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DOMAIN 8 PATHOGENESIS

Molecular Epidemiology of Extraintestinal Pathogenic *Escherichia coli*

JAMES R. JOHNSON[1,2] AND THOMAS A. RUSSO[3]

[1]VA Medical Center, Minneapolis, MN 55417

[2]Department of Medicine, University of Minnesota, Minneapolis, MN 55455

[3]VA Western New York Healthcare System, Department of Medicine, Department of Microbiology and Immunology, Witebsky Center for Microbial Pathogenesis and Immunology, University of Buffalo, Buffalo, NY 14214

ABSTRACT Extraintestinal pathogenic *Escherichia coli* (ExPEC) are important pathogens in humans and certain animals. Molecular epidemiological analyses of ExPEC are based on structured observations of *E. coli* strains as they occur in the wild. By assessing real-world phenomena as they occur in authentic contexts and hosts, they provide an important complement to experimental assessment. Fundamental to the success of molecular epidemiological studies are the careful selection of subjects and the use of appropriate typing methods and statistical analysis. To date, molecular epidemiological studies have yielded numerous important insights into putative virulence factors, host-pathogen relationships, phylogenetic background, reservoirs, antimicrobial-resistant strains, clinical diagnostics, and transmission pathways of ExPEC, and have delineated areas in which further study is needed. The rapid pace of discovery of new putative virulence factors and the increasing awareness of the importance of virulence factor regulation, expression, and molecular variation should stimulate many future molecular epidemiological investigations. The growing sophistication and availability of molecular typing methodologies, and of the new computational and statistical approaches that are being developed to address the huge amounts of data that whole genome sequencing generates, provide improved tools for such studies and allow new questions to be addressed.

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Correspondence: James R. Johnson: johns007@umn.edu

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INTRODUCTION

Extraintestinal pathogenic *Escherichia coli* (ExPEC), the distinctive group of *E. coli* strains that possess an enhanced ability to overcome or subvert host defenses and cause extraintestinal disease in otherwise healthy hosts, are important pathogens in humans and certain animals (1, 2). Molecular epidemiological analyses contributed to the initial recognition of ExPEC strains as being distinct from other *E. coli* (including intestinal pathogenic and commensal variants), and have yielded insights into the ecology, evolution, reservoirs, transmission pathways, host-pathogen interactions, and pathogenic mechanisms of ExPEC, thereby providing an essential complement to experimental assessment of virulence mechanisms. This article first reviews the basic conceptual and methodological underpinnings of the molecular epidemiological approach, then summarizes the main aspects of ExPEC that have been investigated by using this approach.

The concept of ExPEC as representing a discrete microbiological subset of *E. coli* with special relevance to human and animal health has stimulated efforts to identify the bacterial correlates of extraintestinal virulence that determine, whether mechanistically or for classification purposes, a given strain's likelihood of representing ExPEC. As discussed below in "Results of Molecular Epidemiological Studies," such efforts are ongoing, and are increasingly informative thanks to the progressive advances in epidemiological methods and study design, typing technologies, and biostatistical approaches.

METHODOLOGICAL CONSIDERATIONS

Basic Principles of Molecular Epidemiology

Traditional versus molecular epidemiology

Epidemiology is the study of disease as it occurs in populations. In epidemiological studies, structured observations are used to identify host characteristics (that is, risk factors) that predict the occurrence, severity, or clinical manifestations of a particular illness (3–8). By analogy, molecular epidemiology is the study of a disease in relation to selected genetic characteristics of the host and/or the causative agent, such as an infectious disease and the causative microorganisms (9–14). In molecular epidemiological studies that focus on infectious diseases, structured observations are used to identify microbial traits (for example, specific genes, phylogenetic background, or clonal identity) that predict the occurrence, severity, or clinical manifestations of a particular infectious disease, or relevant characteristics of the affected hosts, including age, sex, and underlying predisposing conditions. Molecular epidemiological studies seek insights into the molecular basis for the virulence behavior and host predilections of the pathogen and to identify relevant reservoirs and transmission pathways. Such insights can be useful in developing strategies for managing and preventing infections caused by the particular pathogen.

Strengths and limitations of molecular epidemiology

By virtue of being observational rather than experimental, molecular epidemiological studies exhibit the strengths and limitations inherent to observational studies in general. Their main strength is that they examine "real world" phenomena, that is, wild-type microbes interacting with the natural host in a natural setting, rather than the artificially engineered host-pathogen interactions of experimental studies (15, 16), which can be of uncertain physiological relevance. In addition, molecular epidemiological studies can examine multiple predictor and outcome variables simultaneously, which can be challenging with experimental studies.

The main weakness of molecular epidemiological studies is that they allow the investigator no direct control over the variables analyzed. Consequently, a variable can be isolated only through careful selection of comparison groups so that the groups differ, to the extent possible, only according to the particular variable. Moreover, even with the most carefully selected comparison groups, associations that may emerge still are only that, associations. Although these may reflect causal relationships between the analyzed variables, they also may be due to confounding from other, unmeasured variables (in either the host or the pathogen). Consequently, the better characterized the source subjects and bacteria are, the greater the confidence with which associations between host variables and bacterial traits can be attributed to the particular variables themselves. In addition, because of the considerable variation within human and bacterial populations, large numbers of subjects are needed per group (relative to the number of replicate determinations needed to address variance in experimental studies), appropriate statistical tests are required to assess the significance of any observed differences between groups, and statistically significant findings require confirmation in different populations.

Finally, at least prior to the advent of whole-genome sequencing (WGS: discussed below) and transcriptome analysis, molecular epidemiological studies could assess only known microbial characteristics for which appropriate assays were available. That is, they required prior knowledge of the characteristics to be studied. Therefore, in contrast to exploratory methods such as signature-tagged mutagenesis (17), *in vivo* expression technology (18), and transcriptome analysis (19), they could not be used to discover new virulence factors. However, they provided an important complement to such discovery-based experimental approaches by assessing the epidemiological (that is, population-level) relevance of newly identified traits (20–23). This complementarity between epidemiology and experimentation is implicit in the molecular restatement of Koch's postulates, the first of which is that the trait of interest must be epidemiologically associated with disease (24). Today, WGS makes it possible, in principle, to compare an extensive array of bacterial sequences, many of which may be of unknown

significance or function, with epidemiological variables, thereby facilitating the unbiased discovery of novel bacterial correlates of virulence, which subsequently can be assessed experimentally.

Internal controls

Much stronger conclusions can be drawn from observed between-group differences in molecular characteristics if a study includes an internal control group that is tested in parallel with the clinical group of interest, in particular, if the controls are temporally, geographically, and demographically matched to the clinical isolates, thereby avoiding some of the problems associated with use of external (that is, historical) control groups (25, 26). Concurrent testing of cases and controls using the same methods and reagents, ideally by an operator who is unaware of sample identity, reduces the likelihood that cohort effects, technical factors, or subjective bias could influence the results.

Statistical considerations

Molecular epidemiological studies adhere to the same statistical principles and rely on the same statistical methods as conventional epidemiological studies (27, 28). Between-group comparisons are tested using standard statistical approaches such as a χ^2 test, Fisher's exact test, or univariate logistic regression analysis for dichotomous variables, and an unpaired *t* test or the Mann-Whitney U test for continuous variables. Multiple independent variables can be assessed simultaneously as predictors of an outcome (-dependent) variable by using appropriate multivariate methods. For comparisons involving multiple testing of a particular (bacterial or human) subject, whether for different traits as assessed at the same time or for a given trait as assessed at different times, appropriate tests for paired comparisons must be used, such as McNemar's test for dichotomous variables and a paired *t* test or the Wilcoxon rank-sum test for continuous variables. Additionally, the ever-growing number of bacterial variables that can be determined and analyzed has led to increased use of multidimensional scaling methods, such as principle coordinates analysis and principle components analysis, which summarize the variance within complex data sets using a small number of derived variables, termed coordinates or components. Such methods determine and display graphically the overall extent of similarity or difference between source populations with respect to multiple variables when considered jointly, and the extent of correlation among different variables and between each variable and respective source populations. Other advanced statistical methods that can be used with complex data sets to identify bacterial correlates of epidemiological variables include discriminant analysis (29), classification and regression tree analysis, and machine learning (30).

Type I errors, which are the false conclusion of a difference when none actually exists, are a hazard of the use of multiple comparisons (since the probability of obtaining a "significant" *P* value is proportional to the number of comparisons) and the selective testing of associations suggested by *post hoc* data review (31). However, multiple comparisons are inevitable in molecular epidemiological studies that assess multiple bacterial traits, as increasingly is the practice, and are especially prominent with WGS analysis, which may generate thousands of candidate predictor variables. Statistical adjustment for multiple comparisons, and/or cautious interpretation of putatively significant associations, can be used to address this problem (32). Likewise, *post hoc* data review to discover new associations is an important means for generating new hypotheses. Recognition that such hypotheses require independent confirmation provides a helpful safeguard against false conclusions.

Type II errors, which are the false conclusion of absence of a difference when one actually exists, result from insufficient sample size, which limits statistical power for finding differences (33). However, the seemingly obvious remedy of studying large comparison groups may or may not be an option, depending on the context. This is because, unlike in experimental studies where the number of replicate determinations is largely a matter of investigator choice, in epidemiological studies clinical factors sometimes limit the number of subjects or isolates available for a particular group, thereby imposing insurmountable restrictions on sample size (34). As a consequence, conclusions may need to be tempered to reflect the inherent uncertainty resulting from limited power.

Typing Methods

The various bacterial traits analyzed in molecular epidemiological studies that attempt to define or characterize ExPEC represent a spectrum of levels of organization and complexity, ranging from subgenic DNA sequence (the most basic), through genes, operons, and pathogenicity islands (intermediate), through whole genomes and plasmids (complex), to clones, clonal groups, and phylogenetic groups (highly complex) (35, 36). Each level is

important and informative, and each requires distinctive typing methods.

Sequence analysis

Historically, analysis of sequence diversity within virulence-associated genes or their flanking regions was done by using restriction fragment length polymorphism (RFLP) analysis (now little used) or direct Sanger sequencing of cloned DNA fragments or PCR products (37–44). The increasing accessibility and affordability of next-generation DNA-sequencing methods, e.g., Illumina (short reads) and PacBio (long reads, including for plasmid closure), now allows sequence analysis of whole chromosomes and plasmids (45, 46), providing an alternative or complement to conventional Sanger sequencing. Sequencing technologies, and the associated bioinformatics approaches needed to manipulate and interpret the resulting large-sequence files, are evolving rapidly, and may soon supplant conventional methods even for gene detection, let alone detailed sequence analysis.

Sequence analysis has multiple applications in molecular epidemiological studies (as discussed below). These include determination of the presence/absence of specific putative virulence genes or resistance genes and molecular variants thereof, identification of single-nucleotide polymorphisms in housekeeping genes that correspond with specific ExPEC-associated lineages, and clarification of the genetic architecture of mobile units (e.g., transposons and plasmids) that can transfer virulence and resistance genes between strains and lineages.

Gene detection

Detection of putative virulence markers, historically a mainstay of molecular epidemiology in *E. coli*, can be done using a variety of methods, with the method selected determining the nature of the results, which in turn shapes the conclusions that can be drawn. Conventional membrane or solid-support probe hybridization, which has largely been superseded by newer technologies, relies on complementarity between the probe, often several kilobases in length, and the target region (9, 37, 47). It can readily screen for broad genetic regions if large probes are used (40). Probes can be used either in solution for hybridization with target DNA that has been fixed to a solid substrate, or fixed to a solid substrate (using a macro- or microarray format) for hybridization with target DNA in solution (48).

