Genetic engineering of avian pathogenic *E. coli* to study the functions of FimH adhesin

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Adhesion of pathogen to host cells is an important prerequisite for successful colonization and establishment of the pathogenesis. The aim of this study is to examine the function of FimH adhesin in the adherence of avian pathogenic *E. coli* to porcine intestinal epithelial cell lines (IPEC-J2) and human lung epithelial cell line (A549) in an in vitro infection model. Three strains of avian pathogenic *Escherichia coli* (APEC) and one strain of non-pathogenic *E. coli* were used. The isogenic FimH mutants were constructed by λ Red-mediated recombination system. The wild types and mutants strains were adhered to the host cells with different adherence patterns in certain incubation time. The results demonstrated that the adherence of the isogenic FimH mutants to the porcine intestinal epithelial cells (IPEC-J2) were similar to those of wild types. However, the adherences of isogenic FimH mutants to human lung epithelial cells (A549) were significantly different from the wild types. A549 cell can be used as a type of cell model for colonization of the chicken extraintestinal. FimH offers a unique opportunity to investigate the role of the strength of adhesin independently from the many other factors that may affect surface colonization.

**Keywords:** Avian pathogenic *E. coli*, FimH, Host cell

Avian pathogenic *Escherichia coli* (APEC) are responsible for extraintestinal infections in chickens, turkeys and other avian species. The presence of several putative virulence genes has been positively linked to the pathogenicity of APEC strains1,2. They include the aerobactin iron transport system, K1 capsule, P and type1 fimbriae3,4. The highly pathogenic *E. coli* causing outbreaks at flock level possess virulence factors that are not found in commensal strains5. Type 1 fimbriae are characterized by their ability to bind D-mannose and thus bind to many types of eukaryotic cells including intestinal, lung, bladder, kidney epithelial and various inflammatory cells6. Binding is conferred by the adhesin FimH, which is a minor component of the fimbriae2,7.

FimH consists of a fimbria-associated pilin domain and a mannose-binding lactin domain, receptor-ligand specific adhesion is among the most fundamental of biological phenomena in nature. This phenomenon underlies eukaryotic cell-cell or cell-surface attachment, initiates recognition and signaling events, binds bacteria to target cells and mediates biofilm formation on medical implants8. Usually the adhesion is mediated by protein-protein, protein-lipid or protein-saccharide interactions9. Adhesion has been widely demonstrated to often enable bacteria to carry out initial colonization of host mucosa and to overcome nonspecific defense barriers10. A number of experimental models have been established to evaluate the pathogenicity of *E. coli* for poultry. The model used was focused on the ability of isolates to colonize the host epithelial cell6.

DNA engineering is conducted routinely in *Escherichia coli*, not only for genetic studies in bacteria, but also for constructing DNA molecules to be used in studies of other organisms11. The DNA double-strand break and repair recombination pathway is very efficient in yeast. Functions in this pathway recombine transformed linear DNA with homologous DNA in the yeast12. In contrast to yeast, *E. coli* is not readily transformed by linear DNA, because of the presence of intracellular RecBCD
exonuclease that degrades linear DNA. Inactivation of host RecBCD exonuclease activity, either by mutation or production of the anti-RecBCD Gam function, is required for efficient Red-promoted recombination with linear dsDNA substrates. The λ Red was used to knock-out gene in Salmonella enterica Serovar typhimurium and Salmonella enterica Serovar enteritidis. In the present study isogenic FimH mutant was constructed by Red system, and the adherence properties of the isogenic FimH mutants were compared with avian pathogenic and non-pathogenic E. coli isolates from different ecological niches.

Materials and Methods

**Bacterial strains and culture condition**—Avian pathogenic Escherichia coli (APEC) isolated from the infected chicken belong to the serotypes O78:K89, O1:K89 and O2:K89 and non-APEC strain of serotype O24:K89 from the healthy chicken were selected as a prototype of Escherichia coli type 1 fimbrae. The isogenic FimH mutants for these strains were constructed by λ Red-mediated recombination system. Expressed FimH from all the wild types and non-expressed mutants were confirmed by the ability of agglutination reaction with both guinea pig erythrocytes and yeast cells. All strains were subjected to 48h cultures in LB broth at 37°C to allow a high level of expression of type 1 fimbrae.

