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# Characterization of *Hafnia alvei* by Biochemical Tests, Random Amplified Polymorphic DNA PCR, and Partial Sequencing of 16S rRNA Gene

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***Hafnia alvei* strains which possess the attachment-effacement gene (*eaeA*) may have clinical importance as new diarrhea-causing pathogens and should therefore be differentiated from other *H. alvei* strains. We characterized diarrheal *H. alvei* strains, which were positive in the PCR test for the *eaeA* gene, using biochemical tests not routinely used for identification of members of the family *Enterobacteriaceae*, and compared them with *eaeA*-negative strains isolated from different clinical and nonclinical sources to find characteristics useful for identification. Random amplified polymorphic DNA (RAPD)-PCR and partial sequencing of the 16S rRNA gene were utilized to study the genetic diversity of the isolates. The *eaeA*-positive strains were found to have many characteristic biochemical properties. Negative reactions in the 2-ketogluconate and histidine assimilation tests and a positive reaction in the 3-hydroxybenzoate assimilation test may be useful in routine diagnostics. Nearly identical RAPD-PCR profiles and identical 353-bp fragments of the 16S rRNA genes indicated little genetic diversity among the *eaeA*-positive strains. The low level of homology (92%) in the partial 16S rRNA genes of *eaeA*-positive and -negative *H. alvei* strains raises questions about the taxonomic positioning of *eaeA*-positive *H. alvei*.**

Recent studies have shown that in addition to enterohemorrhagic and enteropathogenic *Escherichia coli* (EHEC and EPEC, respectively) and one biotype of *Citrobacter freundii*, *Hafnia alvei* may also possess the virulence-associated gene *eaeA* (EPEC attaching and effacing) (2, 6, 9, 13, 15). In contrast to most *H. alvei* strains, which are generally not considered pathogens, the *eaeA*-positive *H. alvei* strains may be diarrheagenic. Although the role of the *eaeA* gene is not fully understood, the gene appears to be necessary, but not sufficient, for intimate adherence of the pathogen to epithelial cells and for formation of the characteristic attaching-effacing lesions in the intestinal brush border (9). The *eaeA* gene is the only virulence-associated factor described so far for *H. alvei*. However, we have shown an epidemiological association of *eaeA*-negative *H. alvei* and diarrhea symptoms (13), indicating that other mechanisms may also be involved in the association of *H. alvei* with diarrhea.

In addition to some case reports which implicate *H. alvei* as a new diarrheagenic pathogen (11, 12, 17), more-specific evidence has been demonstrated. In an animal model, *H. alvei* strains isolated from diarrheic children in Bangladesh produced diarrhea and lesions typical of EPEC and gave positive reactions in the fluorescent actin staining test with HEp-2 cells (1, 2, 13). These strains were shown, by DNA hybridization and PCR, to carry a gene homologous to the *eaeA* gene family (2, 13). Recently, Frankel et al. demonstrated that the fusion protein encoded by the *H. alvei eaeA* gene attached to HEp-2 cells with an affinity equal to that of the proteins of EPEC and EHEC (6).

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Differentiating *eaeA*-positive *H. alvei* strains other *H. alvei* strains is important, but it is not routinely done in clinical laboratories. *eaeA*-positive strains give typical reactions in standard biochemical tests used to identify members of the family *Enterobacteriaceae*, e.g., the API 20E commercial biochemical panel (1, 2, 13), and are thus indistinguishable from other *H. alvei* strains. The aim of this study was to characterize diarrhea-causing *eaeA*-positive *H. alvei* strains in comparison with *eaeA*-negative strains to find useful characteristics for the identification of *H. alvei* strains which cause diarrhea through the attaching-effacing mechanism. Useful biochemical reactions were sought by utilizing commercial biochemical identification systems not routinely used for the identification of members of the *Enterobacteriaceae*. Molecular methods, random amplified polymorphic DNA (RAPD)-PCR and partial sequencing of the 16S rRNA gene, were used to estimate the genetic diversity of *H. alvei* strains.

