Environmental Isolates of *Aeromonas* spp. Harboring the *cagA*-Like Gene of *Helicobacter pylori*

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We investigated the presence of *cagA*-like gene of *Helicobacter pylori* in environmental isolates of *Aeromonas* spp. from different water samples of Calcutta, India, by colony hybridization using a *cagA*-specific DNA probe and by PCR with *cagA*-specific primers. Nucleotide sequencing of five PCR products revealed 97 to 98% homology to canonical *cagA* of *H. pylori* 26695 as well as to four clinical *H. pylori* strains from Calcutta. The *cagA*-like gene of the environmental isolates was unstable in laboratory conditions and tended to be lost upon subculturing.

*Helicobacter pylori* is one of the most genetically diverse bacterial species as seen by DNA fingerprinting and in tests of gene content and chromosomal gene order (6). It has been suggested that considerable gene transfer from disparate phylogenetic groups into the *H. pylori* lineage shaped the gene pool of this organism during its evolution (1). Support for this is provided by (i) the abundance of putative restriction and modification genes, many of which are most closely related to those of other bacterial species; (ii) the significant sequence similarity of many predicted *H. pylori* proteins to those from different major taxonomic groups (eukaryotes, *Archaea*, and gram-positive bacteria), and (iii) the presence of transposable elements IS605 to IS608 (1, 7, 8, 9). However, it is not known how ancient these gene acquisitions are: in particular, which elements IS

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The freshly grown colonies on the membrane were first lysed with 0.5 N NaOH and 1.5 M NaCl and subsequently neutralized with neutralization solution (0.5 M Tris-HCl [pH 7.2], 1.5 M NaCl) (15). The DNA was linked to the membrane by UV irradiation, prehybridized at 42°C for 1 h, and hybridized with digoxigenin (DIG)-labeled cagA-specific probe (10 ng/ml) for 14 h at 42°C. The PCR amplicon of the cagA gene of H. pylori 26695 amplified with primers C2 (5’ GAATCAGTATTTTT CAGAC) and Cr2 (5’ GGGTTGTATGATATTTC) (16) was used as a specific probe for cagA. The amplicon was subsequently purified by using the QIAquick purification kit (Qiagen Corporation, Chatsworth, Calif.) and labeled with DIG-11-dUTP by random hexamer labeling using the DIG DNA labeling and detection kit (Boehringer Mannheim, Mannheim, Germany). After hybridization, the membrane was washed thoroughly twice (15 min each) in 2× SSC (1× SSC is 0.15 M sodium chloride and 0.015 M sodium citrate) buffer and 0.1% (wt/vol) sodium dodecyl sulfate (SDS) and twice (30 min each) at 65°C in 1× SSC buffer and 0.1% SDS solution under highly stringent conditions. Next, the membrane was treated with anti-DIG–alkaline peroxidase conjugate and developed with color-developing solution. The colonies that appeared as dark purple spots on the membrane were considered to possess cagA-like gene. The corresponding colonies were selected from dilution plates for further characterization.

The colonies, which appeared as positive for the presence of cagA-like genes, were inoculated in 3 ml of LB broth and incubated overnight at 37°C in a shaker. Bacterial cell lysate was used as the template DNA for PCR using C2 and Cr2 primers, as well as with another set of cagA-specific primers, cagA5 (5’ GGC AAT GTG GGT CCT GGA GCT AGG C) and cagA2 (5’ GGA AAT CTT TAA TCT CAG TTC GG) (11). In both cases PCR was carried out in a 20-μl reaction volume using 10 pmol of each primer per reaction, 0.25 mM of each deoxynucleoside triphosphate, 1 U of Taq polymerase (Takara, Shuzo, Tokyo, Japan), and 3 μl of DNA (lysate) in a standard PCR buffer (Takara). The reaction mixture was subjected to an amplification of 30 cycles, each of which consisted of three steps in the following order: denaturation of template DNA at 94°C for 1 min, annealing of the template DNA at 55°C for 1 min, and extension of the primers at 72°C for 1 min. H. pylori 26695 and E. coli strain DH5α were used as positive and negative controls, respectively. If a positive PCR result was obtained, then the strain was preserved in both nutrient agar stabs in room temperature and LB supplemented with 15% glycerol at −70°C (glycerol stock).

To determine CagA expression, the whole-cell lysate of the PCR-positive environmental isolates and H. pylori strain 26695 were separated by SDS-polyacrylamide gel electrophoresis in 12.5% polyacrylamide gels and transferred electrophoretically to nitrocellulose membranes using a transblot apparatus (Bio-Rad, Richmond, Calif.) at a constant voltage of 40 V for 1 h. Following the transfer, the blotted membrane was sequentially treated with rabbit anti-CagA antibody raised against a high-performance liquid chromatography-purified synthetic peptide homologous to amino acids 411 to 430 of the conserved region of the CagA protein (Tana Laboratories, Houston, Tex.), followed by appropriately diluted alkaline phosphatase-labeled conjugate and developed with color developing solution.

