Induction of Gene Expression in *Escherichia coli* After Pilus-Mediated Adherence

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The induction of cascades of virulence factors after contact between bacteria and host cells was investigated. P-pili mediate the binding of uropathogenic *Escherichia coli* to its host cell receptor. After P-pili binding there was transcriptional activation of a sensor-regulator protein that is essential for the bacterial iron-starvation response. An insertion mutation of the sensor-regulator gene eliminated the ability of uropathogenic *E. coli* to produce siderophores and their iron-regulated membrane receptors, thereby abolishing their ability to grow in urine. These results suggest that P-pilus–mediated attachment may be an important part of the sensor-regulatory process involved in uropathogenic *E. coli* urinary tract infection.

Escherichia coli causes a variety of common bacterial infections in humans and animals, including urinary tract infection, diarrhea, septicemia, and neonatal meningitis. Previous work has shown that P-pilus–mediated adhesion of these bacteria to the P blood group antigens present on host epithelium is essential for the development of acute pyelonephritis (*1*, *2*). High-resolution electron microscopy revealed that P-pili are composite structures consisting of a long, rigid rod and a distal thin, flexible fibrillar structure. The PapG adhesin is located at the tip of the P-pili, and it directs the high-affinity recognition of the host Galα(1→4)Gal–containing globoseries receptors (*3*, *4*). The location of the specific PapG adhesin on the distal end of the P-pili indicates its importance in the initial interaction between the pathogen and its host receptors. This interaction may also be a means for bacteria to sample and consequently elicit the appropriate response upon their arrival at a potential colonization site.

To determine whether P-pili can initiate microbial responses after their specific attachment to the target host receptor, we performed attachment experiments with a uropathogenic *E. coli* strain DS17 (06:K5:H$^-$) and its isogenic mutant strain DS17-8, which expresses P-pili but lacks the PapG tip adhesin (*2*). Human red blood cells (RBCs), which contain the globoside isoreceptor for P-pili-ated DS17, and rabbit RBCs, which lack this receptor, were used for bacterial attachment (*4*). Bacteria were allowed to attach to host cells at 37°C for 30 min, and then total RNA was extracted from the bacteria. Gene expression in *E. coli* under various attachment conditions was examined with the differential display technique (*5*). In this method RNA is reverse-transcribed to cDNA, which is then subjected to polymerase chain reaction (RT-PCR) with a nonspecific set of primers to amplify the RNA products (*6*). Comparison of the amplified RNA identified a number of distinct RNA species expressed only in attachment samples where *E. coli* was able to attach to host cells, that is, in the samples with human RBCs. These RT-PCR products were subcloned and sequenced. The nucleotide and deduced amino acid sequences of these genes were used to search for homologous sequences in GenBank and one was identified as the *barA* gene (*7*). We demonstrate here that *barA* gene transcription is specifically activated by P-pili attachment, and the BarA protein is essential for the iron-starvation response of uropathogenic *E. coli*. Therefore, we propose that it be renamed AirS for attachment and iron regulation sensor.

Specific primers were designed and used for amplification of the *airS* transcription signal from different attachment samples (*8*). RBCs or globoside immobilized on plastic petri dishes were used to induce specific transcriptional activation of the *airS* gene. Transcription of *airS* was detected only after *E. coli* DS17 attached to human RBCs (Fig. 1A) or the globoside-treated dishes (Fig. 1B). These results suggest that *airS* gene transcription was activated only after the attachment of *E. coli* DS17 to the Galα(1→4)Gal containing epitope through the PapG adhesin, because the same transcript could not be detected in *E. coli* DS17 without attachment, or in bacteria incubated with host cells or petri dishes that did not contain the globoside receptor. Moreover, *airS* gene transcription could not be detected if *E. coli* DS17-8, which lacks the PapG tip adhesin, was incubated with human RBCs or treated petri dishes that contained the specific globoside receptor.

