

Isolation of a Nicotinamide-Requiring Clone of *Escherichia coli* O18:K1:H7 from Women with Acute Cystitis: Resemblance to Strains Found in Neonatal Meningitis

Calvin M. Kunin, Tong Hua Hua,
Chandradekar Krishnan, Laura Van Arsdale White,
and Jorg Hacker

From the Department of Internal Medicine, The Ohio State University, Columbus, Ohio; the Central Public Health Laboratory, Toronto, Ontario, Canada; and the Theodor-Boveri-Institut für Biowissenschaften der Universität Würzburg, Würzburg, Germany

During a study of the nutritional requirements of clinical isolates of *Escherichia coli*, we found that 21 (7.0%) of 301 strains required nicotinamide to grow in minimal medium. The nicotinamide-requiring strains were present in 16 (15.8%) of 101 cultures of urine from young women with acute cystitis, in 5 (5.0%) of 100 stool specimens from healthy adults, and in none of 100 blood samples from adult patients with bacteremia. Most of the strains belonged to serogroup O18:K1:H7, were hemolytic, possessed type 1 fimbriae, and exhibited similar patterns of antibiotic susceptibility. Two of the urinary isolates expressed S fimbriae, and all 16 urinary isolates contained the *sfaS* homologue gene on their chromosomes. One of the stool isolates contained the *sfaS* gene. The urinary isolates closely resembled a large clone of *E. coli* that is reportedly associated with neonatal meningitis and sepsis. It may be possible to detect this and related clones by their requirement for nicotinamide and to screen strains for S fimbriae by relatively inexpensive hemagglutination methods, including the use of avian P₁ antigens to detect mannose-resistant, non-P-fimbriated *E. coli*; the agglutination of bovine erythrocytes; and the use of bovine mucin to detect sialyl galactosides in S fimbriae.

Most strains of *Escherichia coli* grow in minimal media containing salts, trace metals, and a simple source of carbon and nitrogen. Naturally occurring auxotrophic variants have been isolated from patients with urinary tract infections, neonatal meningitis, and sepsis [1-7]. The most common nutritional requirement is for nicotinamide [1-3], but some strains need cysteine, thiamine, thymidine, glutamine, various amino acids [4-6], or horse serum [7]. In addition, most naturally occurring temperature-sensitive auxotrophs of *E. coli* require nicotinamide for growth at 42°C [8, 9].

During a study of the nutritional and osmotic requirements of clinical isolates of *E. coli* [9-11], we isolated a group of strains that required nicotinamide for growth in minimal medium from the urine of young women with acute cystitis and from stools of healthy subjects. Most of these strains were hemolytic and belonged to serogroup O18:K1:H7. This serogroup reportedly is associated with neonatal meningitis and sepsis [3, 12] and possesses both type 1 and S fimbriae. In this report we present evidence that the nicotinamide-requiring strains of *E. coli* O18:K1:H7 isolated from the urine of young adults contain either fully expressed S fimbriae or the *sfaS* homologue gene on their chromosomes

and resemble strains causing neonatal meningitis. This observation strongly suggests that these two groups of strains belong to the same clone. The requirement for nicotinamide may provide a useful epidemiological marker for this and related clones.

Materials and Methods

Strains of *E. coli*. Urinary isolates of *E. coli* were obtained from 101 young women with acute urinary tract infection who were seen consecutively at the Ohio State University Student Health Center. All urine specimens contained >10⁵ cfu of *E. coli*/mL. Isolates of *E. coli* were also obtained from cultures of blood from 100 patients; these isolates were provided by the clinical microbiology laboratories of the Ohio State University Hospitals and several local community hospitals. Finally, isolates of *E. coli* were obtained from stool samples from 100 healthy young adults (male and female student volunteers) and laboratory personnel. These samples were collected by rectal swab, diluted in 1 mL of phosphate-buffered saline (PBS, pH 7.2), and inoculated onto MacConkey agar plates. After overnight growth stock cultures obtained from a single colony of each isolate were stored at -40°C in Schaedler's broth containing 15% glycerin (Difco, Detroit).

Media and chemicals. Minimal medium was composed of glucose (2.0 g/L), dipotassium phosphate (10.5 g/L), monopotassium phosphate (4.5 g/L), sodium citrate (0.5 g/L), magnesium sulfate (0.264 g/L), and ammonium sulfate (1.0 g/L) [13]. The pH of the medium was adjusted to 7.2

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Reprints or correspondence: Dr. Calvin M. Kunin, Department of Medicine, The Ohio State University, Starling Loving Hall, Room M110, 320 West 10th Avenue, Columbus, Ohio 43210-1240.