## MATERIALS AND METHODS

**Bacterial strains.** The study included 16 diarrhea-associated *H. alvei* strains isolated in Bangladesh from 16 patients between 1990 and 1993, 43 diarrhea-causing *H. alvei* isolates from 16 Finnish patients, 9 other clinical strains from 9 patients, 42 *H. alvei* isolates from 16 food samples, and 10 *H. alvei* strains from 10 environmental samples, including drinking water, fish farm water, fish intestines, and rabbit intestines. In addition, the following *H. alvei* reference strains were included: ATCC 13337$^T$ (NCTC 8105$^T$), ATCC 29926 (CDC5632-72; DNA hybridization reference strain), and ATCC 29927 (CDC4510-73; DNA hybridization reference strain). *H. alvei* ATCC 29926 and ATCC 13337$^T$ represent hybridization group (HG) 1, and ATCC 29927 belongs to HG 2 (7). *eaeA*-positive bacterial strains *E. coli* E2348/69 (EPEC; O127:H6), obtained from J. P. Nataro (Center for Vaccine Development, University of Maryland School of Medicine, Baltimore), and *E. coli* ATCC 43895 (EHEC) were also included.

**Biochemical reactions.** Biochemical reactions with the commercial API 20E, API rapid ID 32E, and API ID 32GN systems (Biomerieux, Marcy l'Etoile, France) were performed according to the instructions of the manufacturer. Extended incubations (48 h at 37°C and 24 h at 30°C) were tested with three *eaeA*-positive and five *eaeA*-negative strains.

TABLE 1. Results of selected biochemical reactions with the API 20E, rapid ID 32E, and ID 32GN identification systems for *eaeA*-positive and -negative *H. alvei* strains

| Isolate | No. of isolates | % Positive isolates | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | API 20E | | API rapid 32E | | | | | | API ID 32GN | | | | | |
| | | Arginine dihydrolase | Rhamnose fermentation | Arabinose | Malonate | Coumarate | Maltose | Indoxyl-phosphate | Trehalose | L-Alanine assimilation | Histidine assimilation | 3-Hydroxybenzoate assimilation | 2-Ketogluconate assimilation | L-Serine assimilation | L-Proline assimilation |
| Diarrheal *H. alvei* isolates | | | | | | | | | | | | | | | |
| *eaeA* positive | 16 | 86 | 0 | 0 | 0 | 100 | 25 | 0 | 25 | 31 | 0 | 100 | 0 | 31 | 25 |
| *eaeA* negative | 43 | 12 | 84 | 44 | 81 | 26 | 100 | 81 | 98 | 100 | 95 | 0 | 100 | 98 | 98 |
| Nondiarrheal *eaeA*-negative *H. alvei* isolates | | | | | | | | | | | | | | | |
| Clinical | 9 | 0 | 89 | 11 | 44 | 0 | 89 | 33 | 100 | 78 | 89 | 0 | 100 | 89 | 89 |
| Environmental | 10 | 40 | 90 | 10 | 20 | 0 | 90 | 30 | 100 | 100 | 100 | 0 | 100 | 100 | 90 |
| Food originating | 42 | 0 | 90 | 62 | 33 | 10 | 90 | 67 | 98 | 98 | 98 | 0 | 100 | 98 | 95 |
| ATCC strains | 3 | 67 | 100 | 33 | 33 | 67 | 100 | 67 | 100 | 100 | 100 | 0 | 100 | 100 | 100 |
| Total (*eaeA* negative) | 107 | 10 | 88 | 45 | 52 | 16 | 94 | 66 | 97 | 97 | 96 | 0 | 100 | 97 | 95 |
| API profile[a] | | | | | | | | | | | | | | | |
| "*H. alvei* 1"[b] | | 1 | 97 | 71 | 60 | 20 | 90 | 90 | 100 | NA[c] | NA | NA | NA | NA | NA |
| "*H. alvei* 2"[d] | | 1 | 1 | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA |
| "*E. coli* 1"[e] | | 5 | 82 | 87 | 0 | 100 | 99 | 1 | 91 | NA | NA | NA | NA | NA | NA |
| "*E. coli* 2"[f] | | 10 | 35 | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA |

[a] Profiles given by the manufacturer. API ID 32GN profiles are not available.
[b] Used with API 20E only; with API rapid 32E and API ID 32GN, "*H. alvei*". This classification corresponds to "*H. alvei*" of Farmer et al. (5).
[c] NA, not available.
[d] Used with API 20E only. "*H. alvei* 2" corresponds to "*H. alvei* biogroup 1" of Farmer et al. (5).
[e] Used with API 20E and API ID 32GN; with API rapid 32E "*E. coli*."
[f] Used with API 20E and API ID 32GN. "*E. coli* 2" corresponds to "*E. coli*, inactive" of Farmer et al. (5).

