Hafnia paralvei sp. nov., formerly known as *Hafnia alvei* hybridization group 2

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It has been shown previously, based largely on DNA–DNA hybridizations and partial 16S rRNA gene sequencing, that *Hafnia alvei* is genotypically heterogeneous and consists of at least two DNA hybridization groups (HGs). In the present study, the taxonomic status of *H. alvei* HGs 1 and 2 was reassessed. A panel of 24 reference strains and isolates previously assigned to one of the two HGs in *H. alvei* was subjected to (GTG)₅-PCR fingerprinting; this resulted in the delineation of two (GTG)₅-PCR clusters in perfect accordance with the respective HG designations. Based on full 16S rRNA gene sequencing of a selection of reference strains, *H. alvei* HGs 1 and 2 showed internal sequence similarities of 99.8 and 99.5 %, respectively. Between the two groups, sequence similarities ranged from 98.8 to 99.1 %. Mean DNA–DNA hybridization values of 74.7–99.9 % were obtained within each of the two HGs, whereas cross-hybridizations between members of *H. alvei* HG 1 (including ATCC 13337^T) and HG 2 revealed only 32.7–48.7 % DNA–DNA hybridization. Previously published and new phenotypic data revealed that a combination of malonate assimilation and β -glucosidase activity enabled correct assignment of *Hafnia* isolates to one of the two HGs. Collectively, taxonomic data from this study confirm that *H. alvei* comprises at least two taxa at the species level, of which HG 1 corresponds to *H. alvei sensu stricto* because it includes the type strain ATCC 13337^T. Strains formerly classified as members of *H. alvei* HG 2 represent a novel species, for which the name *Hafnia paralvei* sp. nov. is proposed; ATCC 29927^T (=CDC 4510-73^T =LMG 24706^T), the former reference strain of *H. alvei* HG 2, is designated the type strain.

Members of the enterobacterial genus *Hafnia* are commonly isolated from the gastrointestinal tract of humans and animals (including mammals, birds, reptiles, fish and insects) and from foods (including meat and dairy products) (Janda & Abbott, 2006). *Hafniae* are associated with a range of animal infections and have been recovered from human clinical specimens during cases of bacteraemia and gastroenteritis and respiratory tract infections. Their status as primary human or animal pathogens, however, remains controversial because these organisms are usually found together with other pathogens and/or opportunists at the site of infection (Janda & Abbott, 2006). At present,

Hafnia alvei is the only recognized species in the genus *Hafnia* (Skerman *et al.*, 1980). However, several DNA–DNA hybridization studies conducted at the Centers for Disease Control and Prevention (CDC) in the 1970s revealed that *H. alvei* is genetically heterogeneous and consists of at least two DNA hybridization groups (HGs) (Brenner, 1978; Steigerwalt *et al.*, 1976). By convention, HG 1 (in some studies also referred to as DNA group 2) represents *H. alvei sensu stricto* as it contains the type strain ATCC 13337^T, whereas HG 2 was originally delineated using CDC 4360-87 (Steigerwalt *et al.*, 1976), a former reference strain that has not been preserved in the CDC culture collection. In later studies (Janda *et al.*, 2002, 2005; Janda & Abbott, 2006), other strains, including ATCC 29927, were proposed as alternative reference strains for HG 2. The division of isolates that resemble *H. alvei* phenotypically into HGs 1 and 2 has meanwhile also been substantiated by partial 16S rRNA gene sequencing (Janda *et al.*, 2002, 2005) and multilocus enzyme electrophoresis (Okada & Gordon, 2003). Although

Abbreviations: HG, hybridization group; rep-PCR, repetitive DNA element-based PCR.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains ATCC 29926, ATCC 29927 and CCUG 429 are FM179942–FM179944, respectively.

DNA–DNA hybridization data between strains of *H. alvei* HG 1 and 2 are available as supplementary material with the online version of this paper.

biochemical discrimination between the two groups appears to be less straightforward, several potentially useful differential tests have been reported (Janda *et al.*, 2005; Okada & Gordon, 2003). In the present study, reference strains of *H. alvei* HG 1 (including ATCC 13337^T) and HG 2 (including ATCC 29927) were used in new DNA–DNA hybridization experiments aimed at substantiating the position of HG 1 as *H. alvei sensu stricto* and at allocating members of HG 2 to a novel species. In addition, the potential of repetitive DNA element-based PCR (rep-PCR) fingerprinting as a rapid tool to distinguish the two taxa genotypically was investigated.