To further examine the transcriptional

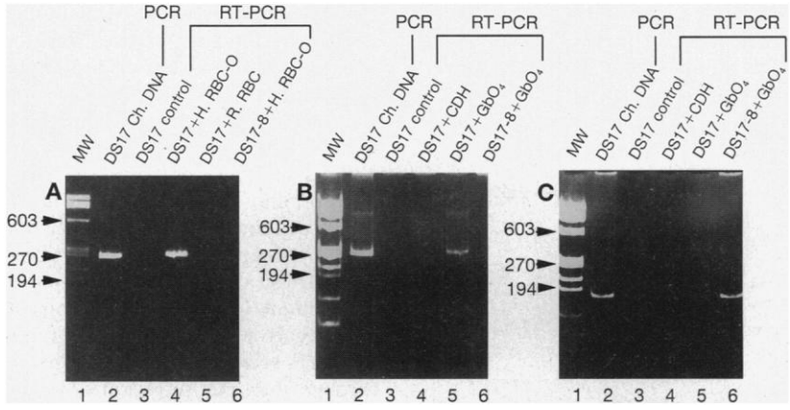

Fig. 1. Specific RT-PCR to amplify the *airS* gene was performed on RNA purified from bacteria after attachment experiments with several different substrates. (**A**) The expected size of the *airS* PCR product is 270 base pairs (bp) when DS17 chromosomal DNA is used as the template (lane 2). Lanes 3 to 5 contained RT-PCR products from DS17 incubated with the following samples: no RBCs (lane 3), human group O RBCs (lane 4), and rabbit RBCs (lane 5). For lane 6, DS17-8, which lacks the tip PapG adhesin for the globoside receptors, was incubated with human group O RBCs as in lane 4. MW, molecular size standard in base pairs. (**B**) Globoside (GbO_4)- or ceramide dihexoside (CDH)–coated plates were used as substrates for attachment experiments with DS17 or DS17-8, and RT-PCR to amplify *airS* was performed. The expected size of the *airS* PCR product is 270 bp when DS17 chromosomal DNA is used as the template (lane 2). Lanes 3 to 5 contained RT-PCR products from DS17, incubated in an uncoated petri dish (lane 3), a CDH-coated petri dish (lane 4), and a GbO_4-coated petri dish (lane 5). In lane 6, RT-PCR was performed on DS17-8 RNA template after incubation in a GbO_4-coated petri dish. (**C**) Samples were the same as in (B) except that a control primer set for the pyruvate dehydrogenase gene was used for RT-PCR to monitor the integrity of the RNA harvested in the experiments. The expected size PCR product is about 150 bp in an 8% polyacrylamide gel.

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activation of the *airS* gene in response to host cell attachment, we cloned the promoter region of the *airS* gene into a promoterless pKK232-8 reporter vector in either orientation to create plasmids KPcat-F or KPcat-R (Fig. 2A). These plasmids were transformed into wild-type DS17 for chloramphenicol acetyltransferase (CAT) assay (Fig. 2B). The observed CAT activity of either KPcat-R–transformed DS17 incubated with human RBCs or KPcat-F–transformed DS17 incubated with rabbit RBCs was the same as the basal amount of CAT activity observed with KPcat-F–transformed DS17 in the absence of attachment. However, when the KPcat-F–transformed DS17 was incubated with human RBCs, a twofold increase in CAT activity compared with the basal amount was observed. Thus, *airS* gene transcription as measured through its promoter and by RT-PCR analysis appears to be activated in uropathogenic *E. coli* in response to P-pilus–mediated attachment to the correct host cell receptor.

To determine the functional importance of *airS* in the interaction of uropathogenic *E. coli* with its target host and to identify the downstream genes it regulates, we created an *airS* mutant containing an insertion of the kanamycin (*kan*) resistance gene cassette in the *airS* gene using the allele replacement method (Fig. 2A). The *airS* mutant, like the wild-type DS17, can express P-pili and remains capable of P-pilus–mediated hemagglutination. It also retains the ability to produce α-hemolysin. However, it exhibits significantly different growth capabilities as compared with the wild-type strain. Unlike the wild-type strain, the *airS* mutant cannot grow in human urine.