PCR detection relies on precise matching between the primers and the target region, and usually is limited to relatively short targets, typically <2 kb (49). Thus, if the relevant sequence polymorphisms are known, PCR can differentiate between minor molecular variants of a particular gene (which may have different disease and/or host associations) and, if multiple primer pairs are used to map an operon, can identify suboperonic deletions (which also may have clinical correlates) (Fig. 1) (50). PCR can be done using either conventional or real-time methods, and with multiplexing of primers for simultaneous detection of multiple targets. Detection of conventional PCR products is usually done using agarose gel electrophoresis, fluorescence or luminescence-based real-time technology, or membrane-bound arrays (51).

WGS analysis is a rapidly emerging technology for detection of known virulence (and other) genes in *E. coli*, and may become the preferred genotyping method as sequencing prices drop and bioinformatics pipelines become increasingly robust, accessible, and user friendly (36, 45, 52–55). For this approach, enormous numbers of sequence reads of various lengths (depending on the particular sequencing method) are generated using one of several available massively parallel sequencing technologies.

Reads are analyzed by using (i) user-selected applications and a private sequence library; (ii) a web-based suite of applications, such as that hosted by the Center for Genomic Epidemiology (Danish Technical University, Copenhagen, DK: http://www.genomicepidemiology.org/); or (iii) a hybrid of these two approaches. Use of web-based platforms offers simplicity and speed, but limits the analysis to whatever markers the particular system happens to include. In contrast, manual analysis allows the user to search for any target sequence of interest, but is more technically demanding. Additionally, as mentioned in an earlier section, by using more sophisticated bioinformatics approaches, it is possible to identify and catalogue sequences of unknown identity that are present in an isolate, for comparison with other isolates from the same or different contexts. The huge amounts of data that WGS generates pose analytical challenges that oblige the development of new computational and statistical approaches (56, 57).

Clones, clonal groups, and phylogenetic groups

Clones and clonal groups (which are groups of closely related clones) can be identified at the molecular level by using typing methods that scan the entire genome.

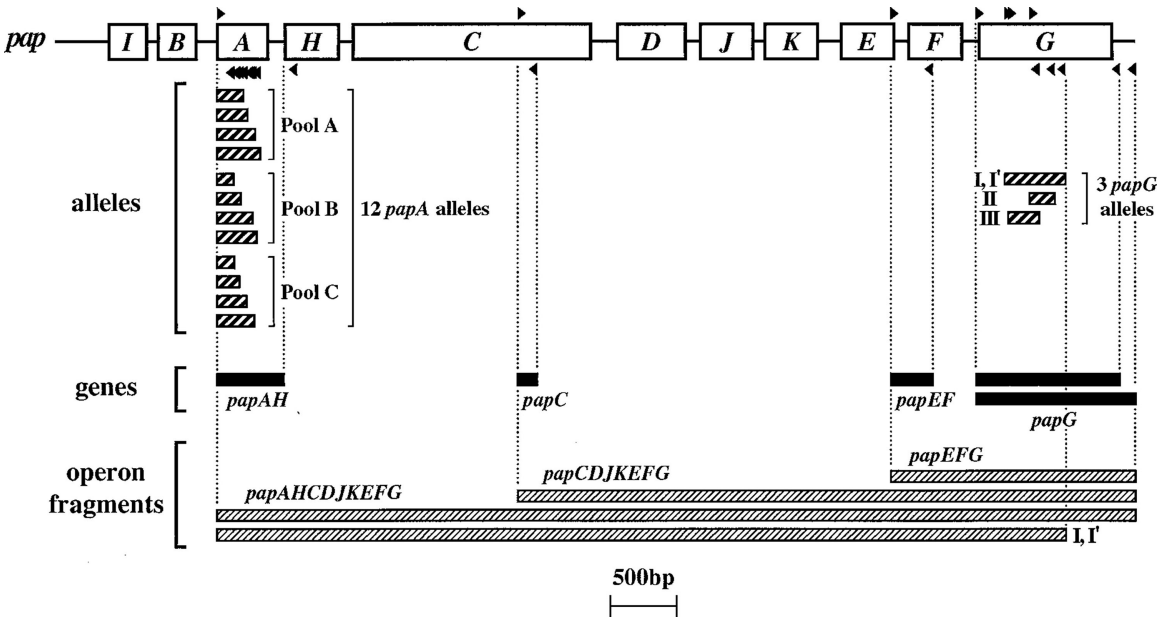
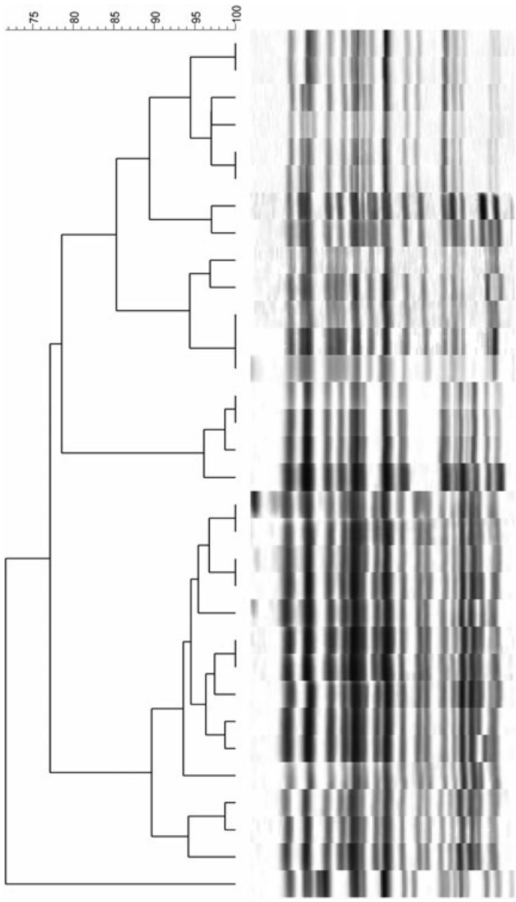


Figure 1 PCR analysis of *pap* operon. Open boxes represent genes within the *pap* operon (including papA, structural subunit; papC, usher; *papEF*, minor tip pilins; and papG, adhesin). Forward and reverse primers (right- and left-pointing black triangles, respectively, above and below the *pap* operon) are used in combinations as shown to yield the indicated PCR products (thin rectangles, below *pap* operon). Heavily striped rectangles, papA and papG allele PCR products. Solid black rectangles, *pap* gene PCR products. Finely striped rectangles, long PCR operon fragments (as generated using either flanking or internal allele-specific papG reverse primers, as illustrated for allele I-I′). Different papA and papG variants are associated with specific lineages, hosts, and clinical syndromes. Intraoperonic deletions that yield a null phenotype (which may be associated with compromised or asymptomatic hosts) can be detected as a truncated long-PCR product. Reprinted from reference 42, with permission.

Of the historical "whole-genome" methods in general use, the most discriminating is pulsed-field gel electrophoresis (PFGE) analysis. This method involves electrophoretic separation of total bacterial DNA that has been digested using a restriction enzyme such as XbaI, which in *E. coli* recognizes a limited number of DNA sites (Fig. 2) (13, 58–60). Use of a second restriction enzyme can further enhance discrimination (61). Identity of two isolates by PFGE analysis implies that they represent the same strain or clone (62). However, PFGE is so discriminating that, beyond a limited level of divergence, it fails to perceive similarity between isolates, making it unreliable for identifying larger clonal groups. It also sometimes paradoxically calls as similar isolates that more precise genetic methods show are only distantly related. Additionally, it requires specialized equipment, is slow, has very limited capacity for data sharing, and is subject to multiple sources of error that limit its ability to reliably and consistently perceive genomic relationships, as compared with WGS-based phylogenetic analysis (see later section) (53, 63).

PCR-based whole-genome profiling methods are less discriminating than PFGE, but are simpler and faster, and can perceive broader clonal group relationships more effectively. Commonly used PCR-based genomic profiling methods include random amplified polymorphic DNA (RAPD) analysis, which uses random or arbitrary primers (Fig. 3) (12), and repetitive element PCR, which uses primers targeting various known chromosomal repeat elements (64). Both methods generate distinctive banding patterns that reflect the spacing of suitable primer sites in the isolate's genome. In addition to allowing simple "same-versus-different" comparisons between isolates, PCR-generated genomic profiles can be subjected to cluster analysis to define quantitative profile similarity relationships, which provides a crude assessment of the underlying phylogenetic relationships (Fig. 4) (65, 66). Such profiling can be used to compare multiple colonies from a particular sample (e.g., feces) with one another to identify putative unique clones, or to compare epidemiologically unrelated clinical, fecal, or environmental isolates with selected reference isolates to allow classification of the unknown isolates as to putative clonal group, e.g., resistance-associated clonal group A (CGA; also known as ST69) by comparison (Fig. 4).

Phylogenetic relationships and membership in specific clonal groups, which is relevant for studies involving



ID	PFGE	Sample	Host	HH no.	Locale	ESBL	H30
CU758	968	Abscess	Child (index)	1	Columbia, MO	-	R1
CU761	968	Fecal	Mother	1	Columbia, MO	-	R1
CD364	968	Urine	Dog (index)	2	Manassas, VA	-	R1
CU797	968	Fecal	Dog (index)	2	Manassas, VA	-	R1
CU790	968	Fecal	Cat #2	2	Manassas, VA	-	R1
CU799	968	Fecal	Cat #1	2	Manassas, VA	-	R1
JJ1886	968	Blood	Sister #2	3	Portland, ME	YES	Rx
JJ1887	968	Urine	Sister #1 (index)	3	Portland, ME	YES	Rx
JJ2547	812	Abscess	Daughter	4	Philadelphia, PA	YES	Rx
JJ2548	812	Blood	Daughter	4	Philadelphia, PA	YES	Rx
JJ2546	812	Urine	Father	4	Philadelphia, PA	YES	Rx
JJ2974	812	Fecal	Mother	4	Philadelphia, PA	YES	Rx
JJ2963	812	Urine	Mother	4	Philadelphia, PA	YES	Rx
HVAST501	806	Fecal	Child (Index)	5	Minneapolis, MN	YES	Rx
HVAST502	806	Fecal	Mother	5	Minneapolis, MN	YES	Rx
HVAST504	806	Fecal	Pet	5	Minneapolis, MN	YES	Rx
JJ2888	806	Fecal	Child (Index)	5	Minneapolis, MN	YES	Rx
HVAST740	903	Fecal	Sibling #3	6	Rochester, MN	YES	Rx
HVAST760	903	Fecal	Sibling #2	6	Rochester, MN	YES	Rx
HVAST656	903	Fecal	Child (index)	6	Rochester, MN	YES	Rx
JJ2913	903	Urine	Child (index)	6	Rochester, MN	YES	Rx
HVAST736	903	Fecal	Child (index)	6	Rochester, MN	YES	Rx
HVAST757	903	Fecal	Mother	6	Rochester, MN	YES	Rx
HVAST758	903	Fecal	Father	6	Rochester, MN	YES	Rx
HVAST695	903	Fecal	Mother	6	Rochester, MN	YES	Rx
HVAST737	903	Fecal	Mother	6	Rochester, MN	YES	Rx
HVAST738	903	Fecal	Sibling #1	6	Rochester, MN	YES	Rx
HVAST709	903	Fecal	Sibling #3	6	Rochester, MN	YES	Rx
HVAST702	903	Fecal	Sibling #1	6	Rochester, MN	YES	Rx
HVAST761	903	Fecal	Sibling #3	6	Rochester, MN	YES	Rx
HVAST697	903	Fecal	Father	6	Rochester, MN	YES	Rx
HVAST503	1883	Fecal	Other adult	5	Minneapolis, MN	YES	Rx

Figure 2 Dendrogram based on pulsed-field gel electrophoresis (PFGE) profiles of 33 clinical and fecal isolates of *Escherichia coli* sequence type 131 (ST131) from the members of 6 households. Profiles are diverse, despite all isolates deriving from the same ST, which reflects the superior resolving power of PFGE over MLST. Isolates from a given household cluster together, consistent with intrahousehold strain sharing. Scale is % profile similarity. All isolates were fluoroquinolone-resistant. H30, clonal subset within the ST131-*H*30 clade (R1 = *H*30R1, Rx = *H*30Rx). Abbreviations: ESBL, extended-spectrum β-lactamase production; HH, household; ID, identifier; PFGE, pulsotype. Reprinted from reference 63, with permission.

potential ExPEC isolates because of the known phylogenetic distribution of extraintestinal virulence in *E. coli* (67, 68), can be resolved with greater accuracy by more focused methods, including PCR-based phylotyping, multilocus sequence typing (MLST), lineage-specific PCR assays, and WGS analysis. PCR-based phylotyping provides a rapid and simple, albeit approximate, method for classifying isolates into one of seven *E. coli* phylogenetic groups (A, B1, B2, C, D, E, F) or a cryptic clade (69). MLST involves DNA sequence analysis of multiple housekeeping genes (typically seven or more, depending on the particular MLST system) that are widely distributed around the chromosome, which enables assignment of an allele designation for the particular sequence variant found at each locus and assignment of a sequence type (ST) based on the isolate's particular combination of alleles (https://enterobase.warwick.ac.uk/species/index/ecoli) (70–72). MLST is now used widely to define both the ST membership of individual isolates and the overall distribution of STs within a collection (i.e., bacterial population). Limitations include the need for a current, well-curated, universally accessible library of STs with reliable associated metadata, plus a system for translating STs into phylogenetic groups.