The nucleotide sequences of cagA amplicon obtained by PCR using primers C2 and Cr2 from five environmental strains and four clinical H. pylori isolates (cultured from the gastric biopsies of duodenal ulcer patients of Calcutta) from our laboratory collection were determined using the BigDye terminator cycle sequencing kit (Perkin-Elmer Applied Biosystems, Foster City, Calif.) on an automated DNA sequencer (API Prism 310). The forward and reverse sequences were edited in a sequence editor using the OMIGA 1.1 software package (version 1.1: Oxford Molecular, Cambridge, United Kingdom), and the consensus sequence was used for further analysis. The sequence data were compared with each other as well as with the published sequences of H. pylori 26695 (GenBank accession number AE000511). The alignments of all the sequences were carried out using the CLUSTAL-X software program with the IUB distance matrix and a gap open penalty of 6.66 (gap extension penalty = 15; transition weight = 0.5) (14).

The biochemical characterization and identification of environmental isolates were performed using the API 20E (Bio Merieux, Marcy l’Etoile, France) identification system, which uses the 23 miniaturized biochemical tests and database. Interpretation of the results was made by referring to the identification table and analytical profile index after 18 h of incubation at 37°C. Resistance to vibriostatic agent, 2,4-diamino-6,7-diisopropylpteridine (0/129), was performed as detailed previously (5). For precise identification, 16S ribosomal DNA (rDNA) sequencing of the strains was done using the MicroSeq 500-16S rDNA bacterial sequencing kit (PE Applied Biosystems). Nucleotide sequences generated were aligned and analyzed for identification of bacterial species using Micro Seq Analysis Software v.1.40 (PE Applied Biosystems). The database comparison, using the full alignment tool of the MicroSeq software, generated a list of the closest matches with a distance score which indicated the percent difference between the given unknown sequence and the database sequence.

During environmental sampling, a total of 41 environmental strains were found to possess cagA-like gene by colony hybridization tests. These colonies had formed on LA plates under normal atmospheric conditions, which preclude H. pylori growth, which requires blood- or serum-supplemented plates and has demanding atmospheric requirements (5% O2; 10% CO2; and 85% N2). Of these 41 strains, 14 yielded the desired 208-bp amplicon with C2 and Cr2 indicating the presence of cagA-like gene. For further analysis, only those cagA-carrying strains were selected that were positive by both PCR and colony hybridization tests. Twelve of these fourteen strains were also positive by PCR using primers cagA5 and cagA2, which gave the appropriate amplicon of 324 bp, while the remaining two yielded negative results. The primer pairs C2 and Cr2 and cagA5 and cagA2 were designed from different regions of the cagA-like gene in these environmental isolates, and hence PCR positivity using both primers strengthened our finding of the presence of a cagA-like gene in these environmental isolates.

Western blot analysis with CagA-specific antibody was used to detect the presence of surface-exposed CagA in the whole-cell protein of PCR-positive strains. However, none of the test environmental strains yielded positive results, although a 128-kDa protein band was observed in positive control strain H. pylori 26695, indicating the presence of CagA.

The negative Western blot result prompted us to repeat the
PCR analysis of the strains with the same pair of primers as used before to determine whether CagA actually was not expressed or whether there was a loss of \textit{cagA} in these strains. Culture material (10 \textsuperscript{6}/H9262 ml) from the glycerol stock of each of the 14 strains was deployed to inoculate 5 ml of LB, and after overnight incubation at 37 °C, about 10\textsuperscript{5} cells from these strains were subjected to PCR analysis with Cf2 and Cr2 primers. Interestingly, no amplification product was observed in six strains that were initially positive by PCR. But for each of the remaining eight strains, the expected 208-bp fragment was detected. However, chromosomal DNA prepared directly from the glycerol stocks of the strains using a DNA extraction kit (Qiagen) was found to retain \textit{cagA} as determined by PCR with primers Cf2 and Cr2. This highlights the possibility of gene loss upon subculturing in the six strains that were negative by PCR for the \textit{cagA} gene. To monitor the frequency of gene loss, 100 \textsuperscript{6}/H9262 ml of glycerol stock of each of the 14 PCR-positive strains was again used to inoculate 5 ml of LB that was incubated overnight. One hundred microliters of a 1:10\textsuperscript{6} dilution of the culture was spread on agar plates, and the colonies were transferred to a Hybond nylon membrane as before and hybridized with DIG-labeled \textit{cagA} probe while another 100 \textsuperscript{6}/H9262 ml of culture was used to further inoculate 5 ml of sterile LB and incubated overnight at 37°C. It was found that none of the six PCR-negative strains yielded positive hybridization result. However, all the eight strains carrying a relatively stable \textit{cagA} gene that was detected during the second PCR yielded positive signal when hybridized with \textit{cagA} probe, and the number of positive colonies on the dilution plates was determined. Similarly, the number of \textit{cagA}-positive colonies was determined upon second subculture, and it was observed that about 75% of the bacterial cells of two strains had lost the \textit{cagA} gene, while for the other six strains the gene was present in ~60% of the cells.

