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Unique chromosomal regions associated with virulence of an avian pathogenic *Escherichia coli* strain

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ABSTRACT **The avian pathogenic *Escherichia coli* strain χ7122 (serotype O78:K80:H9) causes airsacculitis and colisepticemia in chickens. To identify genes associated with avian disease, a genomic subtraction technique was performed between strain χ7122 and the *E. coli* K-12 strain χ289. The DNA isolated using this method was found only in strain χ7122 and was used to identify cosmid clones carrying unique DNA from a library of χ7122 that were then used to map the position of unique DNA on the *E. coli* chromosome. A total of 12 unique regions were found, 5 of which correspond to previously identified positions for unique DNA sequence in *E. coli* strains. To assess the role each unique region plays in virulence, mutants of χ7122 were constructed in which a segment of unique DNA was replaced with *E. coli* K-12 DNA by cotransduction of linked transposon insertions in DNA flanking the unique sequence. The resulting replacement mutants were assessed for inability to colonize the air sac and cause septicemia in 2-week-old white Leghorn chickens. Two mutants were found to be avirulent when injected into the right caudal air sac of 2-week-old chickens. One avirulent mutant, designated χ7145, carries a replacement of the *rfb* locus at 44 min, generating a rough phenotype. The second mutant is designated χ7146, and carries a replacement at position 0.0 min on the genetic map. Both mutants could be complemented to partial virulence by cosmids carrying sequences unique to χ7122.**

Escherichia coli is a diverse species, the majority of strains of which are normal inhabitants of the intestinal tract. However, certain strains are capable of causing intestinal or extraintestinal infections in specific hosts.

An example of strain dependent host specificity is avian pathogenic *E. coli* (APEC), which is the causative agent of airsacculitis, pericarditis, perihepatitis, and colisepticemia in poultry (1). The disease is caused by a limited number of serotypes, the most predominant being O1:K1, O2:K1, and O78 (2–5). The bacteria are thought to enter the respiratory tract of chickens following inhalation of feces contaminated dust, where they colonize the air sac (6). Under certain circumstances, the bacteria are capable of establishing a systemic infection resulting in a fatal septicemia. Prior infection with other pathogens including infectious bronchitis disease virus, Newcastle disease virus, and *Mycoplasma* spp., or stresses such as starvation, overheating, and overcrowding predispose birds to colisepticemia (1, 7, 8). With the increased use of intensive confinement housing, colisepticemia has become the predominant bacterial disease affecting the poultry industry (1). However, little is known about the factors which facilitate colonization of the air sac, entry into the blood stream, or any subsequent steps involved in the virulence of avian pathogenic *E. coli*.

In a number of pathogenic isolates of *E. coli*, the association of specialized unique DNA regions with virulence is well established. The genes for class II capsule synthesis (*kps*) and for O antigen synthesis (*rfb* gene cluster) are either absent or nonfunctional in *E. coli* K-12 (9, 10). Recently, the pathogenicity islands PAI I and PAI II of uropathogenic strains (11, 12), and the locus of enterocyte effacement (LEE) of enteropathogenic and enterohemorrhagic isolates represent examples of large unique regions of chromosome encoding known and/or putative virulence attributes that are not found in *E. coli* K-12 or natural *E. coli* isolates from other sources (13).

It is likely that APEC strains will possess special attributes necessary for colonization and infection of the respiratory tract, especially in avian species. This report describes the use of a genomic subtractive hybridization technique to identify regions of the chromosome likely to be associated with virulence of APEC strains.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Culture Conditions. The naladixic acid resistant virulent APEC strain χ7122 (14) was used in subtractive hybridization, cloning and mutant construction. *E. coli* strains used in this study are listed in Table 1. Cells were grown at 37°C in Lennox broth (L broth) (18) unless otherwise stated. Minimal M9 media (18) was used to compare growth rates of various strains. Solid growth media were made by addition of 1.5% agar. Tetracycline (8 μg/ml), kanamycin (25 μg/ml), chloramphenicol (25 μg/ml), naladixic acid (12.5 μg/ml), and ampicillin (100 μg/ml) were added as required. Plasmid pYA3107 carries the *tsh* gene from strain χ7122 (19).

General Molecular Techniques. Chromosomal DNA was isolated as described by Hull *et al.* (20). ColV plasmid and cosmid DNA isolation and Southern and colony hybridizations were performed by procedures described by Sambrook *et. al.* (21). Hybridizations using the Kohara miniset (22) were performed according to the manufacturers protocol (Takara Biomedicals, Madison, WI). Radioactive probes were generated by random priming of template DNA or, where stated, by extension of a complementary primer using Klenow polymerase (21). Polymerase chain reaction (PCR) for the subtractive hybridization procedure was performed as described by Straus and Ausubel (23) using Amplitaq DNA polymerase (Perkin–Elmer/Cetus) and a 480 DNA Thermal Cycler (Perkin–Elmer/Cetus).