Lineage-specific PCR assays can detect either lineage-defining single-nucleotide polymorphisms within broadly conserved housekeeping genes (typically, those used for MLST) or lineage-specific accessory genes (i.e., genes variably present within the species). Currently, such assays are available for 12 broad subgroups within (ExPEC-associated) phylogenetic group B2 that correspond largely to ST complexes (73), and for specific STs and sub-ST clonal lineages within (ExPEC-associated) groups B2, D,

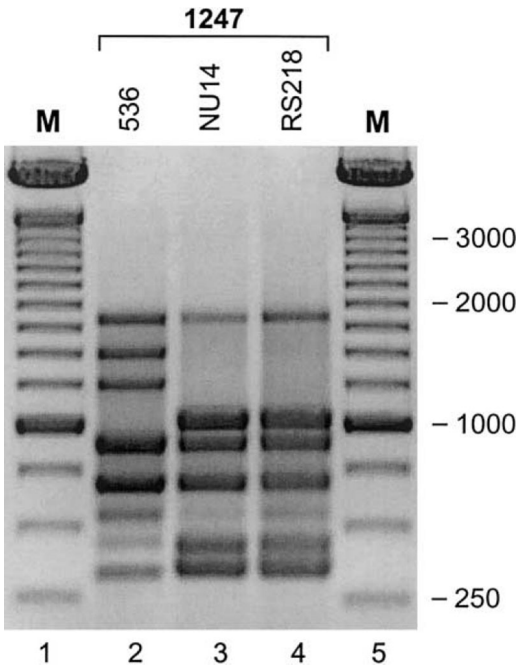


Figure 3 Random amplified polymorphic DNA (RAPD) analysis of *E. coli* strains 536, NU14, and RS218. RAPD profiles generated by using primer 1247 (12) show *E. coli* O18:K1:H7 strains NU14 (cystitis: lane 3) and RS218 (neonatal meningitis: lane 4) to be indistinguishable from one another, but distinct from strain 536 (O6:K15:H31, pyelonephritis: lane 2), illustrating both the broad syndrome capability of certain ExPEC lineages and the clonal diversity of urinary tract infection-causing ExPEC strains. M (lanes 1 and 5), 100-bp marker. Reprinted from reference 160, with permission.

and F (72, 74–81). Occasional misclassification results from genetic alteration of the targeted primer-binding sites.

WGS data, when limited to core genome sequences (i.e., those that are present in all members of the population), can be analyzed using appropriate phylogenetic algorithms to define the phylogenetic structure of a collection (45, 52). Inclusion of reference genomes that represent known phylogenetic or clonal groups allows such phylogenies to be referenced to the established *E. coli* population structure. Unlike the results of other genotyping methods, WGS-based phylogenies are informative across the entire spectrum of resolution within the phylogenetic tree, from the broadest (major phylogenetic groups) to the most resolved (minor sequence variants within individual sub-ST lineages, i.e., clones), as shown in Fig. 5 for the fine structure of the pandemic resistance-associated lineage ST131 (Fig. 5) (36, 63, 80, 82). They allow evolutionary time scale inferences, to clarify the chronology of emergence of epidemic ExPEC clones (36), and permit epidemiologically linked ExPEC isolates to be compared with one another and with unlinked isolates, to assess for clonal reservoirs and transmission (63). Notably, core genome phylogenies by definition ignore variation within the accessary genome (including plasmids, virulence genes, bacteriophages, and resistance genes), which may be important clinically or ecologically and require separate focused analysis.

RESULTS OF MOLECULAR EPIDEMIOLOGICAL STUDIES

Virulence Factors: Associations with Clinical Variables and Phylogenetic Background

Comparisons between clinical isolates and controls

E. coli isolates from the urine, blood, cerebrospinal fluid, etc., of patients with diverse extraintestinal infection syndromes typically exhibit a greater prevalence of specific molecular markers than do fecal isolates from uninfected hosts (9, 83–88). From an epidemiological perspective such virulence markers can be regarded as "virulence factors" (VFs), although this term must be understood as implying "factors associated with" rather than "contributing to" virulence, since epidemiological associations do not necessarily reflect causality (as discussed above).

These VFs can be grouped by functional category, for example, adhesins (fimbrial and nonfimbrial) (89–92), siderophore systems (23, 65, 93–97), toxins (20, 98–101), surface polysaccharides (102–105), invasins (50, 106, 107), serum resistance-associated traits (108, 109), and traits of miscellaneous or unknown function (22, 67) (Table 1, Fig. 6). Clinical isolates often contain multiple VFs from a particular functional category (110, 111); this conceivably may allow for redundancy, synergistic interactions among VFs, and/or adaptability to different environmental niches. Conversely, many seemingly virulent strains lack known representatives of one or more of these functional categories (110, 111). Whether such apparent deficits are compensated for by VFs from other functional categories, or these strains actually do contain unrecognized representatives of the "missing" VF categories, is unknown.

Associations among VFs

Certain VFs commonly occur together among clinical isolates in patterns suggesting either co-selection or direct genetic linkage (112, 113). Extensive genetic linkage of VFs has been demonstrated both within genomic

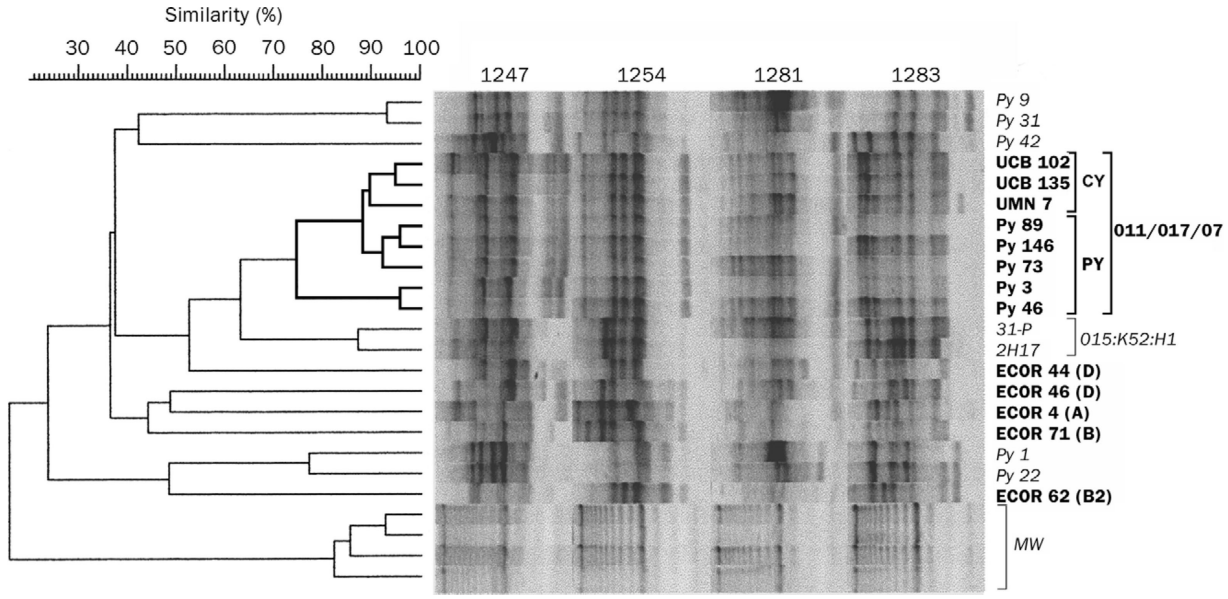


Figure 4 RAPD-based phylogenetic and clonal analysis of *Escherichia coli* isolates. Genomic profiles (shown in computer reconstruction), as generated for each isolate by using RAPD primers 1247, 1254, 1281, and 1283, were concatenated for cluster analysis. Pyelonephritis isolates (n = 10; "Py" strain designations) are labeled in bold if from *E. coli* clonal group A (CGA) (n = 5) and in lightface italic if non-CGA (n = 5). CGA isolates (bold) are bracketed and labeled as to syndrome (CY, cystitis; PY, pyelonephritis) and serogroup (O11/O17/O77) (right), with the corresponding cluster shown in bold (left). The two *E. coli* O15:K52:H1 control strains are bracketed and labeled by serotype. Reference strains from the *E. coli* Reference (ECOR) collection (bold) are identified as to phylogenetic group (right). The depth of the molecular weight ladder cluster (brackets; MW) reflects the intrinsic variability inherent in gel electrophoresis and image analysis, independent of amplification. The CGA isolates cluster together irrespective of clinical syndrome (pyelonephritis, cystitis) and geography (UCB: Berkeley, California; UMN: Minneapolis, MN; Py: multiple centers around the U.S.). Reprinted from reference 248, with permission.

islands, which have been called pathogenicity-associated islands (PAIs) and fitness islands, and can occur on either the chromosome or plasmids, and on plasmids, unassociated with PAIs (114–121). Certain VFs typically occur on the chromosome (for example, *pap*, *sfa/foc*, *hly*, *cnf*, and *fyuA*) (114, 122), others on plasmids (for example, *iss*, *traT*) (123), and some in either context (for example, *afa/dra* and *iuc/iut*) (118, 121). ExPEC strains often contain multiple PAIs, each with a distinctive combination of VFs, which sometimes results in a strain having multiple copies of a particular VF, for example, *pap* (Fig. 7) (114, 124, 125).

This co-occurrence of VFs results in overlapping statistical associations of different VFs with clinical variables, leading to uncertainty as to which VF is primarily responsible for the association. Multivariable analysis can help in this situation, but is not definitive. Moreover, sequence analysis of PAIs, plasmids, and genomes invariably reveals genes of unknown function, some with homology to known VFs in other species (Fig. 8) (114, 119, 125). This suggests that the statistical associations of known VFs with virulence may be mediated through some of these as-yet-uncharacterized VFs; that is, the known VFs, although useful markers, may not themselves be the actual determinants of virulence. The genes of unknown function, once discovered, can become the focus of additional molecular epidemiological studies to clarify the genes' associations with ecological source, clinical syndrome, host groups, lineages, etc.