Urine is a complex body fluid containing a variety of excreted products (9). As one of the nonspecific defense mechanisms against microbial infection, free iron is limited in mammalian biological fluids (*10*). Uropathogenic *E. coli* secretes the low molecular weight, high-affinity iron siderophores aerobactin and enterobactin in response to iron stress (*11*). These siderophores compete effectively with the host iron-binding proteins to obtain iron for bacterial use. Subsequent transport of the iron-siderophore complexes into the bacterium requires cell surface iron-regulated membrane proteins (IRMPs) as receptors specific for each siderophore complex (*12, 13*). Thus, the iron acquisition mechanism is one of the most important adaptive responses for bacterial pathogenesis.

When wild-type DS17 was grown either in human urine or in iron-limited CFA medium, the presence of siderophores in the culture supernatant was detected, indicating that the growth of uropathogenic *E. coli* in human urine was under iron-starvation conditions and that the siderophore system had been activated to overcome the growth-inhibitory effects of iron starvation. Wild-type DS17 also expressed outer membrane IRMPs when grown in iron-limited CFA medium, but not in iron-rich LB medium (Fig. 3). In contrast to the wild-type strain, the *airS* mutant failed to produce siderophores, and it did not express its outer membrane IRMPs in iron-limited CFA medium (Fig. 3). Although the *airS* mutant grew with the same kinetics as the wild-type strain in iron-rich media (Fig. 4A), it grew poorly in the iron-limited media (Fig. 4B). To address whether this poor growth resulted from iron starvation, we examined whether additional iron could restore the growth of the *airS* mutant. Indeed, the addition of 1 mM $FeCl_3$ in iron-limited culture nearly completely restored the growth of the *airS* mutant (Fig. 4B). Together,

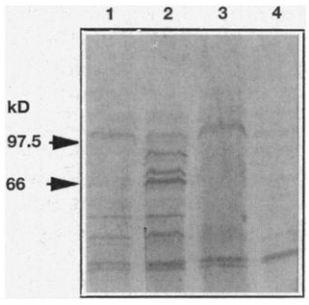

Fig. 3. Outer membrane protein analysis by 8% SDS–polyacrylamide gel electrophoresis. DS17 and the isogenic *airS* mutant strain were grown overnight at 37°C with shaking (250 rpm). Lanes 1 and 2: DS17; lanes 3 and 4: *airS* mutant. In lanes 1 and 3, LB broth (iron-rich) was used as the culture media. In lanes 2 and 4, CFA broth (iron-limited) was used as the culture media. The DS17 iron-regulated outer membrane proteins ranging from 66 to 93 kD were expressed only in iron-limited CFA culture and not in iron-rich LB broth. The *airS* mutant did not express the iron-regulated outer membrane proteins in iron-limited CFA broth.

these data indicate that the *airS* mutant is unable to activate the synthesis of its crucial siderophore iron-acquisition system during iron-starvation conditions.

The mechanism by which the *airS* gene regulates the bacterial siderophore system in uropathogenic *E. coli* is presently unclear. Growth in low-iron media did not increase the level of transcription of the *airS* gene in the absence of P-pilus–mediated attachment (Fig. 2B). Hence, AirS itself may not sense low-iron conditions. Recently, it has been shown that alcaligin, a siderophore produced by *Bordetella brochiseptica* is regulated by the *Bordetella* global virulence regulator BvgS (*14*). It also has