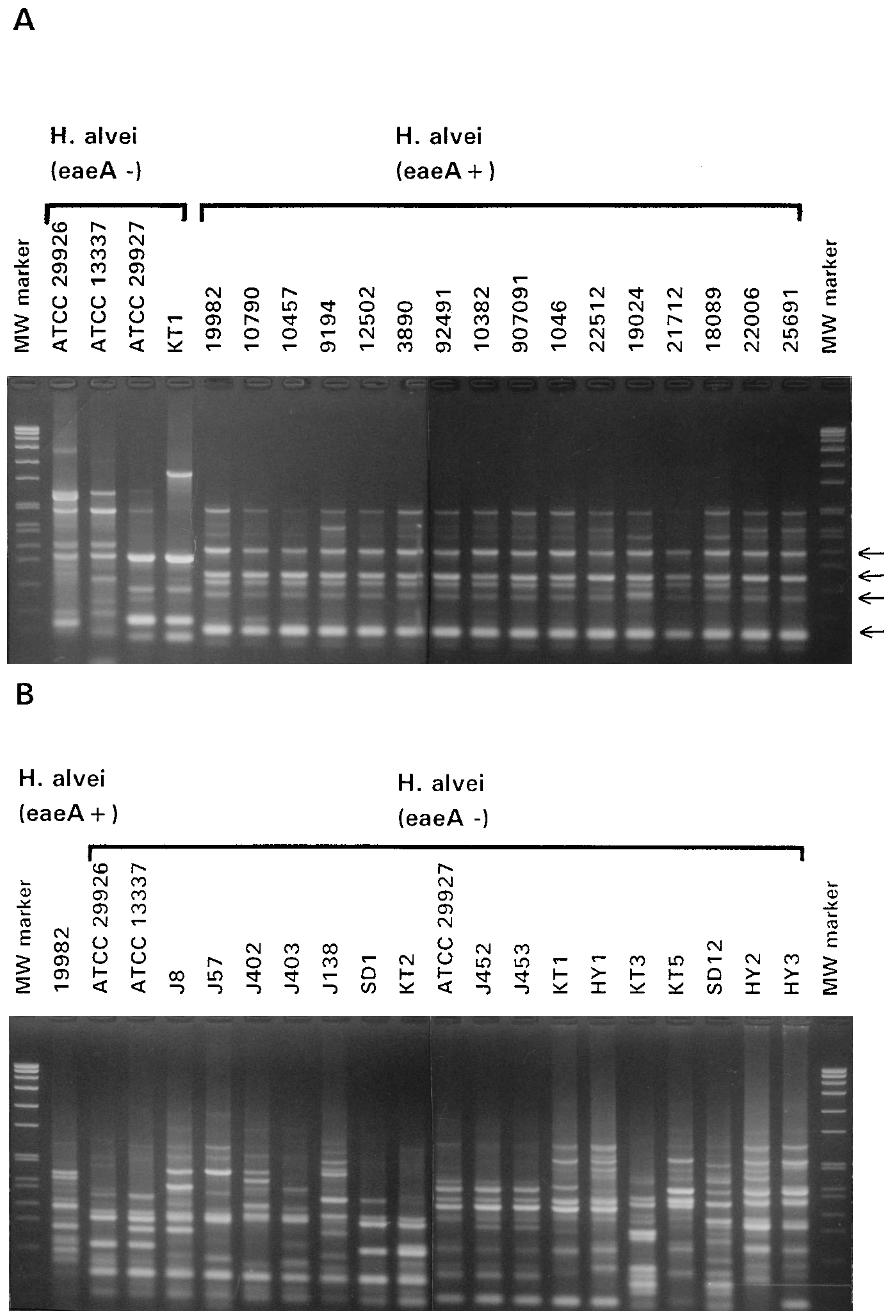


FIG. 1. (A) *eaeA*-positive *H. alvei* strains produce characteristic and homologous profiles in RAPD-PCR analysis using three different primers, as demonstrated here with primer E8b. *H. alvei* ATCC 29926 (DNA reference strain) and ATCC 13337$^T$ represent HG 1, and ATCC 29927 (DNA reference strain) represents HG 2. *H. alvei* KT1 was isolated from a septicemic patient. *eaeA*-positive *H. alvei* strains were isolated from various patients with diarrhea in Bangladesh over several years. The PCR products specific for the *eaeA*-positive strains are indicated (arrows). (B) *eaeA*-positive strains did not show any detectable homology with the 107 *eaeA*-negative *H. alvei* strains when three primers were used, as demonstrated here with primer E7. *eaeA*-negative *H. alvei* strains formed polymorphic RAPD profiles, but two profile types, resembling HG 1 and 2, dominated. Strains SD1, KT3, KT5, and SD12 are diarrheal isolates; strains KT1, KT2, HY1, HY2, and HY3 are from other clinical samples; and strains J8, J57, J138, J402, J403, J452, and J453 are from minced meat. Boehringer Mannheim marker VII was used as a molecular weight (MW) marker.

**PCR methods.** Seventy-eight strains not previously tested for the *eaeA* gene were screened by PCR as described previously (13).

RAPD-PCR analysis of all 123 strains was performed basically as described by Williams et al. (18), with factors affecting reproducibility carefully observed (10, 18). Amplifications were performed with a DNA thermal cycler (PTC-100; MJ Research, Watertown, Mass.) using the following conditions: 45 cycles of 30 s at 94°C, 30 s at 36°C, and 1 min 30 s at 72°C with a 3-min initial denaturation at 94°C and a 5-min final extension at 72°C, with a 25-μl volume containing 25 ng of purified DNA and 0.25 U of Dynazyme DNA polymerase (Finnzymes, Espoo, Finland) in a buffer provided by the manufacturer (containing 3 mM $MgCl_2$, 10 mM Tris-HCl [pH 8.8], 50 mM KCl, 0.1% Triton X-100, 200 μM [each] dATP, dCTP, dGTP, and dTTP, and 0.5 μM