Phylogenetic group and clonal groups

According to molecular analyses, certain ExPEC clonal groups, as identified traditionally based on their O:K:H serotypes (for example, O18:K1:H7, O6:K2:H1, and O7:K1:H–) (126), which correspond closely with specific STs according to MLST, are disproportionately represented among clinical isolates in comparison with controls (110, 127, 128). These virulent clonal groups derive primarily from phylogenetic group B2 (e.g., STs 12, 14, 73, 95, 127, and 131), and to a lesser extent group D (e.g., STs 69, 394, and 405), which explains the observed predominance of groups B2 and D among clinical isolates (83, 110, 112, 129). Most of the traditionally recognized extraintestinal virulence markers (for example, *pap*, *sfa/foc*, *hly*,

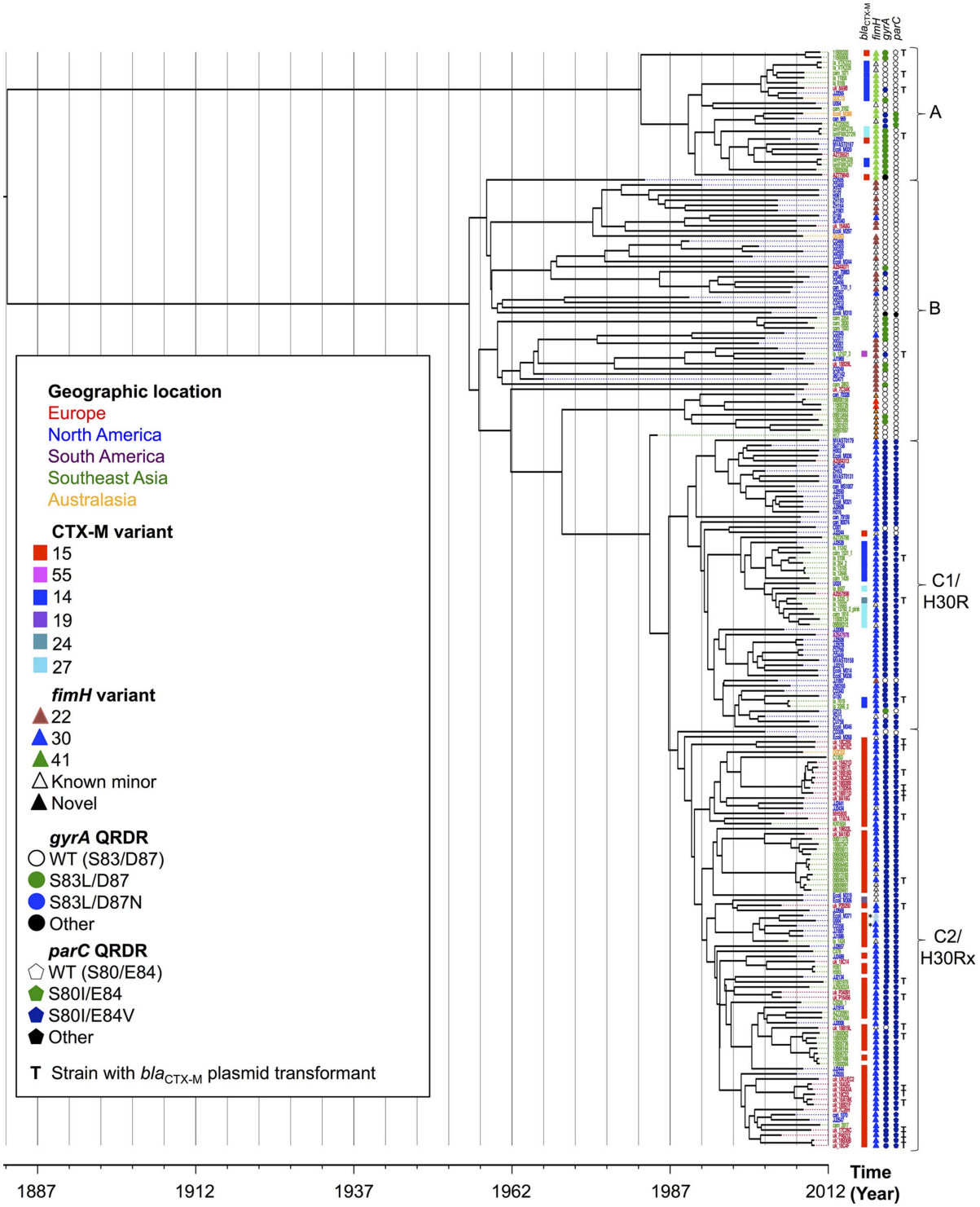


Figure 5 Time-scaled whole genome sequence (WGS)-based phylogeny of ST131 *Escherichia coli* (*n* = 215). Meta-data include allelic variants of $bla_{\text{CTX-M}}$ (extended-spectrum beta-lactamase gene) and *fimH* (type-1 fimbriae adhesin gene), plus mutations in the quinolone resistance-determining region (QRDR) of *gyrA* (WT, wild-type). Brackets identify defined ST131 clonal subsets. Branch tips are colored by geographic region, per the key. **T**, *bla* plasmid transformant generated for strain; *, cases with putative deletions in the assembled *bla* gene. Geographic clustering is evident, some of it linked with specific accessory gene variants; e.g., within the C1/H30R clonal subset, the Southeast Asian isolates (green) are largely confined to a specific clade that consists of two subclades, one characterized by $bla_{\text{CTX-M-14}}$ and the other by $bla_{\text{CTX-M-27}}$. Reprinted from reference 36, with permission.

Table 1 Virulence-associated traits of extraintestinal pathogenic *Escherichia coli* (ExPEC) by functional category[a]

Category	Gene(s) or operon	Comment	Evidence for trait as a virulence factor (reference[s])	
			Epidemiological[b]	Experimental (*in vivo*)[c]
Adhesins[d]	*afa/dra*	Dr antigen-binding adhesins (AFA I-III, Dr, F1845)	Yes (176, 266, 267)	Yes (268)
	afaE-8	Afimbrial adhesin VIII	Yes (175)	No
	auf	Surface structure of unclear function	Yes (269)	No (269)
	bmaE	Blood group M-specific adhesin	Yes (270)	No
	clpG	CS31A adhesin (K88–related)	Yes (271)	No
	csgA	Curli	Yes (272)	Yes (272)
	ecp	Pilus	Yes (273)	No
	fim	D-mannose-specific adhesin, type-1 fimbriae	Yes (267, 274, 275)	Yes (89, 276)
	foc	F1C fimbriae	Yes (277)	No
	gafD	GlcNac-specific (G, F17c) fimbriae adhesin	Yes (270, 271)	No
	iha	Iron-regulated-gene-homologue adhesin	Yes (21, 111, 267)	Yes (96)
	nfa	NFA-1, -2, -3, -4 (nonfimbrial adhesins)	No (278–280)	No
	pap	Pilus associated with pyelonephritis (P fimbriae)	Yes (9, 151, 267)	Yes (281); no (282)
	pil	Type IV pilus	Yes (283)	Yes (284)
	sfa	S fimbriae (sialic acid-specific)	Yes (111)	Yes (285)
	sfa/foc	S and F1C fimbriae	Yes (286)	Yes (285)
	yad	Fimbria	Yes (283)	Yes (283)
	ygi	Fimbria	Yes (283)	Yes (283)
	yvc	Fimbria	Yes (283)	No
Toxins[d]	*astA*	EAST1, heat-stable enteroaggregative *E. coli* cytotoxin	Yes (79, 287)	No (189, 288)
	cdtB	Cytolethal distending toxin, CDT	Yes (111)	No
	cnf1	Cytotoxic necrotizing factor 1, CNF-1	Yes (289)	Yes (98, 290, 291)
	hly	α-Hemolysin (Hly)	Yes (292)	Yes (100, 293)
	pic	Protein associated with intestinal colonization, PIC	Yes (294)	Yes (18, 284, 294, 295)
	sat	Secreted autotransporter toxin (serine protease)	Yes (20, 267)	Yes (296)
	tsh	Temperature-sensitive hemagglutinin, TSH	Yes (68, 294)	Yes (18, 294)
	upxA (*tosA*)	RTX toxin	Yes (297)	Yes (298)
	vat	Vacuolating autotransporter toxin	Yes (299, 300)	Yes (284)
Nutrition	*argC*	Arginine synthesis	N/A[e]	Yes (301)
	aro	Shikimate synthesis	N/A[e]	Yes (284)
	chuA	Heme receptor	Yes (268)	Yes (97)
	dppA-oppA-sapA	Uptake of short peptides and amino acids		Yes (284, 302)
	dsd	Serine utilization	Yes (303)	No (304)
	entF	Enterobactin synthesis	No	Yes (65)
	fyuA, irp	Yersiniabactin (siderophore) receptor, synthesis	Yes (122, 267, 268)	Yes (305)
	guaA	Guanine synthesis	N/A[e]	Yes (301)
	ireA	Iron-regulated element (catecholate siderophore)	Yes (23)	Yes (23)
	iroN	Salmochelin (siderophore) receptor	Yes (21, 306)	Yes (65, 95)
	iuc, iutA	Aerobactin (siderophore) synthesis, receptor	Yes (268, 307)	Yes (97)

(continued)

Table 1 Virulence-associated traits of extraintestinal pathogenic *Escherichia coli* (ExPEC) by functional category[a] *(continued)*

Category	Gene(s) or operon	Comment	Evidence for trait as a virulence factor (reference[s])	
			Epidemiological[b]	Experimental (*in vivo*)[c]
	pckA	Gluconeogenesis		Yes (302)
	sdhB	TCA cycle		Yes (302)
	tonB	Siderophore uptake	N/A[e]	Yes (65)
Protectins	*iss*	Increased serum survival (outer membrane protein)	Yes (307, 308)	Yes (309)
	kpsMT II	Group II capsule synthesis (e.g., K1, K5, K12)	Yes (112, 126, 267, 310)	Yes (311)
	kpsMT III	Group III capsule synthesis (e.g., K3, K10, K54)	Yes (112, 126, 310)	Yes (312)
	proP	Osmoprotection; proline permease	N/A[e]	Yes (313)
	rfc	O4 lipopolysaccharide (LPS) synthesis	Yes (314, 315)	Yes (316)
	traT	Surface exclusion; serum resistance-associated	Yes (307)	No (317)
TCSS[f]	*barA-uvrY*	Regulation of VFs, e.g., hemolysin, LPS		Yes (318)
	cpxAR	Regulation of VFs, e.g., hemolysin		Yes (319)
	envZ-ompR	Regulation of VFs, e.g., osmoprotection		Yes (320)
	kguRS	Regulation of VFs, e.g., nutrient acquisition	Yes (321)	Yes (321)
	qseBC	Regulation of VFs, e.g., adhesins, flagella		Yes (322)
Invasins	*aslA*	Cellular invasion (arylsulfatase-like gene)	N/A[e]	Yes (323)
	ibeA-C	Invasion of brain endothelium IbeA (Ibe10), B, and C	Yes (IbeA) (111)	Yes (323)
	nlpI	Invasion of brain endothelium, complement resistance	No	Yes (324, 325)
	ompA	Outer membrane protein A (cellular invasion)	N/A[e]	Yes (323)
	traJ	Cellular invasion (F-like plasmid transfer region homologue)	No	Yes (323)
Misc.[g]	*cvaC*	Microcin (colicin) V; on plasmids with *traT*, *iss*, *iuc/iut*	Yes (307)	Yes (326)
	fliC	Flagella: motility, ascent to the kidneys		Yes (327)
	malX	Pathogenicity island marker (from strain CFT073)	Yes (130, 132, 267)	No (139)
	ompT	Outer membrane protein T (protease)	Yes (328)	Yes (unpublished, JRJ)
	pga	Extracellular polysaccharide (poly-GlcNac), biofilm		Yes (284)
	rfaH	Transcriptional antitermination: O antigen, capsule, Hly, ChuA		Yes (329)
	usp	Uropathogenic-specific protein (bacteriocin)	Yes (22, 120, 267)	Yes (330)
	ydd-pqq	ABC transporter, inner-outer membrane proteins		Yes (284)

[a]Note: list is not comprehensive even for recognized markers, and additional markers remain to be identified and characterized. Conversely, not all the listed traits necessarily contribute to virulence; some are only epidemiologically linked with virulence or confer *in vitro* phenotypes that are suspected of promoting virulence. Additionally, some of the listed traits are prominent also among intestinal pathogenic *E. coli*, e.g., cytolethal distending toxin, certain Dr-binding adhesins, and EAST1 (*astA*).

