype 2, and two new biotypes 3 and 4, constitute only ~25% of the isolates tested. Likewise, unusual *H. alvei* fermentation patterns previously described by Gavini and others [27] account for <10% of all isolates studied, similar to the findings of Greipsson and Priest [14] and Sakazaki and Tamura [2]. Thus, an easy biotyping system to subgroup strains of *H. alvei* implicated in human disease does not exist.

In agreement with previous studies [12, 13], the results of the present investigation provide further evidence that the ICDDR strains of Albert and colleagues [7, 8] that have been implicated in diarrhoeal disease are not members of the genus *Hafnia*. ICDDR strains produced glutamate decarboxylase, an activity only associated with *E. coli* and some *Shigella* strains in the family Enterobacteriaceae [18]. Furthermore, partial 16S rDNA sequence data clearly indicate that all five ICDDR strains are most closely related to *Escherichia/Shigella* and exhibit high sequence divergence (~8%) from the type strain for *H. alvei*, ATCC 13337^T.

True *Hafnia* strains – such as D46-NF and D67-NF, that were epidemiologically associated with outbreaks of gastro-enteritis – lacked the *eaeA* gene, and no other recognisable virulence factors that might play a causal role in bacterial diarrhoea were identified [29]. This indicates that there is no credible laboratory evidence at present to link *H. alvei* to individual case reports [10, 30] or outbreaks [9, 16] of diarrhoeal disease. Currently, *H. alvei* can only be considered a rare opportunist extra-intestinal pathogen in selected patient populations [6].

The results of 16S rDNA sequencing of 22 *H. alvei* isolates support the findings of DNA pairing studies by Steigerwalt *et al.* [3] and Brenner [4] that two genomospecies exist within *H. alvei*. As ATCC 13337^T is the type strain for *H. alvei*, DNA group 1 is by definition *H. alvei sensu stricto*. DNA group 2, which accounted for 17 (77%) of the 22 isolates analysed in this study, represented the majority of clinical isolates. Brenner [4] found that no single test could accurately predict DNA group assignments for individual isolates. The single best discriminatory test in that study was motility at 24 h, with 9% of DNA group 1 isolates and 100% of DNA group 2 isolates positive. However, in the present investigation, motility at 24 h did not yield

as high a discriminatory value (20% versus 71%) as in the study of Brenner [4]. This may be due to the low number of group 1 isolates identified by 16S rDNA sequencing (n = 5) or the deliberate selection of isolates with unusual phenotypic properties used in this study for sequence analysis. What is clear from the results shown in Table 4 is that neither Barbe biotypes nor distinct taxa as defined by Gavini *et al.* [27] based upon unusual sugar fermentation patterns correlates with presently defined DNA groups. Brenner [4] indicated that *Hafnia* DNA groups 1 and 2 could be phenotypically separated from one another by a battery of biochemical tests which included those tests suggested by Barbe plus inulin fermentation, sodium acetate utilisation and urease production. However, exact numbers of strains analysed with phenotype patterns were not provided. As seven or more tests appear to be necessary to define each DNA group, and there appears to be no clinical reason to warrant separation of the two genomospecies, *H. alvei* will continue to exist as a complex for the foreseeable future. However, further studies characterising the two genomospecies within *Hafnia* and their role in human disease seem warranted.

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