Cosmid Library Construction. A genomic library of χ7122 was constructed in the low copy number vector pYA3174. Plasmid pYA3174 carries an ampicillin resistance gene, a pSC101 replicon, dual *cos* sites from pCos2embl, a *Bam*HI cloning site flanked by symmetrical *Eco*RI and *Not*I sites, and the *mob* site from pGP704, permitting mobilization of cosmids by conjugal transfer from strain SM10λ*pir* (16). Chromosomal DNA from χ7122 was partially digested with *Sau*3A and size

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Abbreviations: APEC, avian pathogenic *E. coli*; PAI, pathogenicity island; LEE, locus of enterocyte effacement; L broth, Lennox broth; cfu, colony-forming units.

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Table 1. *E. coli* strains

Strain	Genotype or characteristics	Source, construction, or ref.
χ7122	O78:K80:H9	14
χ289	W1485 λ⁻ *glnV44* F⁻	This laboratory
MG1655	Wild type	15
SM10λ*pir*	*thi thr leu tonA lacY supE* λ(*pir*R6K) *recA*::RP4-2-Tc::Mu Km	16
χ2819	F⁻ *lacY1 glnV44 galK2 galT22* λ(*cI857 b2 red*β*3 S7*) *recA56* Δ*thyA57 metB1 hsdR2*	17
CAG12093	MG1655 *car-96*::Tn*10*	15
CAG18425	MG1655 *thr-3091*::Tn*10kan*	15
CAG18447	MG1655 *proAB81*::Tn*10*	15
CAG18633	MG1655 *zag-3198*::Tn*10kan*	15
NK5526	*his*G213::Tn*10*, λ⁻, IN(*rrnD-rrnE1*)	N. Kleckner
CAG12176	MG1655 *zee-3189*::Tn*10kan*	15
CAG18604	MG1655 *zgd-3156*::Tn*10kan*	15
CAG18472	MG1655 *nupG3157*::Tn*10*	15
χ7176	χ7122 *car-96*::Tn*10*	P1(CAG12093) → χ7122
χ7177	χ7176 *thr-3091*::Tn*10kan*	P1(CAG18425) → χ7176
χ7146	χ7122 (χ289:*thr-car*)	P1(χ289) → χ7177
χ7180	χ7122 *zag-3198*::Tn*10kan*	P1(CAG18633) → χ7122
χ7181	χ7180 *proAB81*::Tn*10*	P1(CAG18447) → χ7180
χ7148	χ7122 (χ289:*proAB-zag*)	P1(χ289) → χ7181
χ7178	χ7122 *zee-3189*::Tn*10kan*	P1(CAG12176) → χ7122
χ7179	χ7178 *hisG*::Tn*10*	P1(NK5526) → χ7178
χ7145	χ7122 (χ289:*hisG-zee*)	P1(χ289) → χ7179
χ7175	MG1655 *zgd-3156*::Tn*10kan* *nupG3157*::Tn*10*	P1(CAG18472) → CAG18604
χ7147	χ7122 (MG1655:*zgd-nupG*)	P1(χ7175) → χ7122

fractionated through a 10–25% NaCl gradient (20). DNA fragments of 35–50 kb were ligated to pYA3174 digested with *Bam*HI and *Pvu*II. The ligation mixtures were packaged *in vitro* and transduced into *E. coli* K-12 strain χ2819, followed by *in vivo* amplification and repackaging (17). Clones capable of complementing *E. coli* K-12 strains carrying auxotrophic mutations in *thr* (at 0.0 min), *purE* (at 12.0 min), *trp* (at 28.0), *his* (at 45.0 min), *thyA* (at 63.8 min), and *purA* (at 95.0 min) were obtained at frequencies between 10^{-3} and 10^{-6} per cosmid-bearing λ particle, indicating that DNA inserts from around the chromosome were present and that the library is therefore representative of the genome of χ7122.

Conjugal Transfer of Cosmid DNA. *In vivo* packaged cosmid clones were transferred to *E. coli* strain SM10λ*pir*, and then to mutant derivatives of χ7122 via conjugation (16).