primer and overlaid with 1 drop of sterile paraffin oil). Genomic DNAs from *H. alvei* HG 1 (ATCC 29926) and 2 (ATCC 29927) and the type strain (ATCC 13337) were included as controls in each of the experiments. Three primers based on the enterobacterial repetitive intergenic consensus sequence were used: E7 (5′-AGTAAGTGACTGGGGTGAG CG-3′), E8 (5′-ATGTAAGCTCCTGGGGATTCAC-3′), and E8b (5′-ATGTA AGCTCCTGGGGATTCA-3′). The reproducibility of the method was tested by repeating the amplifications two or three times.

**Partial sequencing of the rRNA genes.** The 16S rRNA genes from 14 *H. alvei*

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                         60                   80                   100                  120
                          |                    |                    |                    |
E.coli           ACACATGCAA GTCGAACGGT AACAGGAAGA AGCTTGCTTC TTTGCTGACG AGTGGCGGAC GGGTGAGTAA TGTCTGGGAA
EPEC             .......... .......... ........AC ........G. .......... .......... .......... ..........
H.alvei(eaeA+)   .......... .......... ........AC ........GT ..C....... .......... .......... ..........
H.alvei(eaeA-)   .......... ......G... .G..CA.GAG ........CT C.G.G..... ..C....... .......... ..........
H.alvei 13337    .......... ......G... .G..CA.GAG ........CT C.G.G..... ..C....... .......... ..........

                         140                  160                  180                  200
                          |                    |                    |                    |
E.coli           ACTGCCTGAT GGAGGGGGAT AACTACTGGA AACGGTAGCT AATACCGCAT AACGTCGCAA GACCAAAGAG GGGGACCTTC
EPEC             .......... .......... .......... .......... .......... .......... .......... ..........
H.alvei(eaeA+)   ......C... .......... .......... .......... .......... .......... .......... ..........
H.alvei(eaeA-)   .......... .......... .......... .......... .......... G.....TTCG ........T. ..........
H.alvei 13337    .......... .......... .......... .......... .......... G.....TTCG ........T. ..........