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before use; the osmolarity was 238 mOsm/kg. In some experiments minimal medium was supplemented with 0.1–1.0 M NaCl, or minimal essential medium containing nonessential amino acids (20 mL/L), or minimal essential medium containing essential amino acids (40 mL/L) or individual amino acids, nicotinic acid, nicotinamide, quinolinic acid, trigonelline, and N¹ methylnicotinamide (Sigma Chemical, St. Louis). Chicken eggs were purchased from a local store. Cockatiel and pigeon eggs were obtained from local breeders. Other media used included trypticase soy broth, trypticase soy agar, brain-heart infusion broth (BBL, Cockeysville, MD), and CFA agar (composed primarily of casamino acids and yeast extract) [14].

Growth conditions. A single colony was isolated on MacConkey agar plates and grown overnight at 37°C in minimal medium broth. The inoculum, consisting of a 1:20 dilution of a 0.5 McFarland standard (final concentration, $\sim 5 \times 10^5$ cfu/mL), was added to tubes containing 0.9 mL of minimal medium. The tubes were incubated at 37°C and 42°C for 48 hours. Optical density was determined at 600 nm with a Spectronic 601 spectrophotometer (Milton Roy, Rochester, NY).

Serological typing. One hundred of the strains were characterized by O, H, and K1 antigens and by hemolysin production at the Central Public Health Laboratory of the Ontario Ministry of Health in Toronto.

Identification of type 1, P, and S fimbriae. For the identification of type 1 mannose-sensitive fimbriae, *E. coli* was grown in brain-heart infusion broth for 48 hours at 37°C. The cells were harvested by centrifugation, washed twice, and prepared as a suspension containing $\sim 5 \times 10^{10}$ cfu/mL. Guinea pig blood was collected in heparinized tubes. The erythrocytes were washed twice in PBS and resuspended at a 3% concentration. Hemagglutination was measured at room temperature by gentle mixing for 5 minutes on a glazed white ceramic tile in the presence or absence of an equal volume of 3% D-mannose.

For the identification of P fimbriae, *E. coli* was grown overnight at 37°C on CFA agar plates. The cells were scraped from the surface of the plates, washed twice in PBS, and prepared as a suspension containing $\sim 5 \times 10^{10}$ cfu/mL. Human O erythrocytes were prepared from blood collected in heparinized tubes. The cells were washed three times in PBS and resuspended at a final concentration of 5%. Hemagglutination was evaluated on glass slides rotated at 60 revolutions per minute over ice, with or without an equal volume of 5% α -methyl-D-mannoside or other inhibitors. The presence of mannose-resistant P fimbriae was confirmed by use of latex particles on which α -D-Gal(-4)- β -D-Gal-disaccharide was covalently bonded (PF test, Orion Diagnostica, Espoo, Finland) [15].

Mannose-resistant *E. coli* was considered to lack P fimbriae if the PF test with digalactoside-coated latex beads gave negative results and if hemagglutination was blocked by pi-

geon and cockatiel (but not chicken) egg white [16]. Each experiment included the following controls: (1) boiling of the strain for 5 minutes to destroy fimbrial proteins; (2) testing of a strain of *E. coli* O27:H31 that contained type 1 fimbriae only; and (3) testing of a mannose-resistant strain of *E. coli* O2:H7 that possessed P fimbriae detected by the PF test.

Evidence that the strains possessed S fimbriae was based on the ability of fetuin and bovine submaxillary mucin (Sigma Chemical) to block mannose-resistant hemagglutination. The presence of S fimbriae was confirmed by hemagglutination of bovine erythrocytes with and without 2% mannose [17] and by whole-cell ELISA [18] employing a monoclonal antibody (mAb A1) specific for the S adhesin SfaS obtained by K. Jann Freiburg [19]. A DNA probe specific for the gene *sfaS*, which codes for the sialic acid-specific adhesin, was generated by polymerase chain reaction with a suitable oligonucleotide primer pair on the basis of the nucleotide sequences of *sfaS*. The DNA probe was labeled with ³²P by means of the random priming system [20] (Boehringer Mannheim, Germany). Colony dot hybridization was undertaken as described previously [21]. *E. coli* strains 536 (O6:K15) and IHE3034 (O18:K1) as well as the clone HB101/pANN801-13 carrying the cloned *sfa* determinant were used as controls.

Antimicrobial susceptibility testing and identification of species. Tests of antimicrobial susceptibility and species identification were performed in standardized microwell dilution plates prepared by the Clinical Microbiology Laboratory of the Ohio State University. The panel contained 16 antimicrobial drugs, including aminoglycosides; ampicillin, amoxicillin, and ticarcillin with and without clavulanate or sulbactam; mezlocillin; cefazolin; ceftizoxime; ceftazidime; imipenem; nitrofurantoin; and trimethoprim-sulfamethoxazole.