Subtractive Hybridization. Chromosomal DNA from the prototrophic *E. coli* K-12 strain χ289 was mixed with λ DNA in a ratio of 100:1, sheared to a length of 1–3 kb, and biotinylated using photoactivatable biotin (Clontech). The resulting biotinylated DNA mixture (10 μg) was hybridized with *Sau*3A-digested χ7122 DNA (0.5 μg) in a subtractive hybridization procedure as described by Straus and Ausubel (23), except that hybrids were removed using streptavidin-coated magnetic beads (Promega). After five rounds of subtractive hybridization, adaptors were ligated to the *Sau*3A ends of the unbound fraction, and the resulting ligation was PCR amplified (23). The PCR products were then radioactively labeled with [α-^{32}P]dCTP by extension with one of the strands of the adaptor using DNA polymerase (Klenow fragment) (21).

Bacteriophage Transduction and Replacement of Unique Regions. Bacteriophage P1 *clr100* Cm (24) was used for transduction of *E. coli* (25). Optimum transduction of χ7122 was obtained following growth at 30°C to an OD_{600} of 0.3.

To replace the unique DNA of χ7122 with *E. coli* K-12 DNA using P1 *clr* 100 *Cm*-mediated transduction, two approaches were used. In one approach, *E. coli* K-12 strains were constructed with Tn*10* and Tn*10kan* insertions flanking the position of the unique DNA sequence as determined by hybridization to the Kohara miniset (see below). The transposons were cotransduced into strain χ7122, followed by selection for tetracycline and kanamycin resistance.

An alternate approach was used when hybridization to multiple Kohara clones made positioning of the unique region less precise. An individual Tn*10kan* insertion was transduced into χ7122, and the resulting strain was used as the recipient for transduction of a linked Tn*10* insertion. Transductants were screened for loss of kanamycin resistance to ensure that DNA extending to the position of the Tn*10kan* insertion was cotransduced.

In either approach, to ensure that insertions in auxotrophic markers did not affect virulence, mutant strains were transduced with lysates made on χ289 and prototrophic derivatives were selected. The resulting strains thus have *E. coli* K-12 DNA in place of the unique DNA segment of χ7122 and are deleted for the specific unique DNA region.

Virulence Studies. For LD_{50} determinations, bacteria were grown to OD_{600} of 0.4 in L broth, pelleted by centrifugation, and resuspended in phosphate-buffered saline (PBS) to a density of 10^9 colony-forming units (cfu)/ml. Suspensions (100 μl) of 10-fold dilutions were injected into the right caudal air sac of 2-week-old specific-pathogen-free white Leghorn chickens. *In vivo* selection of cosmid clones that restore virulence to avirulent mutants was performed by injecting ≈10^8 cfu of pools of 15 cosmid clones in the avirulent mutant into the right caudal air sac of 2-week-old birds. Birds were euthanatized 3 days after inoculation, and heart blood was drawn and plated on L agar containing naladixic acid and ampicillin.

RESULTS

Enrichment of Sequences Unique to χ7122. The prototrophic *E. coli* K-12 strain χ289 is avirulent ($LD_{50} > 10^8$) in

2-week-old chickens and is thus lacking some of the attributes present in the virulent APEC strain χ7122 ($LD_{50} = 9.4 \times 10^5$ cfu). To isolate DNA that is present in χ7122 but not in *E. coli* K-12, a subtractive hybridization was performed in which DNA fragments of χ7122 that hybridize with DNA from strain χ289 or λ are selectively removed. Southern hybridization experiments using radioactively labeled λ DNA to probe digests of the genome of χ7122 showed that this strain carries lambdoid prophage sequences, and since strain χ289 does not, these lambdoid sequences would be represented among unique DNA following subtractive hybridization. Exogenous λ DNA was therefore included in the subtractive hybridization procedure to remove lambdoid sequences of χ7122 that would otherwise result in high background signals in subsequent hybridization experiments using cosmid vectors and the lysogenic *E. coli* strain χ2819. Following five rounds of subtraction, PCR amplification of the χ7122 unique DNA gave a product ranging in size from <0.3 kb to 3.0 kb (data not shown).

To verify that sequences common to χ7122 and χ289 were removed by the subtraction procedure, a Southern blot of *Hin*cII-digested genomic DNA from χ289 and χ7122 was probed with the radioactively labeled PCR product. The probe hybridized to DNA fragments of χ7122 varying in size from 0.5 kb to 15 kb (Fig. 1). No hybridization was detected with DNA of χ289.