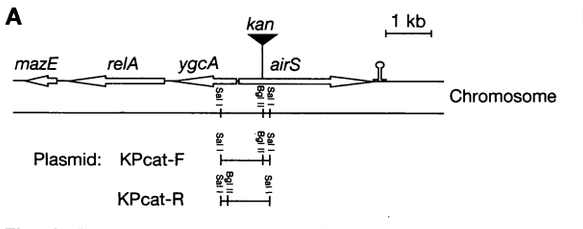

Fig. 2. Restriction map of the *airS* locus and construction of reporter plasmids for detection of attachment activation of the *airS* gene by chloramphenicol acetyltransferase (CAT) assay. (**A**) Restriction map of the *airS* locus. The arrows show the direction of transcription. The hairpin symbol indicates the putative transcriptional termination site. An *airS::kan* mutant was created by insertion of a kanamycin resistance gene cassette into the Bgl II site of the *airS* gene by the allele replacement method (*20*). For construction of reporter plasmids, DNA of λ phage clone 8B9, which contains the *airS* gene (*21*), was digested with Sal I endonuclease, and the 1.1-kb DNA fragment containing the promoter region of *airS* was cloned into the Sal I site of the pKK232-8 promoter selection vector (*22*). (**B**) The plasmid KPcat-F contains the Sal I DNA fragment with the *airS* gene promoter in the forward orientation in the plasmid pKK232-8, and the plasmid KPcat-R contains the Sal I DNA fragment in the reversed orientation in plasmid pKK232-8. Plasmids were transformed into wild-type DS17, grown in LB or CFA broth, and attachment experiments were performed as described in (*8*). Bacteria were lysed by sonication, and the cell extracts were used for CAT assay as described in (*23*).

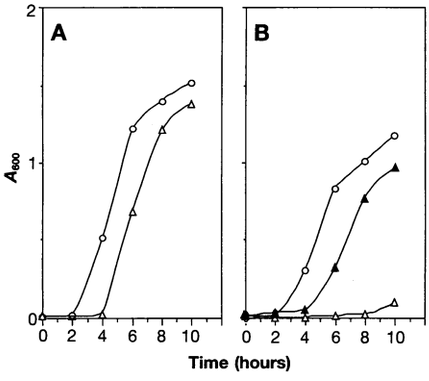

Fig. 4. Growth curves of DS17 and the isogenic *airS* mutant in different culture media at 37°C. (**A**) DS17 (open circles) and the *airS* mutant (open triangles) grown in LB media. (**B**) DS17 (open circles) and the *airS* mutant (open triangles) grown in CFA media, and the *airS* mutant (closed triangles) grown in CFA media supplemented with 1 mM $FeCl_3$. Absorbance (*A*) was measured at a wavelength of 600 nm.

been shown that the expression of the siderophore receptor PfeA is mediated by both the Fur (ferric uptake regulation) protein and a classic two-component regulatory system in *Pseudomonas putida* (*15*). The AirS protein has amino acid sequence similarities to the sensor-regulator proteins BvgS (54%) of *Bordetella pertussis* and LemA (56%) of *Pseudomonas syringae* (*16*). Structurally, AirS is also similar to BvgS and LemA as they all contain both an autophosphorylated histidine site (transmitter module) and a phospho-accepting aspartate site (receiver module) in their primary amino acid sequences (*7*). As part of a global regulatory network, BvgS and LemA have each been shown capable of responding to various environmental signals encountered, resulting in the coordinate regulation of transcription of their virulence factors during the bacterial infectious cycle (*14, 16*).

The ability to obtain adequate iron from the environment is closely linked to bacterial virulence. Clinical isolates of bacteria with iron-acquisition systems have enhanced virulence in animals. Additionally, aerobactin-producing strains of uropathogenic *E. coli* are more lethal in an animal model of ascending urinary tract infection than non–aerobactin-producing strains (*17*). In vivo, aerobactin has been detected in urine from patients with *E. coli* urinary tract infection (*18*). Furthermore, serum from patients with *E. coli* bacteriuria have antibodies against IRMPs (*19*). The rapid transcriptional activation of *airS* upon P-pili attachment and the requirement of *airS* for activation of the siderophore system indicate that *airS* may direct the coordinate regulation of uropathogenic *E. coli*'s iron-acquisition machinery in the urinary tract of the host.