**Construction of APEC mutant strains**—Luria-Bertani (LB) media was used for growth of bacteria and the recovery of transformants. Bacterial strains were routinely grown at 37°C except for strains containing the temperature sensitive plasmids, pCP20 or pKD46, which were grown at 30°C. Primer P1 5'-ATG AAA CGA GTT ATT ACC CTG TTT GCT GTA CTG CTG ATG GGC TGG TCG GTT GTG TGA GCT GGA GCT GCT TCG-3' and primer P2 5'-TTA TTG ATA AAC AAA AGT CAC GCC AAT AAT CGA TTG CAC ATT CCC TGC CAT ATG AAT ATC CTC CTT AG-3' have internal overlap with the chloramphenicol resistance cassette (Cm) and external overlap with the FimH gene to be deleted. Primers P3 5'-ATG AAA CGA GTT ATT ACC CTG TT -3' and primer P4 5'-CGC CAA TAA TCG ATT GCA CAT T-3' were only flank the target FimH gene for PCR verification. Primers were synthesized by Shanghai Gene Core Biotechnology Company.

Chromosomal mutations were created using the λ Red recombination method as described by Datsenko and Wanner. Briefly, P1 and P2 primers were used to amplify FRT flanked antibiotic resistance cassettes of plasmids pKD3; the resulting PCR products were purified. Avian pathogenic E. coli strains containing the plasmid pKD46, which carried the λ recombination genes gam, bet, and exo were grown at 30°C until OD600 of 0.6. L-arabinose was then added to a final concentration of 0.2% and induced for 1h. The competent cells were prepared and electroporated with purified PCR products, 1ml of LB were added and incubated for 1h at 37°C, cell were plated on LB media with Cm at 37°C and incubated overnight. PCR was carried out by P3 and P4 primers to confirm that FRT flanked Cm antibiotic resistance cassettes (cat) were integrated in the correct region on the chromosome. Thereafter, PCR products were cloned into pGEM-T Easy vector and sequenced. To eliminate the Cm cat, mutants containing a FRT Cm were transformed with the helper plasmid pCP20, and ampicillin resistance transformants were selected at 30°C, after which a few were colony-purified once non-selectively at 42°C and then tested for loss of antibiotic resistances.

**Cell line and cell culture**—Human lung epithelial cell line (A549) and porcine intestinal epithelial cell line (IPEC-J2) were grown in Dulbecco’s modified eagle medium (DMEM)/Ham’s F-12 (1:1) medium supplemented with 10% calf sera and 1% non-essential amino acids (Gibco). Cells were maintained in an atmosphere of 5% CO₂ at 37°C, and passaged by trypsinization every 3-4 days.

**Bacterial adhesion**—The A549 and IPEC-J2 cells were seeded in 24 well tissue culture plates at concentration of 4×10⁵ cells/well and grown for 20h. Cell monolayers were washed three times with sterile phosphate-buffered saline (PBS, pH 7.2). Each well monolayer was infected at a multiplicity of infection (MOI) of 100 bacteria/epithelial cell in 1ml of cell culture medium without antibiotic. After 1 or 4h of incubation at 37°C with 5% CO₂, the infected monolayers were gently washed thrice with PBS to remove loosely adherent bacteria. Cells were lysed with 1ml of 1% triton x-100 (Sigma) for 15min. Samples were serially diluted and plated on MacKonkey agar plates to determine the number of colony-forming units, the assay was performed in triplicates.

**Data analysis**—All values are presented as the mean±SE, and the significant difference between wild type and FimH isogenic mutants of APEC in the
Results

Construction of APEC mutant strains—The Red system includes three genes of Gam, Bet and Exo: Gam inhabits the host RecBCD exonuclease so that Bet and Exo can gain access to DNA ends to promote recombination. In the present study four isogenic FimH mutants of APEC were constructed using the λ Red recombination system. A1.1kb of chloramphenicol resistance cassette was amplified from the pKD3 plasmid as a template. Avian pathogenic E. coli strains containing the plasmid pKD46, which induced the λ recombination genes, were electroported with the purified PCR product. The resulting colonies characterized by PCR were 1114bp with Cm insertion (Δ fimH:: Cat) compared to 883bp of FimH. A temperature sensitive helper plasmid pCP20 was used to eliminate the antibiotic resistance cassette, the loss of Cm cat was confirmed by PCR and the mutant strains were re-named Δ fimH