[b]Statistically associated with clinical isolates or specific host characteristics, or highly prevalent in a particular extraintestinal infection syndrome.

[c]Based on animal model infection studies, not necessarily using isogenic strains or complemented mutants.

[d]Certain adhesins and toxins function as invasins, e.g., type-1 fimbriae, some Dr-binding adhesins, and CNF-1 (115, 145, 197).

[e]N/A, not applicable (ubiquitous within *E. coli*, which precludes epidemiological comparisons).

[f]TCSS, two-component secretion system.

[g]Misc., miscellaneous.

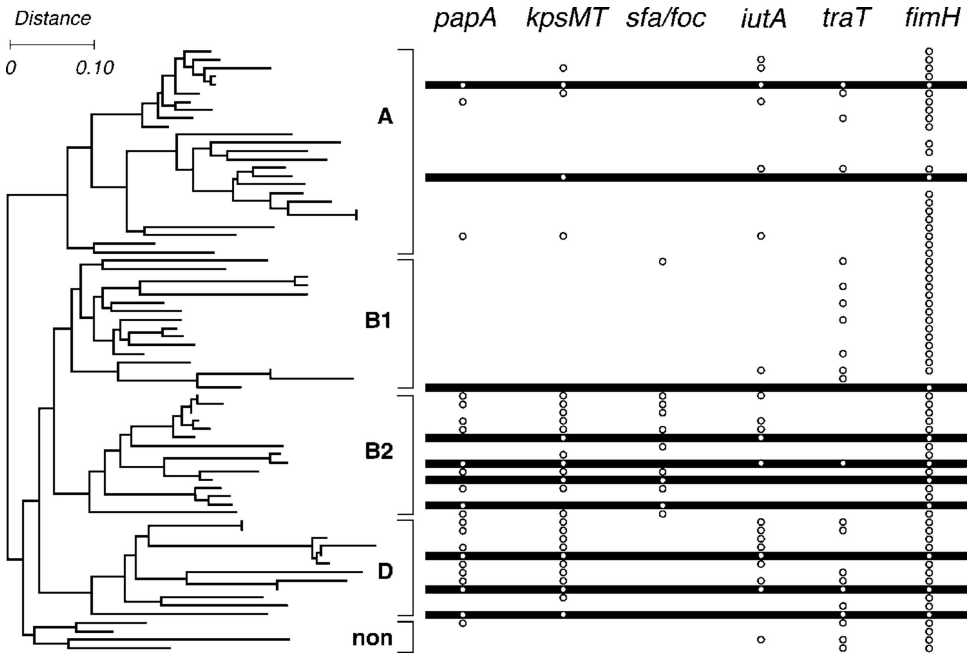


Figure 6 Phylogenetic distribution of extraintestinal pathogenic *Escherichia coli* (ExPEC)-associated virulence traits. Dendrogram at left depicts phylogenetic relationships for the 72 members of the *E. coli* Reference (ECOR) collection, as inferred based on multilocus enzyme electrophoresis (67). The four traditional major *E. coli* phylogenetic groups, i.e., A, B1, B2, and D (now split into groups D and F). The nonaligned ("non") strains (now called group E) are bracketed and labeled. Bullets at right indicate presence of putative virulence genes (*papA*, P fimbriae; *kpsMT*, group II capsule synthesis; *sfa/foc*, S and F1C fimbriae; *iutA*, aerobactin system; *traT*, serum resistance; and *fimH*, type 1 fimbriae). Horizontal bars at right indicate the 10 ECOR strains isolated from humans with symptomatic UTI. The remaining strains, except for one asymptomatic bacteriuria isolate, are fecal isolates from healthy human or animal hosts. Note the concentration of (chromosomal) ExPEC-defining virulence genes *papA*, *kpsMT*, and *sfa/foc* within phylogenetic groups B2 and D, but their occasional joint appearance also in distant lineages, consistent with coordinate horizontal transfer, giving rise to ExPEC strains in historically non-ExPEC lineages. The more scattered phylogenetic distribution of *iutA* (ExPEC-defining) and *traT* is consistent with these two genes' typically plasmid location, although *iutA* also can be chromosomal. *fimH* is nearly universally prevalent, consistent with its presence in other species of *Enterobacteriaceae*, presumably reflecting an origin in a shared enterobacterial ancestor. Note the concentration of UTI isolates within phylogenetic groups B2 and D and the concentration of virulence genes among UTI isolates. Note also the appreciable minority of fecal isolates with multiple virulence genes, reflecting a fecal reservoir of ExPEC. Reprinted from reference 67, with permission.

and *kps*) are typically concentrated within these virulent clonal groups and, hence, within phylogenetic groups B2 and/or D, whereas others (for example, *afa/dra*, *iuc/iut*, and *traT*) are more broadly and/or sporadically distributed across the species (Fig. 6) (35, 130). These divergent patterns of phylogenetic distribution correspond with vertical (within-lineage) versus horizontal (between-lineage) transmission, respectively, and reflect the typically chromosomal versus plasmid location of the respective sequences, as discussed above.

Considerable variation in VF profiles is evident at every level within the phylogenetic tree, including among the major phylogenetic groups, among the various clonal groups within these phylogenetic groups, and even among subclones within individual clonal groups. This is consistent with extensive ongoing remodeling of PAIs and/or virulence plasmids, in addition to acquisition and loss of entire PAIs or plasmids. Such evolutionary processes presumably result in the continuous emergence of new pathotypes upon which selective forces can act (110, 131).

Several studies have compared clinical isolates with fecal isolates from the same hosts, as opposed to fecal isolates from a separate control population (132–134). This strategy ensures a degree of matching for associated host characteristics greater than that provided by a traditional unpaired study design. The results of such studies, like those of most traditional comparison studies, suggest that special pathogenicity (as indicated by the presence of multiple VFs) rather than simple prevalence (that is, quantitative predominance in the fecal flora) is necessary for a fecal strain to cause urinary tract infection (UTI).

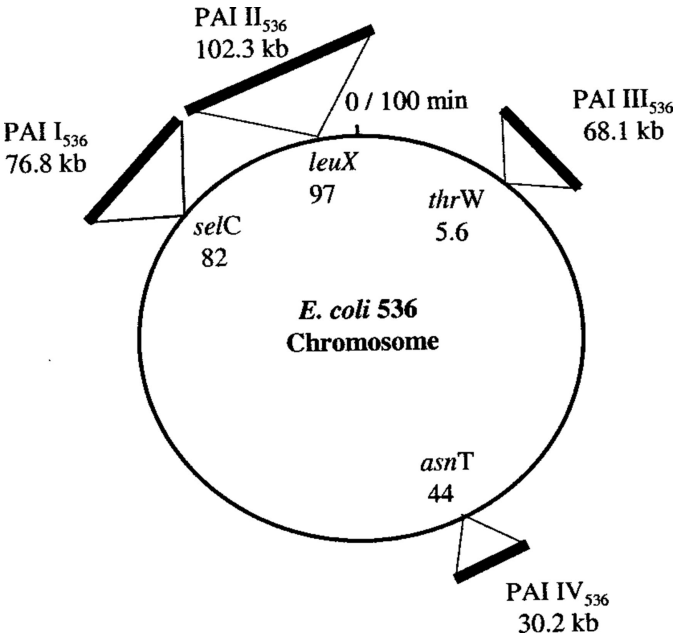


Figure 7 Genome map of ExPEC strain 536. The map is based on the chromosome of *E. coli* MG1655 (K-12). Pathogenicity islands (PAIs) are indicated according to their chromosomal insertion sites next to tRNA-encoding genes. Contents, by PAI, include: PAI I (α-hemolysin, F17-like fimbriae, CS12-like fimbriae); PAI II (α-hemolysin, P fimbriae with *papG* III); PAI III (S fimbriae, *iro* siderophore system, Tsh-like hemoglobin protease); PAI IV (yersiniabactin system). Many additional smaller DNA insertions compared to K-12 are present (not shown). Linkage of virulence genes in PAIs contributes to statistical associations between different virulence genes and between specific virulence genes and the lineages within which the corresponding PAIs tend to occur. Reprinted from reference 121, with permission.

Phylogenetic background versus VFs

The overlapping associations of VFs and phylogenetic background with clinical virulence call into question which of these bacterial characteristics, VFs or phylogenetic background, more directly determines virulence, i.e., a strain's ExPEC status. Several studies in which both phylogenetic group and VF profiles were analyzed have shown that VFs are statistically more closely associated with clinical virulence (132, 135, 136). However, phylogenetic group exhibits a residual association with virulence even after known VFs are accounted for (135). This is consistent with the existence of as-yet-undefined virulence-associated traits that are both phylogenetically distributed and incompletely linked with known VFs.

Comparisons among syndromes and host groups

Molecular epidemiological comparisons are not limited to *E. coli* populations from infected versus uninfected hosts. Comparisons also can be made between isolates (i) from patients with different clinical syndromes (to identify syndrome-specific, versus conserved, VFs or clonal groups) (113, 137, 138), (ii) from infected hosts who possess or lack particular predisposing conditions (to identify bacterial traits that may interact with specific host defense mechanisms or host receptors) (139–142), and (iii) from different host species (to identify species-specific, versus broad host range, VFs or pathogens) (106, 140–148).

The results of such studies support certain general conclusions regarding ExPEC strains, including which bacterial traits typify them, which host factors interact with these traits, and to what extent do these relationships vary with host species, sex, age, anatomical site of infection, and illness severity. First, invasive clinical syndromes, such as pyelonephritis, bacteremia, prostatitis, and meningitis, as compared with less invasive syndromes, such as cystitis and asymptomatic bacteriuria, on average usually involve strains with greater molecular virulence, as reflected in the number of VFs and a group B2 background. Second, various forms of host compromise significantly decrease the requirement for bacterial virulence within a defined clinical syndrome. This is exemplified by the reduced prevalence of *pap* among pyelonephritis isolates from patients with, versus those without, vesicoureteral reflux, that is, spontaneous retrograde flow of urine from the bladder back up to the kidneys (132, 149), and the reduced prevalence of *pap* and chromosomal aerobactin determinants among blood isolates from patients with urosepsis who have, versus those who lack, underlying anatomical or medical conditions predisposing to UTI (47). Third, although there is some syndrome and host specificity of VFs and clonal groups, there also is considerable commonality among syndromes and host groups, whereas tremendous diversity is apparent within each syndrome and host group. Examples of relative syndrome and host specificity that have been identified include the statistical association of *sfaS* (S fimbriae) with neonatal meningitis (83, 111); of *pap* with pyelonephritis (150, 151); of *papG* allele III, *hly*, and *cnf* with canine UTI (132, 143); and of the F11 variant of *papA* (P fimbriae structural subunit) with avian septicemic *E. coli* (152). However, each of these associations is incomplete, since the same VFs or clonal groups occur to various degrees also in other syndromes and host groups, as exemplified by the prominence of the O18:K1:H7 clonal group (ST95) in both neonatal meningitis (as traditionally recognized) and uncomplicated cystitis in women (as more recently appreciated)