Analysis of data. The data were entered into the Epi Info program, Version 5 (Centers for Disease Control, Atlanta), and were analyzed by the χ^2 test [22].

Results

Isolation of nicotinamide-requiring strains of *E. coli*. The 301 isolates obtained from blood, urine, and stool were tested for their ability to grow at 37°C in minimal medium with and without added 0.1 M increments of NaCl. Isolates that failed to grow were retested in medium containing amino acid mixtures, yeast extract, thiamine, or nicotinamide. Twenty-one (7.0%) of the clinical isolates failed to grow in minimal medium, even with added NaCl, unless 0.1 mM nicotinic acid or nicotinamide was added. These isolates were quite distinct from osmo-remedial variants of *E. coli* that require nicotinamide to grow in hypotonic medium [10].

Nicotinamide-requiring strains of *E. coli* were isolated from 16 (15.8%) of the 101 urine samples from women with acute cystitis and from five (5.0%) of the 100 stool specimens

Table 1. Compounds that supported the growth in minimal medium of nicotinamide-requiring strains of *E. coli* O18:K1:H7.

Compound	Minimal concentration (M) supporting growth
Nicotinamide	10 ⁻⁷
Nicotinic acid	10 ⁻⁶
N ¹ methylnicotinamide	10 ⁻⁴
Quinolinic acid	10 ⁻³
Trigonelline	10 ⁻³

NOTE. Aspartic acid, glutamic acid, glutamine, and oxalacetic acid (10⁻³ M) failed to support the growth of these strains in minimal medium.

from healthy adults ($P < .0001$). No such isolates were recovered from the 100 blood samples tested. All of 15 nicotinamide-requiring strains tested were found to be hemolytic and to belong to serogroup O18:K1:H7. These 15 isolates were uniformly susceptible to several β -lactam antibiotics, aminoglycosides, tetracycline, nitrofurantoin, and ciprofloxacin.

The intermediate metabolites of nicotinamide that supported the growth of these strains are shown in table 1. Nicotinamide was the most active; next were nicotinic acid, N¹ methylnicotinamide, quinolinic acid, and trigonelline.

Characterization of fimbriae of nicotinamide-requiring strains. That all nicotinamide-requiring strains of *E. coli* O18:K1:H7 contained type 1 fimbriae was shown by mannose-sensitive agglutination of guinea pig erythrocytes. The lack of P fimbriae in these strains was documented with digalactoside-coated beads. Twelve (75%) of the 16 urinary isolates and 2 of the 5 stool isolates exhibited mannose-resistant hemagglutination of human O erythrocytes. Hemagglutination was not blocked by a 1:8 dilution of cockatiel or pigeon egg white, which is known to contain P₁ antigens [16]. In contrast, hemagglutination of P-fimbriated strains of *E. coli* O2:H7 was completely blocked by 1:32 and 1:64 dilutions of cockatiel and pigeon egg white, respectively, but not by undiluted chicken egg white. Bovine submaxillary mucin containing a final concentration of 1.1 mM free *N*-acetylneuraminic acid (NANA) and fetuin containing 1.6 mM free NANA blocked hemagglutination of four representative strains in the presence of 2.5% α -methyl-D-mannoside. These studies provided preliminary evidence that the strains possessed S fimbriae based on the finding of non-P, mannose-resistant adhesins that were blocked by sialyl galactosides [15, 23].

To confirm the presence of S fimbriae or their chromosomal gene, all of the strains were examined for their ability to agglutinate bovine erythrocytes; an ELISA with S fimbria-specific monoclonal antibodies and hybridization with an *sfaS* gene probe were used. Two of the urinary isolates possessed S fimbriae detectable by both agglutination of bovine erythrocytes and ELISA. All 16 urinary isolates contained

the *sfaS* gene. None of the five stool isolates expressed S fimbriae, and only one contained the *sfaS* gene.

Other clinical isolates and auxotrophic strains. Several other strains of *E. coli* O18 that did not require nicotinamide for growth were isolated. These strains were of serogroups O18:H1 (four strains) and O18:NM (one strain). They possessed neither the K1 antigen nor P fimbriae and were not hemolytic.

Twelve (4.0%) of the 301 clinical isolates (serogroups O27:K1:H31 and O2:H7) were osmo-remedial; that is, they failed to grow in minimal medium unless the osmolality was raised by addition of NaCl, sucrose or mannitol, or nicotinamide. These strains are characterized in a separate report [10]. The nutritional requirement of one additional strain could not be determined. This was a blood isolate of *E. coli* O1:H4 that grew only in trypticase soy broth or in 1% yeast extract broth; it was resistant to trimethoprim-sulfamethoxazole and to gentamicin but not to any of the other antimicrobial drugs tested.