                         220                  240                  260                  280
                          |                    |                    |                    |
E.coli           GGGCCTCTTG CCATCGGATG TGCCCAGATG GGATTAGCTA GTAGGTGGGG TAACGGCTCA CCTAGGCGAC GATCCCTAGC
EPEC             .......... .......... .......... .......... .......... .......... .......... ..........
H.alvei(eaeA+)   .......... .......... .......... .........T ..T...A... .......... ..A....... ..........
H.alvei(eaeA-)   .......AC. .....A.... .......... .......... .......... ...T...... .......... ....T.....
H.alvei 13337    .......AC. .....A.... .......... .......... .......... ...T...... .......... ....T.....
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FIG. 2. Alignment of the partial 16S rRNA genes of *E. coli* as determined by Brosius et al. (3), EPEC E2348/69, 10 identical *eaeA*-positive *H. alvei* strains (*H. alvei eaeA*$^+$), 4 identical *eaeA*-negative *H. alvei* strains (*H. alvei eaeA*$^-$-, including strains SD1 and SD12 from Finnish diarrheic patients, strain J6 from minced meat, and the *H. alvei* DNA reference strain ATCC 29927), and the *eaeA*-negative type strain *H. alvei* ATCC 13337, obtained from GenBank (accession no. M59155). Numbering is according to Brosius et al. (3).

and 2 *E. coli* strains were amplified by using primers pA-B (5′-biotin-AGAGTTTGATCCTGGCTCAG-3′) and pC* (5′-CCCACTGCTGCCTCCCGTAG-3′) (4), yielding a PCR product of 353 bp from the 5′ end of the gene. Templates were prepared by the boiling method as described elsewhere (14). The amplification was performed in a 100-μl volume by using 10 μl of boiled bacterial lysate as a template, 1 μM primer, 1 U of Dynazyme DNA polymerase, and the buffer provided by the manufacturer with 33 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min 20 s. The amplified PCR product was sequenced by the solid-phase method as described previously (8). Sequencing reactions were done with the AutoRead kit (Pharmacia, Uppsala, Sweden), and the results were analyzed with an automated laser fluorescent DNA sequencer (Pharmacia). The alignment of the obtained sequences was done by using the PC-Gene program (Intelligenetics, Geneva, Switzerland).

**Nucleotide sequence accession numbers.** Partial sequences of 16S rRNAs from EPEC strain E2348/69 and *eaeA*-positive *H. alvei* 10457 have been deposited in the EMBL data bank under accession no. Z47359 and Z47360, respectively.

## RESULTS

In addition to the 6 Bangladeshi diarrheal *H. alvei* isolates previously shown to be *eaeA* positive (13), 10 new Bangladeshi diarrheal isolates generated a PCR product of the size predicted for the *eaeA* gene.

*H. alvei* strains possessing the *eaeA* gene had 2 unique biochemical reactions (assimilation of 2-ketogluconate and 3-hydroxybenzoate) and 11 other characteristic reactions among the 83 reactions tested; the remaining reactions did not differentiate between the *eaeA*-positive and -negative strains (Table 1). Some of the characteristic reactions were typical of *E. coli*, although many reactions in the three identification systems were against *E. coli* (Table 1). The *eaeA*-negative strains isolated from different sources did not differ markedly from each other in their biochemical properties.

The API 20E species identification system classified all *eaeA*-positive strains as "*H. alvei*2," with identifications of 91.9% for 2 strains and 70.1% for 14 arginine-positive strains. Extending the incubation time to 48 h did not have any effect on the biochemical reactions, nor did lowering of the incubation temperature to 30°C. The API rapid ID 32E system gave identifications of "*H. alvei*" of 83.4 to 98.3% for 12 strains and 49.6% for 4 strains. The next most common identification for all strains was *Serratia marcescens*. The API ID 32GN system identified 12 of the *eaeA*-positive strains as "*E. coli* 2" with identification of 98.6 to 99.3%; 2 strains as "*E. coli* 2" with an identification of 49.6%, though the next most common classification was "*H. alvei*" with an identification of 49.0%; and 2 strains as "*H. alvei*" with an identification of 76.2%. All 107 *eaeA*-negative *H. alvei* strains were identified as "*H. alvei*" by all three identification systems. With API 20E, 95 strains were named as "*H. alvei* 1" and 12 rhamnose-negative strains were named "*H. alvei* 2". Extending the incubation time to 48 h for the API ID 32GN system influenced the identification only once, when an unacceptable profile giving an identification of "*Shigella* sp." was turned into an acceptable profile giving an identification of "*H. alvei*."