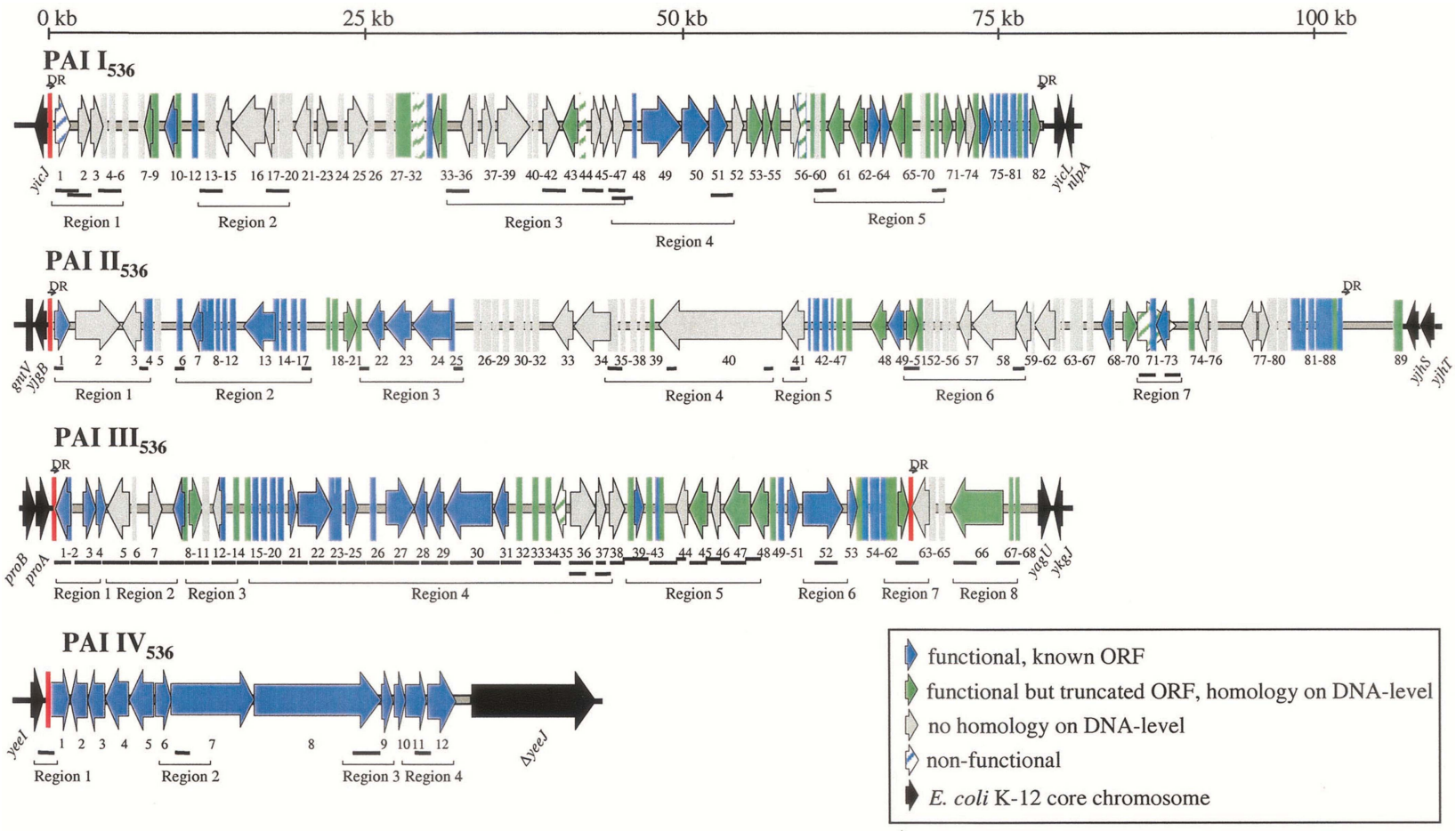


Figure 8 Maps of pathogenicity-associated islands (PAIs) from ExPEC strain 536. Known or putative open reading frames (ORFs) are grouped according to the following characteristics: blue, functional, known ORFs; green, truncated ORFs with a start codon and a stop codon; gray, as-yet-unidentified ORFs without homologues on the DNA level. Nonfunctional ORFs (e.g., due to internal stop codons or frameshifts) are indicated by hatched symbols. ORF numbers are indicated below the corresponding ORF symbols. Functional or truncated tRNA-encoding genes are marked in red. Direct repeat (DR) structures flanking PAIs are indicated. Thick black lines below the PAIs represent regions that were detected by PCR. Several PAI-specific PCRs were grouped into PAI regions. The molecular epidemiology of novel ORFs that are discovered through sequence analysis of PAIs can be investigated in subsequent studies. Reprinted from reference 121, with permission.

(Fig. 3) (1, 91, 119, 128, 153, 154). To what extent high-resolution phylogenetic analyses such as those based on WGS data will identify host or syndrome-specific subclades within certain clonal groups and STs that previously were regarded as unitary entities remains to be seen (53, 80).

Regulation of expression

The relative lack of syndrome-specific VFs does not necessarily negate the concept that selected VFs are critical for infection at selected sites. It may reflect instead the broad environmental flexibility of *E. coli* both within and outside the human host, which could be manifested by site-specific regulation and expression of genes or sets of genes. Therefore, in addition to the simple presence or absence of a particular gene, molecular epidemiological studies may also need to consider gene expression, since expression obviously is required if the genotype is to influence the virulence phenotype. Expression can be assessed through a variety of phenotypic tests, which moves beyond the realm of strict molecular epidemiology. However, in the instance of the *fim* operon, expression is regulated by an invertible switch element in the promoter region, the position of which ("on" versus "off") can be defined for a bacterial population via a simple PCR assay (57, 124, 155, 156). Differences between UTI versus control isolates, and between cystitis versus pyelonephritis isolates, with respect to their *fim* switching bias can be demonstrated, supporting the concept that regulation of *fim* expression may influence not only overall pathogenicity but also anatomical site tropism (155, 156). Such a molecular epidemiological approach is particularly relevant for *fim* since, although expression of type 1 fimbriae appears from experimental studies to be quite important for UTI pathogenesis, the nearly uniform presence of *fim* in *E. coli* (and other enterobacteria) all but precludes demonstration of a virulence association for *fim* through conventional presence-absence comparisons between clinical isolates and controls (130).

Site-specific expression of VFs and core "housekeeping" genes directed by environmental cues and mediated by regulatory elements (e.g., two-component signal systems; Table 1) is a more global mechanism that enables growth/survival within a variety of challenging niches (157). Molecular epidemiological associations of regulatory elements may prove to be a fruitful area for additional study.

Molecular variants

Another potentially important consideration is the particular variant of a virulence gene present in an isolate. Molecular variation within a gene may produce pathogenetically important phenotypic alterations in the encoded peptide, such as the shifts in preferred receptor sugars or glycolipids that are associated with polymorphisms in *fimH* (type 1 fimbrial adhesin) and *papG*, respectively. Diverse single-nucleotide polymorphisms (SNPs) in *fimH*, which can be detected by sequence analysis or with SNP-specific PCR primers, cause single-amino-acid changes in the FimH peptide that produce a shift from a (commensal-associated) trimannose-binding phenotype to a (UTI-associated) monomannose-binding phenotype (158). Interestingly, the monomannose-binding variants, although at an advantage within the pathogenic niche (for example, because of their enhanced binding to bladder epithelium), also are more susceptible to inhibition by monomannose residues, such as are found in salivary glycoproteins; this presumably makes them less effective as gut colonization factors (158). An additional point mutation in a monomannose-binding *fimH* variant, resulting in a single FimH amino acid substitution (Ser-62-Ala), can further strengthen monomannose binding and also confer type IV collagen binding (Fig. 9), which may be important in the pathogenesis of neonatal meningitis (159, 160). Such mutations have been termed pathoadaptive, since they represent minor modifications of genes already present in nonpathogenic members of the species, with the mutations conferring enhanced fitness in the pathogenic niche (161).

More extensive alterations within *papG*, which can be detected by using specific primers or probes, or sequence analysis, are evident among the several known allelic variants of *papG* (42, 90, 162–164). The respective peptide products of the *papG* alleles bind preferentially to various forms of Gal(α1-4)Gal-containing glycolipids (165–168). Because of the varied distribution of these particular glycolipids by anatomical site and host species, the distinctive binding preference of the PapG variants may underlie their somewhat divergent associations with clinical syndromes and host groups, such as the associations of PapG II with pyelonephritis and of PapG III with cystitis, dogs and cats, and compromised hosts with urosepsis (84, 143, 168–173). Other examples of epidemiologically significant molecular variation within a particular VF, or VF family, include the *sfa/foc* (S and F1C fimbriae) family (174), the *afa/dra* (afimbrial and Dr-binding adhesins) family (92, 175, 176), the group 2

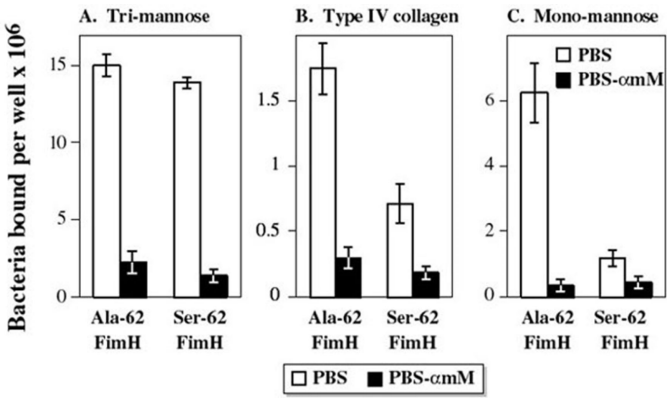


Figure 9 Receptor binding specificity of type 1 fimbrial adhesin (FimH) variants. *In vitro* binding of isogenic recombinant strains expressing the Ala-62 or Ser-62 FimH variants (from strains NU14 and 536, respectively) to (A) a trimannose substrate (bovine RNAse B), (B) human collagen type IV, and (C) a monomannose substrate (yeast mannan). Both variants bind equally well to trimannose, but the Ala-62 variant exhibits stronger type IV collagen and monomannose binding than does the Ser-62 variant. (Commensal-associated FimH variants exhibit equally strong trimannose binding but minimal binding to type IV collagen or monomannose [not shown].) Open columns, bacteria incubated without α-methyl mannoside (αmM); solid columns, bacteria incubated with 50 mM αmM. Data are mean + SEM (n = 4) of number of bacteria bound per well. Molecular epidemiological studies can be used to elucidate the likely clinical relevance of such genetic and phenotypic variation within different virulence factors. Adapted from reference 160, with permission.

and group 3 *kps* (capsule) families (177, 178), and *cnf1* and *cnf2* (cytotoxic necrotizing factor I and II; associated with extraintestinal and intestinal virulence, respectively) (179). Likewise, *papA* (P fimbriae structural subunit) exhibits 13 known alleles (180, 181). These tend to be clonally distributed, such that they can be used, in conjunction with other VFs or seroantigens, as markers for particular clones (110, 181). They also exhibit somewhat distinctive associations with specific host groups or syndromes, for example, F10, F12, and F48 with canine UTI (132, 143, 182).

VFs as predictors of clinical outcomes

Apart from their role in pathogenesis, VFs also have been studied as potential clinical predictors that could be used to guide patient management. For example, P fimbriae (or *pap*) testing has been proposed as a way to identify boys at risk for renal scarring (183), adults with pyelonephritis or urosepsis who have unrecognized predisposing conditions (184), and patients whose household members should be screened for colonization with the patient's UTI strain (185). Other proposed clinical applications of such testing include determining length of therapy for children with UTI (186) and identifying pregnant women at high risk for developing pyelonephritis (187). However, the true clinical utility and cost-effectiveness of such clinical applications of VF testing are unconfirmed (188), such that at present they cannot be recommended outside a research setting.

Antimicrobial resistance and virulence

Acquired resistance to therapeutically important antimicrobial agents is increasingly prevalent among clinical *E. coli* isolates, making management of *E. coli* infections more difficult and costly (189, 190). The relationship between resistance and virulence or phylogenetic background has been explored in multiple molecular epidemiological studies. Older data indicated that among *E. coli* isolates from patients with urosepsis, resistance to historical antimicrobial agents such as ampicillin, sulfonamides, tetracycline, and streptomycin is negatively associated with virulence and a group B2 phylogenetic background, but is positively associated with host compromise (191). This is consistent with a scenario wherein resistance provides a greater fitness advantage than do traditional VFs or a group B2 background for infections in compromised hosts, who have weakened defenses but are frequently exposed to antimicrobial agents.