With the whole-cell protein of these six stable strains, the Western blot experiment was repeated again, but as before, none of these strains expressed CagA in spite of the presence of the \textit{cagA}-like gene. The \textit{cagA}-specific PCR products of five out of the above six strains were sequenced directly and compared with the published sequence of strain 26695 (GenBank accession number AE000511) in the GenBank database and four \textit{cagA} sequences of clinical \textit{H. pylori} strains of Calcutta, also determined in this study. All the sequences obtained were 99 to 100% similar to each other and 97 to 98% identical to the corresponding portion of \textit{cagA} of 26695 and Calcutta \textit{H. pylori} strains (Fig. 1).

For presumptive identification of the environmental strains, oxidase tests were first performed, and all the six strains yielded positive results. The results of the API 20E test identified the strains as \textit{Aeromonas hydrophila}; analytical profile indices being 3047124 (for three strains) and 3047125 and 1047124 for each of the remaining three strains. All the strains were also resistant to a 150-\textmu g/ml concentration of vibriostatic agent, 2,4-diamino-6,7-diisopropylpteridine (0/129). Five strains were chosen for precise identification using the 16S rDNA sequence and data analysis system. Of the five strains, one (C-15) was identified as \textit{Aeromonas veronii}, while another (C-8) was closely related to it (Fig. 2). However, the other three strains (C-45, C-92, and C-92) grouped within the cluster of \textit{Aeromonas enteropelogenes} and \textit{Aeromonas caviae} in phylogenetic analysis (Fig. 2).

In this study, evidence has been furnished that \textit{Aeromonas} spp. from aquatic sources in Calcutta, India, were found to

**FIG. 1.** Alignment of 169-bp sequence within \textit{cagA} gene of five environmental isolates of \textit{Aeromonas} spp. (C-8, C-15, C-45, C-92, and CE3) and four clinical \textit{H. pylori} strains (I-18, I-30, I-47, and I-77) determined in this study and \textit{H. pylori} reference strain 26695 (GenBank accession number AE000511). The alignments of all the sequences were carried out using the CLUSTAL-X software program.
harbor the cagA-like genes as demonstrated by colony hybridization, PCR analysis, and nucleotide sequencing. However, Western blot analysis indicated that these strains did not express CagA. The presence of a cagA-like gene in Aeromonas spp. raises the question of whether gene transfer between H. pylori and Aeromonas is directly responsible for its presence. Since both H. pylori and Aeromonas spp. are known to survive in raw water and since both are human enteric pathogens (3, 5, 10), such a possibility could not be ruled out.

During preliminary screening through colony hybridization testing with specific probe, 41 environmental strains were found to be positive for cagA. But the PCR-negative results for some probe-positive isolates might be attributed to the sequence variation in the binding region of the PCR primers or to gene loss. The sequence variation may be a part of the greater allelic variation of the gene, and it might be that different alleles of cagA are in circulation in the environmental isolates of Aeromonas spp.
An interesting observation made in this study is the loss of the \textit{cagA} gene in the environmental isolates of \textit{Aeromonas} after subcultivation. It is difficult to explain why the gene is retained by a few strains and lost by the others. Such rapid gene loss (even after a single subculture) was also observed in other organisms under different experimental conditions (4, 12). One explanation for the reduction in the copy number of the gene might be that a \textit{cagA} gene-harboring cell does not pass on the gene to all of its progeny. However, the apparently complete loss of genes after subcultivation in some strains suggest that there must be fewer than 10 copies per 10^5 cells; otherwise we would have detected them by PCR. Clearly, the in vitro instability of \textit{cagA} genes requires further experiments on the DNA regions flanking the \textit{cagA} genes to ascertain whether insertion sequences were present at the ends that could mediate gene transfer. In fact such instability may reduce the frequency of detection of strains harboring \textit{cagA} from the environment. Also, for this reason, screening and confirmatory tests should be carried out on isolates after the minimum possible number of subcultures.

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