A total of 24 reference strains and isolates of *H. alvei* were included in this study (Fig. 1), all of which were characterized phenotypically using conventional biochemical tests as described previously (Janda *et al.*, 2005) except that malonate, aesculin and salicin tests were read for 48 h and that D-arabinose was read for 96 h. In addition to these previously described tests, β -glucosidase activity was tested using 4-methylumbelliferone fluorescent discs (Key Scientific), which were read at 24 h according to the manufacturer's instructions. Previously, 20 strains were assigned to HG 1 or 2 by partial 16S rRNA gene sequencing (Janda *et al.*, 2002) and included ATCC 13337^T (=LMG 10392^T) and ATCC 29926 (=CDC 5632-72=LMG 24702) of HG 1 and ATCC 29927 (=CDC 4510-73 =LMG 24706) and CCUG 429 (=LMG 24707) of HG 2. The remaining four isolates were isolated from the intestinal tract of Siberian sturgeon (*Acipenser baerii*) at the Laboratory for Aquatic Ecology, Katholieke Universiteit Leuven (Leuven, Belgium), and were allocated phenotypically to *H. alvei* HG 1 or 2 using conventional biochemical tests (Janda *et al.*, 2005). All isolates were grown routinely on nutrient agar (Oxoid) at 28 °C (fish isolates) or 37 °C (clinical isolates) under aerobic conditions.

All isolates were first examined by rep-PCR fingerprinting using the (GTG)₅ primer, i.e. (GTG)₅-PCR. DNA extraction,

(GTG)₅-PCR and data processing were performed essentially as described previously (Gevers *et al.*, 2001). Clustering analysis of (GTG)₅-PCR band patterns grouped the 20 strains previously characterized by partial 16S rRNA gene sequencing into two clusters that were in total agreement with their respective HG designations (Fig. 1). In addition, this grouping also confirmed the allocation of the four sturgeon isolates to their respective HG predicted by conventional biochemical tests. Of these, isolates LMG 24715 and R-25912 (HG 1) displayed identical (GTG)₅-PCR fingerprints, indicating that they probably represent the same genotype. These results demonstrate that (GTG)₅-PCR analysis offers a simple tool to assign genotypically uncharacterized *H. alvei* isolates rapidly to one of the two HGs delineated in the species. Because it allows discrimination at the infraspecific level, the method can also be used to study the epidemiology and environmental distribution of *H. alvei* HGs and to recognize potential replicates among large sets of isolates.

The complete 16S rRNA gene sequence of strain ATCC 13337^T was determined previously (GenBank accession no. M59155); sequences of strains ATCC 29926, ATCC 29927 and CCUG 429 were determined in this study. Genomic DNA was extracted as described by Pitcher *et al.* (1989). Amplification, purification and sequencing were performed according to Vancanneyt *et al.* (2006). Sequences were aligned using CLUSTAL_X version 1.81 and imported in BioNumerics version 5.1 (Applied Maths) for global cluster analysis. Unknown bases were discarded for analysis. Pairwise comparison of strains ATCC 13337^T and ATCC 29926 (HG 1) and strains ATCC 29927 and CCUG 429 (HG 2) revealed 16S rRNA gene sequence similarities of 99.8 and 99.5%, respectively. Between the two groups, sequence similarities ranged from 98.8 to 99.1%. These results confirm previous observations on the basis of partial 16S rRNA gene sequencing indicating that HGs 1

Fig. 1. Dendrogram showing clustering of digitally inverted (GTG)₅-PCR fingerprints of 24 *H. alvei* reference strains and isolates. The dendrogram was obtained by UPGMA using Pearson's product-moment similarity coefficient (expressed as a percentage). Strains ATCC 29926, 829-7-70, 506-1-72, 84A-1383, ATCC 29927 and CCUG 429 (indicated by asterisks) have been deposited in the BCCM/LMG Bacteria Collection (Ghent University, Gent, Belgium; <http://bccm.belspo.be/about/lmg.php>) as LMG 24702–LMG 24707, respectively. HGs were determined previously by partial 16S rRNA gene sequencing (Janda *et al.*, 2002, 2005) except for isolates LMG 24715, LMG 24716, LMG 24717 and R-25912, where HG classification was based on conventional biochemical methods (this study).

and 2 can be differentiated phylogenetically (Janda *et al.*, 2002, 2005). In the latter study, sequence divergences of $\leq 0.09\%$ from ATCC 13337^T and $\leq 0.19\%$ from ATCC 29927 were reported for members of HGs 1 and 2, respectively. Analysis of the full sequences obtained in the present study, however, suggests that the internal divergence range of HG 2 is somewhat broader, spanning up to 0.5%.