In RAPD-PCR analysis, the *eaeA*-positive strains produced nearly identical profiles which were different from those of the *eaeA*-negative strains (Fig. 1). The majority of the *eaeA*-negative strains shared some homology with the representative(s) of either HG 1 or HG 2 (Fig. 1).

The 353-bp fragments from the 5′ end of the 16S rRNA genes of all 10 *eaeA*-positive *H. alvei* strains tested were identical and differed from the fragments from the five *eaeA*-negative *H. alvei* strains in 27 nucleotides, giving a homology of 92% (Fig. 2). A search of the database demonstrated that the *eaeA*-positive *H. alvei* strains showed closest homology to *E. coli*, with a difference of 10 nucleotides (Fig. 2). The best homology observed was with EPEC E2348/69, which was sequenced in this study and had only seven differing nucleotides (Fig. 2), giving a homology of 98%. EHEC strain ATCC 43895 was homologous with the *E. coli* sequence reported by Brosius et al. (3), but EPEC strain E2348/69 differed from their *E. coli* sequence by three nucleotides.

## DISCUSSION

*eaeA*-positive *H. alvei* strains may have clinical importance as diarrheal pathogens, and therefore, their differentiation from other *H. alvei* strains may have diagnostic usefulness. Neither conventional biochemical tests nor the commercial API 20E system reliably differentiates between *eaeA*-positive and -negative *H. alvei* strains. Therefore, we tested the usefulness of

biochemical reactions in the API rapid ID 32E and API ID 32GN systems and characterized the genetic diversity of the isolates by molecular methods.

The new biochemical tests introduced in this study simplify the identification of *eaeA*-positive *H. alvei* strains in routine clinical laboratory tests. When *H. alvei* is suspected as a causative agent of diarrhea, assimilation of 2-ketogluconate, 3-hydroxybenzoate, and histidine should be included in the biochemical tests. Biochemically typical isolates can then be further tested for the attaching-effacing property. At the moment, little is known about the relative importance of *eaeA*-positive *H. alvei* strains as diarrheal pathogens. *eaeA*-positive *H. alvei* strains are frequently isolated from diarrheal feces in Bangladesh. However, in our previous study, which included 189 diarrheal Finnish patients, no *eaeA*-positive strains were detected, although *H. alvei* was isolated from the stools of 14 patients (13). To our knowledge, the occurrence of *eaeA*-positive *H. alvei* in countries other than Bangladesh and Finland has not been studied. These new data should help to clarify the possible role of *H. alvei* as a causative agent of diarrhea.

Results with the molecular methods indicate that *eaeA*-positive *H. alvei* strains have little genetic diversity, in contrast to the other *H. alvei* strains. The present RAPD-PCR results are in accordance with the previous observation that *H. alvei* consists of at least two genospecies (16). RAPD-PCR is very sensitive to genetic variation and suitable for characterizations within a species. However, RAPD-PCR is also sensitive to minor changes in the reaction conditions (18), and great care is needed to achieve reproducible results. Although the taxonomy of *H. alvei* is beyond the scope of this article, the low level of homology (92%) between rRNA genes of the *eaeA*-positive and -negative strains and the identifications produced with the API ID 32GN system call into question the present taxonomic position of this organism. Studies including sequencing of the entire 16S rRNA genes and DNA-DNA hybridization are in progress to determine the taxonomic status of *eaeA*-positive *H. alvei*.

In conclusion, this study showed that the *eaeA*-positive *H. alvei* strains associated with diarrhea form a distinct and genetically homologous group which is identifiable by biochemical methods.

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