Subsequent studies regarding fluoroquinolone resistance that were done prior to the emergence of ST131-*H*30, the currently dominant fluoroquinolone-resistant *E. coli* lineage (which is from group B2), demonstrated similar negative associations between resistance and VFs or a B2 phylogenetic background (192–195). Although these were interpreted as suggesting that VFs may be lost concomitant with mutation to fluoroquinolone resistance (195), that explanation did not account for the concomitant resistance-associated phylogenetic shifts away from group B2, which suggested instead that resistant isolates derive predominantly from distinct, less virulent bacterial populations (192, 193). Therefore, selection for antimicrobial resistance within different host and bacterial populations, rather than loss of virulence genes in exchange for resistance, may have produced the observed VF differences between susceptibility groups. Indeed, fluoroquinolone resistance has been shown to be associated with host compromise. Thus, among clinical isolates selection factors similar to those that historically produced statistical associations between low virulence and resistance to older antimicrobial agents may be operative also

with fluoroquinolones. Notably, the current prominence of ST131-*H*30, with its broad virulence gene repertoire despite extensive multidrug resistance, has largely erased the historical negative associations of resistance with virulence genes and group B2 (79, 196).

Other findings confirm that antimicrobial resistance does not necessarily equate with reduced virulence. For example, among fecal *E. coli* from diseased cattle and swine, and from retail meat products (which strains likely derive from the source animal hosts), settings in which most of the organisms are nonpathogens, resistance to extended-spectrum cephalosporins or fluoroquinolones is associated with minimal shifts in VF profile (192, 197–204). Likewise, two notable epidemic multidrug-resistant clonal groups that preceded ST131-*H*30 by several decades, i.e., *E. coli* "clonal group A" (ST69) and the O15:K52:H1 clonal group (STc31), are replete with VFs, which presumably contributed to these clonal groups' success as pathogens among otherwise healthy hosts. Resistance and virulence presumably are uncoupled for the animal-source isolates by the absence of selection for virulence, and for human clinical isolates from clonal group A and the O15:K52:H1 clonal group (as for those from ST131-*H*30) by the uniform requirement for virulence, irrespective of resistance. Further studies clearly are needed to clarify the relationship between virulence and resistance, taking into account ecological source and relevant selection factors.

VFs versus colonization factors

Paradoxically, certain molecular epidemiological (205–207) and experimental (208–212) data suggest that at least some of what traditionally have been regarded as VFs in ExPEC may also promote intestinal survival and colonization, and that virulence may even be a by-product of commensalism (213). This hypothesis provides a more plausible mechanism for the evolution of these traits than does the enhanced pathogenicity the traits confer, since the ability to persist and flourish within the host intestinal tract represents a more obvious survival advantage than does the ability to cause sporadic and usually self-limited or even fatal disease.

Moreover, although many of the traditionally recognized extraintestinal VFs are encoded on what have been designated PAIs, which implies that pathogenicity is their raison d'être, this terminology is evolving. The newer, more inclusive designation "fitness island" reflects the recognition that similar accretions of genes encoding related functions, with associated insertion sequences and other mobility-promoting elements, occur in non-pathogens, including even environmental (non-host-associated) organisms (214), and that even in ExPEC strains PAIs may function as colonization factors (215). However, the hypothesis that VFs have evolved primarily as colonization factors does not explain why ExPEC are not the dominant clone(s) within the intestinal tract in most human hosts, as would be expected if they truly have a fitness advantage in this niche (113, 216). Additional epidemiological and experimental studies are needed to clarify the relationship between specific bacterial traits, including recently discovered putative VFs, and intestinal colonizing ability.

Genomic Profiling

In most of the studies discussed above, *E. coli* isolates were analyzed in the aggregate, with conclusions being based on comparisons between groups of isolates, without particular regard to the constituent clones. In contrast, genomic profiling (also called molecular fingerprinting or clonal typing) allows individual clones or strains to be resolved and analyzed. This approach underlies a distinct branch of molecular epidemiology, one that focuses on the individual clone rather than on the group. Topics that have been addressed using this approach include the reservoirs, colonization patterns, transmission pathways, and clinical diagnostics of ExPEC.

Fecal-vaginal-urethral hypothesis

According to the fecal-vaginal-urethral hypothesis, *E. coli* strains causing UTI usually derive immediately from the host's own fecal and perineal flora. This model, which was first suggested based on O serogroup data (217), is now supported also by molecular data showing that, in most episodes of acute cystitis or pyelonephritis in women, prostatitis in men, or UTI in dogs, the urine organism is also the host's predominant fecal strain (60, 63, 132, 218, 219). This concept was also demonstrated by a study that used daily sampling to obtain high-resolution tracking of colonization patterns leading up to an acute UTI episode (220). Over the week prior to the UTI episode, the causative UTI strain's prevalence in the vaginal reservoir rose rapidly, preceding the strain's appearance in urine cultures, which in turn preceded the onset of UTI symptoms. This is relevant to prevention efforts, since it suggests that fecal (and vaginal) colonization with a urovirulent organism is a potentially modifiable risk

factor for subsequent UTI. This provides a rationale for studying the determinants of intestinal (and vaginal) colonization with particular *E. coli* strains and for searching for external reservoirs of virulent *E. coli* that might be acquired by the host as intestinal (and vaginal) colonizers.

Same- versus different-strain recurrent UTI

Molecular fingerprinting also has been used to assess the same- versus different-strain nature of recurrent UTI isolates, in comparison with index isolates. The findings have been quite variable, with same-strain episodes accounting for from 25 to 100% of recurrences in different studies (60, 63, 64, 221–224), with differences in selection criteria and patient populations contributing to the variability. In one study, 30/44 (68%) of recurrent UTIs were caused by a strain previously identified in that person (60). This percentage rose with the number of recurrences per person-year, from 55% (6/11) among patients with two recurrences, through 72% (17/24) among those with four or five recurrences, to 78% (7/9) among those with six or more recurrences. Analysis of a subset of subjects established that most recurrent UTIs were due to same-strain reinfection, not overt persistence within the urinary tract, and suggested that the colonic flora was the reservoir for these reinfecting strains (although intracellular persistence within the urinary tract, as discussed below, could not be ruled out). As observed in this study and others, some patients experience multiple same-strain recurrences, some of which can occur months or years after the initial episode, occasionally with intervening UTI episodes due to unrelated strains (60, 63, 64, 221, 224, 225).

The biological relevance of this sort of analysis is that different-strain recurrence implies an independent infection episode whereas same-strain recurrence implies either relapse from a persisting endogenous focus or reintroduction of the strain from a persisting external reservoir in the host or the environment. This distinction may have clinical relevance for prevention and treatment efforts, since the occurrence of multiple independent infection episodes suggests an underlying host predisposition to infection (which may be amenable to intervention, for example, through a change in contraceptive method) (66), whereas the presence of a persisting reservoir (whether external or internal) suggests a need to identify and eradicate the reservoir.

Potential endogenous reservoirs include the long-term intracellular persistence of a strain within the bladder epithelium that seems to underlie the intermittent episodes of recurrent bacteriuria that occur in experimentally infected mice following apparent resolution of the initial infection (226). Limited clinical evidence supports that this phenomenon occurs also in humans, although its proportional contribution to same-strain recurrent UTI is undefined (227). For external reservoirs, the host may be persistently colonized with a strain in the intestine and/or vagina or may repeatedly reacquire it from the (animate or inanimate) external environment (221). In either situation, efforts to identify and eliminate the external reservoir conceivably could be protective.

Strain sharing between associated hosts

Environmental sources of uropathogenic or antimicrobial-resistant *E. coli* have been investigated by using genomic profiling, with or without VF detection and phylogenetic analysis. Within-household strain sharing has been demonstrated by PCR-based genomic profiling, PFGE, and whole-genome analysis between adult sex partners (in some instances, accompanied by symptomatic UTI in one or both individuals) and between parents and children, siblings, and even humans and pets (Figs. 2, 10, and 11) (63, 148, 224, 228–237). Likewise, several hospital-based pyelonephritis outbreaks have been documented in which health care workers seemingly transmitted a virulent strain to patients (238–240). Interestingly, some evidence suggests that what classically have been regarded as VFs also predict co-colonization of epidemiologically associated hosts, implying that these traits may promote person-to-person transmission as well as infection (224, 233, 234, 237, 241, 242).

However, that person-to-person transmission actually occurs is inferential except in the hospital setting: the underlying mechanism for the observed within-household strain sharing remains to be defined. Sexual transmission, a favored hypothesis, is supported by epidemiological evidence associating certain sexual practices with co-colonization of adult sex partners and by anatomical coincidences such as colonization of the male partner's urethra or glans penis with a strain also found in the female partner's vagina and urine (229, 241, 243). However, sexual transmission is unlikely to explain co-colonization involving children, pets, and nonsexually associated adults (63, 148, 230, 236, 237, 242, 244); other modes of host-to-host transmission (whether direct or indirect) must be considered, as must be possible coordinate acquisition from a common external source, such as the food supply.

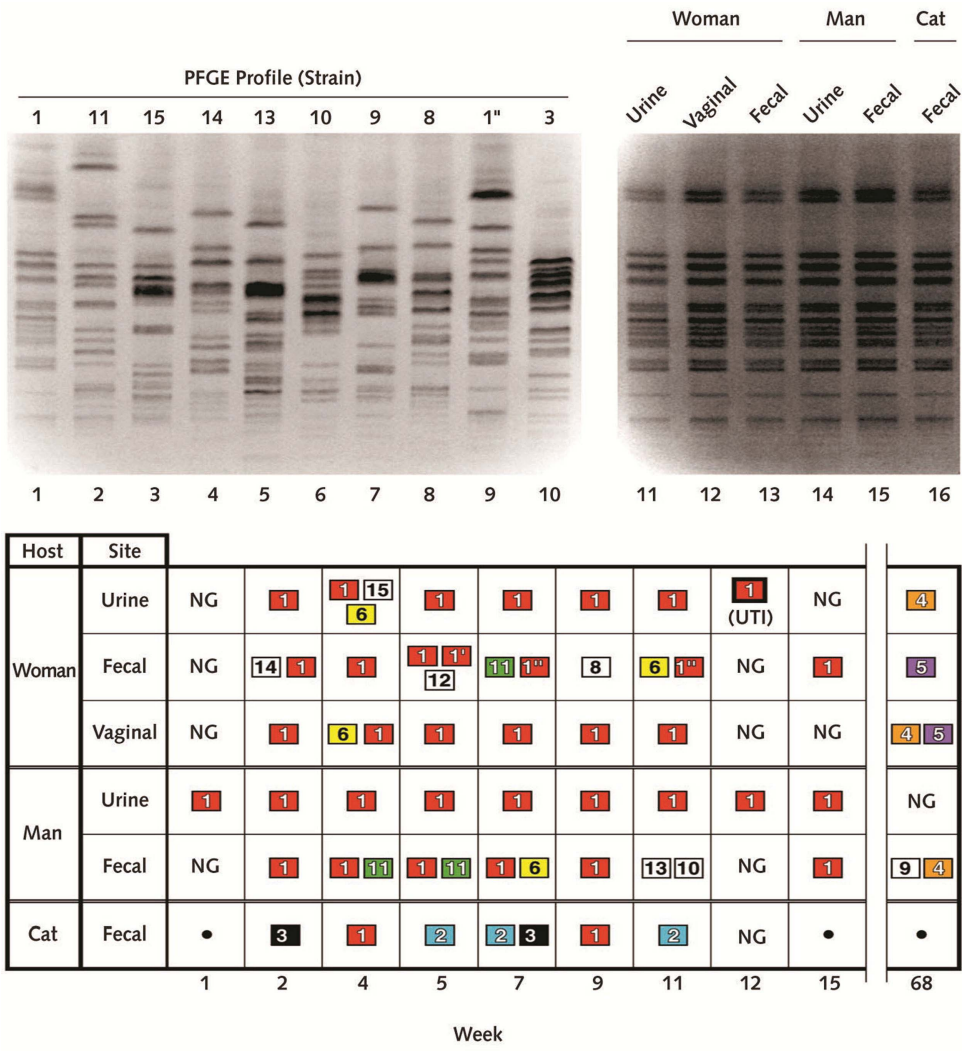


Figure 10 Pulsed-field gel electrophoresis (PFGE) profiles and colonization patterns of *Escherichia coli* isolates from three household members (man, woman, and pet cat). (Top panel) PFGE profiles. Lane numbers are shown below gel images. Lanes 1 through 10, profiles of nine of the unique strains, with strain designations shown above gel lanes, plus subtype 1″) (lane 9). Lanes 11 through 16, profiles of independent isolates of strain 1, as recovered from various anatomical sites from the woman (lanes 11–13), man (lanes 14 and 15), and cat (lane 16). (Bottom panel) Distribution of 14 unique *E. coli* strains over time (week of sampling shown below grid), as recovered from various anatomical sites from the three household members. NG, no growth; •, no sample. Strains isolated more than once appear in colored boxes, with a unique color for each strain. Strains isolated only once appear in colorless boxes. Week 12, which coincided with symptoms of acute urinary tract infection (UTI) in the woman, yielded strain 1 from the woman's urine specimen (boldface box). There is no strain 7. Strain 1, the woman's UTI strain, was the most extensively shared and persistent strain, and had the most virulence genes of the 14 strains. Reprinted from reference 242, with permission.