The DNA G+C contents of *H. alvei* HG 1 strains ATCC 13337^T and ATCC 29926 and HG 2 strains ATCC 29927 and CCUG 429 were determined using the enzymic degradation method described by Mesbah *et al.* (1989). The nucleoside mixture was separated by HPLC using a Waters SymmetryShield C8 column maintained at 37 °C. The solvent was 0.02 M (NH₄)H₂PO₄ (pH 4.0) with 1.5% acetonitrile. Non-methylated λ -phage DNA (Sigma) was used as the calibration reference. The G+C contents of HG 1 strains ATCC 13337^T and ATCC 29926 were 51.5 and 49.0 mol%, respectively, and those of HG 2 strains ATCC 29927 and CCUG 429 were 49.8 and 49.2 mol%, respectively.

To redetermine the relatedness among members of *H. alvei* HGs 1 and 2, DNA–DNA hybridizations were performed using ATCC 13337^T and ATCC 29926 as reference strains of HG 1 and ATCC 29927 and CCUG 429 as reference strains of HG 2. Total genomic DNA was prepared using a combination of the protocols of Marmur (1961) and Pitcher *et al.* (1989) as described previously (Goris *et al.*, 1998). DNA–DNA hybridizations were performed with biotin-labelled probes in microplate wells according to Ezaki *et al.* (1989) with modifications by Goris *et al.* (1998) using an HTS7000 Bio Assay Reader (Perkin Elmer) for fluorescence measurements. The hybridization temperature was 37 °C, in the presence of 50% formamide. Reciprocal experiments were performed for every pair of strains and standard deviations ranged from 1 to 12%. Hybridizations were repeated three times and means of the resulting values were determined. Reciprocal DNA–DNA hybridization values between *H. alvei* strains ATCC 13337^T and ATCC 29926 (HG 1) and ATCC 29927 and CCUG 429 (HG 2) were in the ranges 81.7–96.6% and 74.7–99.9%, respectively (Supplementary Table S1, available in IJSEM Online). Results of cross-hybridizations between the aforementioned members of *H. alvei* HG 1 and HG 2 ranged from 32.7 to 48.7%. In a comparable range, Steigerwalt *et al.* (1976) reported 51–55% DNA–DNA binding between members of the two groups. Data from the new DNA–DNA hybridizations thus clearly reinforce these previous observations and, in conjunction with other molecular evidence presented in this study and elsewhere, confirm that the species *H. alvei* consists of at least two HGs. Taking into account the proposed threshold of $\geq 70\%$ DNA–DNA binding to allocate bacterial isolates to the same species (Wayne *et al.*, 1987), it can be concluded that both HGs deserve species status. As suggested previously (Janda *et al.*, 2005), HG 1 corresponds to *H. alvei sensu stricto* because it includes the type strain ATCC

13337^T. To accommodate strains formerly classified in *H. alvei* HG 2, a novel species should be created, for which the name *Hafnia paralvei* sp. nov. is proposed. The former reference strain of *H. alvei* HG 2, ATCC 29927, is designated the type strain of this novel species.