Discussion

A clone of hemolytic, nicotinamide-requiring *E. coli* O18:K1:H7 that possessed type 1 and S fimbriae or the *sfaS* gene was isolated from the urine of 15.8% of young women with acute cystitis. A similar strain was recovered from stool samples from five of 100 healthy individuals but was not represented among 100 blood culture isolates of *E. coli*. This unusual clone is of special interest because of its association with neonatal meningitis and sepsis. Korhonen et al. [12] reported that *E. coli* O18:K1:H7 was the clone isolated most often from children with these diseases. The strains from these children possessed both type 1 and S fimbriae but did not produce hemolysin. A requirement for nicotinamide was not assessed. Both the K1 capsule and S fimbriae appear to be important virulence factors in *E. coli* neonatal bacteremia and meningitis [12, 24, 25]. S fimbriae bind to the vascular endothelium of the brain and to the epithelial lining of the choroid plexuses and brain ventricles [26, 27]. The frequent association of this clone with neonatal meningitis raises the possibility that children of mothers who carry this strain in their urine or stool may be at increased risk of meningitis [24].

We were surprised to find that only two of the 16 urinary isolates of *E. coli* O18:K1:H7 expressed S fimbriae on their surface, but all contained the *sfaS* gene. It is possible that S fimbriae undergo phase variation and that the relevant genetic information was not transcribed under the conditions in which the strains were isolated and grown. Phase variation is well described for type 1 fimbriae [28] and may be important for S fimbriae as well.

We confirmed the observations of Johnson et al. [16] that cockatiel and pigeon egg white blocks hemagglutination by P-fimbriated *E. coli*. This method provides a relatively inex-

pensive means of detecting mannose-resistant, non-P-fimbriated strains. Bovine mucin is also helpful for the preliminary identification of mannose-resistant S-fimbriated strains [23].

We were somewhat surprised to isolate a relatively large number of strains of *E. coli* O18 from women with acute cystitis. The most common O serogroups of *E. coli* in outpatients with urinary tract infection are 1, 2, 4, 6, 7, 25, 50, and 75 [29–33]. Group O18, although less common, is represented in several large collections of isolates from the urinary tract [3, 32, 33]. *E. coli* O18 accounted for 18 (3.3%) of 539 blood culture isolates in one study in Denmark [34]. Nine of these strains (1.7% of the total) were of serogroup O18:H7. Thus it is not surprising that we did not find this serogroup among blood culture isolates.

Nicotinamide-requiring strains of *E. coli* are the most common auxotrophs in humans [1–3]. In a survey of naturally occurring auxotrophic mutants of *E. coli*, Robeson et al. [1] found that 9% of 548 strains were auxotrophs. Of these, 77% were niacin-requiring strains isolated mostly from patients with urinary tract infections. In view of the relative abundance of nicotinamide-requiring strains of *E. coli* in urinary tract infections, urine would be expected to contain substances supporting their growth. Microbiologically active nicotinic acid and nicotinamide are present in human urine [35]. One of the major urinary metabolites is of nicotinamide N¹ methyl nicotinamide. Trigonelline is also found in urine; its presence may be related to smoking and to intake of coffee and legumes. All of these compounds as well as quinolinic acid (a precursor of nicotinic acid) supported the growth of the nicotinamide-requiring strains in our study.

Achtman et al. [3] described three nicotinamide-requiring clones of *E. coli*. One of these was a hemolysin-producing O18:K1 strain (membrane pattern 6) isolated from patients with neonatal meningitis, urinary tract infection, or sepsis. A second clone of O18:K1 (membrane pattern 9) was nonhemolytic and was not associated with these diseases. A third clone was of the O1:K1 serogroup (membrane pattern 9); this nonhemolytic clone was isolated from patients with urinary tract infections and from patients with neonatal meningitis as well as from the feces of healthy individuals. The authors noted that “the apparent association of hemolysin production or nicotinamide-requirement with disease may reflect the existence of a limited number of bacterial clones among disease isolates and the coincidental expression of certain properties by these clones.”

Although most of the isolates of *E. coli* O18:K1:H7 described in this study were from the urine of young women with acute cystitis, the reservoir for such strains is more likely to be in the stool. Since these strains can cause urinary tract infections and can be obtained in relatively pure culture from the urine, it is reasonable to assume that they can be isolated more readily from patients with urinary tract infections than from the mixed flora of the stool. More detailed

studies are needed to determine the endemicity of this clone in human populations.

It may be possible to use several inexpensive and readily available methods to screen for strains of *E. coli* that have the potential to produce neonatal meningitis and sepsis. These methods include assessment of the requirement for nicotinamide for growth in minimal medium; detection of mannose-resistant, non-P fimbriae with pigeon or cockatiel egg white [16]; and assay for agglutination of bovine erythrocytes. The ultimate value of these markers as epidemiological tools needs to be determined.

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