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6. Attachment and RT-PCR procedures were as follows: DS17 or DS17-8 were grown at 37°C in LB broth overnight, and 1 ml of overnight culture was inoculated into 9 ml of fresh LB broth. Cultures were incubated for 3 hours more, and 0.5 ml of 10% glutaraldehyde-stabilized human group O RBCs or rabbit RBCs were added to the cultures. After 30 min to allow for attachment, cells were pelleted, and the pellets of bacteria and RBCs were lysed in 1 ml of TRIzol total RNA isolation reagent (Gibco BRL). Total RNA isolation was continued according to TRIzol instructions. Approximately 2 mg of total RNA harvested from each sample was reverse-transcribed and then used as templates for PCR with a nonspecific set of primers as described in (*5*). The PCR parameters were 94°C for 1 min, 42°C for 1 min, and 72°C for 1 min for a total of 40 cycles, followed by 10 min of elongation at 72°C.
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8. Specific attachment and RT-PCR procedures were as follows: Total RNA isolations were as described in (*6*). Approximately 2 mg of total RNA harvested from each sample was reverse-transcribed and then used as templates for PCR with the *airS* primer set. The primer sequences were as follows: airS-1 (5′-CAGCACGCTGGATGAAGTCGT-3′) and airS-2 (5′-AGCGCGATTGATCGCGTTCAGGAAT-3′). For Fig. 1, B and C, petri dishes (100, 15 mm in diameter) were coated overnight at 4°C with 200 mg of GbO_4 or CDH dissolved in 3 ml of methanol. The DS17 or DS17-8 strains were grown in LB broth overnight at 37°C, and 1 ml of overnight culture of each was inoculated into 9 ml of fresh LB broth. Bacteria were grown for another 3 hours and then centrifuged. Nine milliliters of culture supernatant was removed, and the bacterial pellets were resuspended in the remaining 1 ml of culture media. The bacterial suspensions were added to the pre-coated petri dishes at 37°C for 30 min with gentle shaking (60 rpm). Bacteria were lysed on the plates with 5 ml of TRIzol solution, and total RNA was isolated as above. Specific *airS* primers are listed above. The pyruvate dehydrogenase primers were pdh-1 (5′-TTGCTCGAAACACGACACGCCCT-3′) and pdh-2 (5′-ACGGCAATCTGATACTGGAGTAC-3′). PCR products were resolved on 8% polyacrylamide gels.
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Pycnodysostosis, a Lysosomal Disease Caused by Cathepsin K Deficiency

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Pycnodysostosis, an autosomal recessive osteochondrodysplasia characterized by osteosclerosis and short stature, maps to chromosome 1q21. Cathepsin K, a cysteine protease gene that is highly expressed in osteoclasts, localized to the pycnodysostosis region. Nonsense, missense, and stop codon mutations in the gene encoding cathepsin K were identified in patients. Transient expression of complementary DNA containing the stop codon mutation resulted in messenger RNA but no immunologically detectable protein. Thus, pycnodysostosis results from gene defects in a lysosomal protease with highest expression in osteoclasts. These findings suggest that cathepsin K is a major protease in bone resorption, providing a possible rationale for the treatment of disorders such as osteoporosis and certain forms of arthritis.

Recent interest in developing treatments for osteoporosis and certain forms of arthritis has stimulated efforts to identify the gene defects underlying certain inherited sclerosing skeletal dysplasias. Among these, pycnodysostosis (Pycno) is a rare, autosomal recessive trait characterized by osteosclerosis, short stature, acro-osteolysis of the distal phalanges, bone fragility, clavicular dysplasia, and skull deformities with delayed suture closure (*1*). The disease has gained attention as the French painter, Henri de Toulouse-Lautrec, was retrospectively diagnosed as having Pycno, a diagnosis that has been the subject of recent debate (*2*).

Bone resorption, a process mediated by osteoclasts, is characterized by the solubilization of inorganic mineral and subsequent proteolytic degradation of organic matrix, primarily type I collagen. In Pycno, osteoclast numbers are normal as are their ruffled borders and clear zones, but the region of demineralized bone surrounding individual osteoclasts is increased (*3*). Ultrastructural examination of these osteoclasts reveals large, abnormal cytoplasmic vacuoles containing bone collagen fibrils. These findings

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