Members of *H. alvei sensu stricto* and the newly proposed *H. paralvei* can be easily differentiated by multilocus enzyme electrophoresis (Okada & Gordon, 2003) and by DNA-based methods such as (partial) 16S rRNA gene sequencing (Janda *et al.*, 2002, 2005; this study) and (GTG)₅-PCR fingerprinting (this study; Fig. 1). Due to the considerable phenotypic heterogeneity among *Hafnia* strains, which has led to the description of four biotypes (Janda *et al.*, 2002) and the differentiation between typical (clinical) *H. alvei* and brewery isolates (Farmer *et al.*, 1985), no single biochemical test has so far been reported that is completely discriminatory for *H. alvei* and *H. paralvei*. However, based on a literature survey of several taxonomic studies on the DNA groups of *H. alvei*, Janda & Abbott (2006) reported cumulative data for 12 phenotypic tests that are useful in distinguishing HG 1 (*H. alvei*) and HG 2 (*H. paralvei*). As a result, it was found that the overall phenotypic profile based on malonate assimilation, aesculin hydrolysis and fermentation of D-arabinose and salicin allows correct assignment of *Hafnia* isolates to one of the two taxa in >95% ($P < 0.001$) of cases (Table 1; Janda *et al.*, 2005). Of these tests, however, only malonate assimilation allows clear-cut differentiation between the two HGs. In a subsequent attempt to find a second fully discriminatory character, a set of 13 *Hafnia* strains not included in the present study but which were previously allocated to HG 1 or 2 by 16S rRNA gene sequencing were tested with the MicroLog 1 System (Biolog) (J. M. Janda and S. L. Abbott, unpublished data). Of the 95 substrates, only two proved useful for separating the two HGs within *Hafnia*: malonic acid (malonate), which had already been identified as a differential characteristic, and methyl β -D-glucoside. When the 24 strains included in this study were tested for β -glucosidase using 4-methylumbelliferone fluorescent discs (Key Scientific), this character was positive for all *H. alvei*

Table 1. Phenotypic differentiation of *H. alvei* and *H. paralvei* sp. nov.

Data for malonate utilization, D-arabinose and salicin fermentation and aesculin hydrolysis were taken from Janda *et al.* (2005); data for β -glucosidase were obtained in this study using 4-methylumbelliferone fluorescent discs. +, 90–100% of strains positive; v, 16–89% of strains positive; –, 0–15% of strains positive.

Phenotypic trait	<i>H. alvei</i>	<i>H. paralvei</i>
β -Glucosidase	+	–
Malonate utilization	+	–
D-Arabinose fermentation	–	v
Salicin fermentation	v	–
Aesculin hydrolysis	v	–

strains (11 strains positive after 24 h and one positive after 72 h), but negative for 10 of the 12 *H. paralvei* strains. In combination with the malonate test, β -glucosidase thus allows reliable classification of almost 100 % of the *Hafnia* strains into one of the two species (Table 1).

Description of *Hafnia paralvei* sp. nov.

Hafnia paralvei [par.al've.i. Gr. prep. *para* (also shortened to *par*) besides; L. gen. n. *alvei* of a beehive and also a bacterial epithet; N.L. gen. n. *paralvei* organism similar to *H. alvei*].

This description is based on data reported previously by Farmer (2003), Janda *et al.* (2005), Okada & Gordon (2003) and data from this study. Except where mentioned otherwise, the phenotypic profile of the type strain corresponds to the species description. Gram-negative, oxidase-negative, non-spore-forming, peritrichously flagellated rods. Optimal growth occurs after 24 h at 28–37 °C on nutrient agar and various other non-selective nutrient-rich media. Facultatively anaerobic, non-pigmented, produces nitrate reductase and positive for the enterobacterial common antigen. Positive for lysine and ornithine decarboxylases. Negative for production of indole and H₂S, arginine dihydrolase, phenylalanine deaminase, lipase, DNase and degradation of gelatin, mucin and polypectate. Produces acid from D-glucose, L-arabinose, trehalose, maltose, D-mannitol, D-xylose and glycerol, but not from adonitol, amygdalin, D-arabitol, dulcitol, *myo*-inositol, lactose, melibiose, D-sorbitol, sucrose or methyl α -D-glucoside. Most strains (including the type strain) are positive for the Voges–Proskauer reaction, motility, acid production from D-arabinose and hydrolysis of *p*-nitrophenyl β -galactoside. Strains are negative for either malonate utilization (including the type strain) or β -glucosidase or both. Together with *H. alvei*, the novel species can be distinguished from other enterobacteria based on lysis by the *Hafnia*-specific phage 1672 after overnight incubation at 37 °C. Results for tests proposed to discriminate between *H. alvei* and *H. paralvei* are listed in Table 1. Strains have been isolated from human clinical specimens, mammals, reptiles and freshwater fish.

The type strain is ATCC 29927^T (=CDC 4510-73^T =LMG 24706^T), which was isolated from a human clinical specimen. The DNA G+C content of the type strain is 49.8 mol%.

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