Community-wide dissemination of ExPEC

Transmission pathways of ExPEC within the larger community are relevant to the dissemination of virulent clonal groups within the human population. Older examples of this phenomenon include the O15:K52:H1 clonal group, which (as mentioned above) first came to attention when it caused the mysterious South London outbreak of 1986 and 1987 (245–247), and *E. coli* clonal group A, which (as also mentioned above) has emerged as a prominent cause of drug-resistant UTI across the United States (10, 61, 248) and appears to have undergone point-source spread within at least one community (61). The most recent and dramatic example is ST131-*H*30, which first emerged around 2000 and expanded rapidly thereafter to become, by 2010, the leading cause of fluoroquinolone-resistant and ESBL-associated *E. coli*

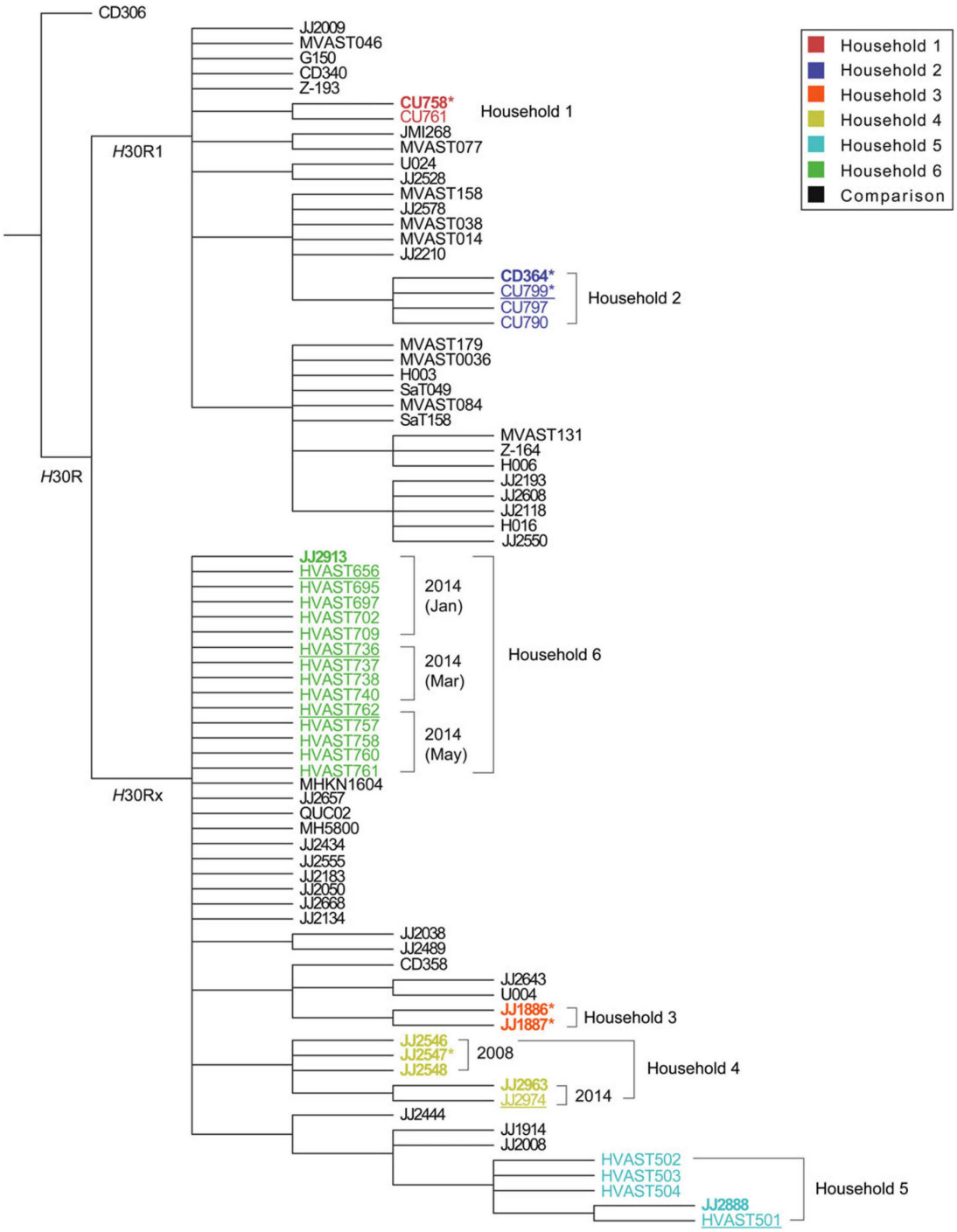


Figure 11 Bootstrapped consensus core genome phylogeny for 33 household isolates and 47 comparison isolates of *Escherichia coli* sequence type 131 (ST131). The tree was based on 1000 bootstrapped maximum likelihood trees, retaining only those nodes that appeared in >70% of trees, and was rooted with strain CD306. Branch lengths are meaningless. Household isolates (as in Fig. 2) are color-coded by household (1–6); comparison isolates are shown in black. For the household isolates: boldface indicates clinical isolates; regular font indicates fecal isolates; underlining indicates fecal isolates from a clinical isolate's source host; and asterisks indicate the 6 household isolates that were included in Price et al (11), i.e., JJ1886, JJ1887, JJ2547, CU758, CU799, and CD364 (which in Price et al was labeled as CD449). Dates are shown for the 2 households that underwent serial sampling (households 4 and 6). Clustering by household supports within-household transmission (strain sharing); near identify of clinical and fecal isolates within each household supports the fecal reservoir as the source for infection-causing strains; and variation in a given strain within its source household over time (households 4 and 5) suggests microevolution during long-term host colonization. Reprinted from reference 63, with permission.

infections worldwide (249, 250). Several other resistance-associated ExPEC clonal groups likewise have recently emerged and disseminated (78, 251, 252).

The seeming point-source outbreak behavior of clonal group A, mentioned above, prompted the suggestion that, by analogy to *E. coli* O157:H7, *Campylobacter*, and *Salmonella*, contaminated food products might serve as a transmission vehicle also for ExPEC (61). Indeed, retail foods are commonly contaminated with antibiotic-resistant *E. coli* and ExPEC, along a descending prevalence gradient from poultry (highest) through other meats (beef and pork) to produce and miscellaneous foods (lowest), and a subset of these isolates resembles human clinical isolates according to virulence genes and genomic profiles (192, 197–204). The human health implications of such contamination remain to be fully defined. Insights into this question conceivably could derive from higher-resolution molecular epidemiological comparisons of ExPEC isolates from retail foods versus colonized and infected humans to ascertain the extent of commonality between these populations. Indeed, preliminary results from a WGS-based comparison of temporally and geographically matched *E. coli* isolates from retail meat products and patients with UTI in a relatively isolated community (Flagstaff, Arizona) suggest that 11 to 20% of UTI isolates derive from genome clades that are dominated by meat-source isolates, implying a food animal source (L. B. Price, personal communication). Such cross-sectional ecological studies could be complemented by longitudinal molecular epidemiological surveillance of individuals and the foods they consume, thereby identifying temporal patterns of genomic commonality of *E. coli* strains suggesting food-borne transmission.

Within-host epidemiology in a single sample or across samples

Various molecular typing methods have been used to investigate the clonal composition of a single fecal or clinical sample or of concurrent paired samples (e.g., fecal or blood versus urine) from a given subject, and to track this over time by analyzing serial samples, or to compare same-clone and different-clone isolates from a given host. Such studies have shown that what conventionally would be regarded as monoclonal infections may actually involve multiple clones (253–255) or a single clone that has developed microheterogeneity (including for antimicrobial resistance gene content) *in vivo*, likely during adaptation to the infection niche (255). Plasmid segregation can occur *in vivo* or in the clinical laboratory, producing genetic variants with discordant resistance profiles that could conceal the underlying clonal commonality and, thus, potentially obscure the likely source of bacteremia (256). Subtle genetic differences between concurrent or sequential same-clone isolates from different niches or disease contexts (e.g., feces, cystitis, bacteremia) in a single host, or closely associated hosts, may (257, 258) or may not (255, 259) have apparent virulence-related implications, so may or may not be responsible for the isolates' divergent clinical behaviors. Finally, at the time of an acute urinary tract infection the disease-causing clone often not only predominates in the host's feces, consistent with both the "fecal-urethral" and "prevalence" hypotheses, but also exhibits more virulence-associated traits (e.g., VFs and group B2 background) than do the host's concurrent "fecal-only" clones, consistent with the "special pathogenicity" hypothesis (132, 134, 260, 261).

Clonal diagnostics

The well-established associations of specific *E. coli* lineages (as defined at the level of ST complexes, STs, and sub-ST clades, e.g., ST131-*H*30) with antimicrobial resistance, clinical manifestations, and host characteristics, together with the increasing availability of rapid molecular detection methods, allow for the development of clone-specific diagnostic tests to predict, in real time, both antimicrobial resistance and clinical phenotype (262–265). Emerging data suggest that if such tests were to be used clinically to inform empirical treatment choices, this could reduce significantly the frequency of "drug-bug" (i.e., antimicrobial versus organism) mismatch and use of overly broad-spectrum therapy, thereby improving both clinical outcomes and antimicrobial stewardship (264). Clinical studies are needed to determine the real-world effectiveness of such tests.

SUMMARY AND CONCLUSIONS

Molecular epidemiological analyses of ExPEC, which are based on structured observations of *E. coli* strains as they occur in the wild, provide an important complement to experimental assessment. Fundamental to the success of molecular epidemiological studies are the careful selection of subjects and the use of appropriate methods for genotyping and statistical analysis. To date, molecular epidemiological studies have yielded numerous important insights into host-pathogen relationships, phylo-

genetic background, reservoirs, clinical diagnostics, and transmission pathways of ExPEC, including antimicrobial-resistant strains, and have delineated areas in which further study is needed. The rapid pace of discovery of new putative VFs and the increasing awareness of the importance of VF regulation, expression, and molecular variation should stimulate many future molecular epidemiological investigations. The ever-increasing sophistication and availability of molecular typing methodologies, and the new computational and statistical approaches that are being developed to address the huge amounts of data that WGS generates, provide improved tools for such studies and allow entirely new questions to be addressed.

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