Association of avian pathogenic E. coli with host cells—The adhesion of pathogenic bacteria to intestinal epithelial cells is an important prerequisite for their colonization and the establishment of the pathogenesis. In the present study colony forming units (CFU) counting assay was used to investigate the difference between the isogenic FimH mutant and wild type in adherence to porcine intestinal epithelial cell (IPEC-J2) and human lung epithelial cell line (A549). The results showed that the adhesion of all isogenic FimH mutants and wild types to host cells were increased with certain incubation time. The wild type of APEC serotype O78:K89 was significantly (P<0.05) associated with IPEC-J2 cell compared with FimH mutant. However, no significant in vitro adhesion differences were observed between the isogenic FimH mutants and wild-types avian pathogenic Escherichia coli on IPEC-J2 cell for serotypes O1:K89 and O2:K89 (Fig. 1). The results showed that FimH was not required for colonization of the intestinal porcine epithelial cell in vitro by E. coli; it may be concluded that FimH is not a major characterization of a porcine intestinal epithelial cell line for in vitro studies of microbial pathogenesis in swine, and that it is not a major determinant of bacterial pathogenicity. The A549 cell is the human lung epithelial cell line; in the present study it has been shown that the interaction between APEC and host cells (A549) can be used as a model for the natural colonization of the respiratory surface by the APEC and provide information about the response pathways involved. Since, the wild type strains of all serotypes demonstrated significantly (P<0.05) a higher association with A549 cells than isogenic FimH mutants did (Fig. 2). This is because the adherence of APEC in this in vitro model depends on the expression of type 1 fimbriae.

Discussion

The construction of FimH mutant gene was performed by Red recombination system1. The technical advance of the system came from replacing the E coli RecBCD gene cluster with the red genes14. The genes gam, beta and exo expressed at moderate levels under control of the lac promoter from multicopy plasmid21. Red system promoted recombination between the bacterial chromosome and linear dsDNA molecules introduced into the cell via

Fig. 1—Association of wild type and isogenic fimH mutant strains with IPEC-J2 cell [Avian pathogenic E. coli serotype O2:k89, O78:k89 and O1:k89 and their isogenic fimH mutants ΔfimH were used at multiplicity of infection (MOI) of 100 bacteria per cell, infection was performed for 1 and 4h, and the adhesion levels were expressed as the number of colony-forming units (CFU) per well]
electroporation, the system had been used to replace gene in Salmonella, enteropathogenic E. coli and entrohemorrhagic E. coli. Thus, we have succeeded to knockout FimH adhesin gene from three avian pathogenic E. coli and one non-pathogenic E. coli, and this will help to study the function of FimH adhesin and his interaction with host cell.

APEC strains adhere to chicken epithelial cells of the pharynx and trachea by means of the type 1 fimbriae, comprising a major structural subunit, FimA and a minor subunit, FimH that mediates the attachment to D-mannose residues. The IPEC-J2 cell line is a non-transformed intestinal cell line originally derived from jejunal epithelia isolated from a neonatal, is suitable as host for a number of pathogenic and non-pathogenic bacteria. In the present study no significant in vitro adhesion differences were observed between the isogenic FimH mutants and wild-types avian pathogenic Escherichia coli on IPEC-J2 cell. The non-significant difference of adherence between wild type and mutants may be due to that type 1 fimbriae have mainly involved in the upper respiratory colonization instead of intestinal in the present study. The adhesion patterns and lysis of human and porcine epithelial cells by pathogenic E. coli varied broadly depending on the origin of the bacteria and species origin of the epithelial cells.

In the present study the wild type strains demonstrated significantly a higher association with A549 cells than isogenic FimH mutants for all strains studied. Similarly, Mellata et al. indicated that the wild type APEC strain demonstrated a high association with phagocytes (macrophage and heterophils) than FimH mutant strain. Because FimH mutant strains were less able to survive in the lungs of chickens than the parent strains, and unable to adhere in vitro to chicken epithelial pharyngeal or tracheal cells. FimH through specific ligand-receptor interactions can trigger various responses in cells involved in host defenses such as macrophage, mastocytes, lymphocytes B and neutrophils. FimH mediates weak adhesion at low flow but strong adhesion at high flow. There is evidence that this occurs because FimH forms catch bonds, defined as bonds that are strengthened by tensile mechanical force. The physiological advantage to the weak adhesion demonstrated by commensal variants of FimH may be to allow rapid surface colonization. Duncan et al. indicated that the distinct binding specificities of different enterobacterial type 1 fimbriae cannot be ascribed solely to the primary structure of their respective FimH subunits, but are also modulated by the fimbrial shaft on which each FimH subunit is presented, possibly through conformational constraints imposed on FimH by the fimbrial shaft.

It is clear in the present study that FimH plays similar biological roles for commensal bacteria because they also have to colonize specific niches and overcome the host's natural clearing mechanisms. Schierack et al. indicated that the commensal E. coli isolates had a broad spectrum of adhesion to IPEC-J2, ranging from 0 to more than 100 bacteria per cell due to difference in the FimH genes. It is thought that commensal and some pathogenic Escherichia coli strains use type 1 pili or curli to colonize human and animal tissues. Commensal bacteria block adhesion of pathogens by competition for common receptors and aid in maintaining the physiological balance of the intestinal tract. It can be concluded that FimH offers a unique opportunity to investigate the role of the strength of adhesion independently from the many other factors that may affect surface colonization.

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