Identification of Cosmid Clones Carrying Unique DNA. A cosmid library representing the genome of χ7122 in pYA3174 was probed with the radioactively labeled χ7122 unique PCR product. From a total of 1000 cosmid clones, 145 hybridized to the unique DNA probe (data not shown). To verify that known unique sequences were represented among the positive clones, colony hybridizations were performed using the ColV plasmid from χ7122, and a *Sca*I fragment from pYA3107 that contains the *tsh* gene as probes (19, 26). Since the *tsh* gene and the ColV plasmid are present in χ7122 but not in χ289, these sequences should be represented among clones identified as carrying unique DNA. The *tsh* gene and ColV plasmid hybridized to 19 (13.1%) and 62 (42.7%) of the 145 clones, respectively (data not shown), indicating that the unique probe was able to identify cosmid clones carrying known unique sequences.

Localization of Unique Regions on the *E. coli* Chromosome. Some of the cosmid inserts will carry DNA that represent junctions between a unique DNA sequence and DNA that is homologous to the *E. coli* K-12 chromosome. The portion of the cosmid clone that is homologous to *E. coli* K-12 DNA facilitates mapping the position at which unique DNA sequences of strain χ7122 are inserted in the chromosome by hybridization to an ordered lambda library of the *E. coli* K-12 genome (Kohara miniset) (Fig. 2).

To map the positions of unique DNA on the *E. coli* chromosome, a series of cosmid DNA pools were used to probe the Kohara miniset. Since the ColV plasmid is not chromosome associated, clones identified as carrying ColV plasmid sequences were omitted. The remaining 83 cosmid clones carrying unique DNA were divided into six pools of 15 clones. To avoid background signal due to hybridization of pYA3174 to the λ vector of the Kohara clones, individual cosmid DNA pools were digested with *Not*I to isolate the insert DNA from the vector. The insert DNA was radioactively labeled by random priming, and used to probe the Kohara miniset (Fig. 3). Probes generated from six pools hybridized to a total of 33 Kohara clones, representing 12 separate positions on the *E. coli* chromosome. The numbers of the Kohara clones that hybridized to the probe pools and their position on the *E. coli* K-12 map are listed in Table 2.

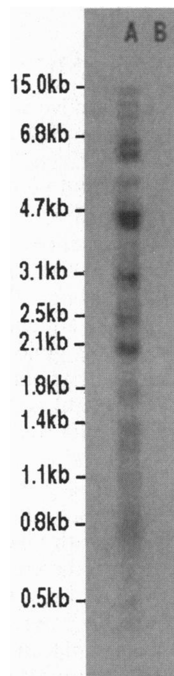

FIG. 1. Results of probing Southern blots of *Hin*cII-digested genomic DNA of strains χ7122 and χ289 with the radioactively labeled PCR product from the subtractive hybridization technique. Equal amounts (5 μg) of DNA were loaded in each lane. Strains are labeled as follows: lane A, χ7122; lane B, χ289. Molecular sizes are given in kilobases on the left.

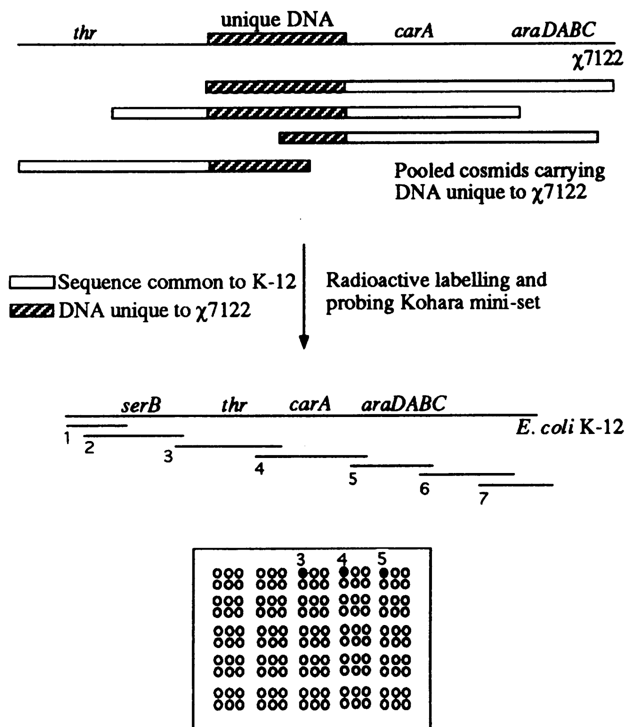

FIG. 2. (*Upper*) Schematic representation of the approach used to determine the approximate position of χ7122 unique regions with respect to the *E. coli* K-12 chromosome. Cosmid clones carrying both unique DNA (striped bars) and DNA present in *E. coli* K-12 (open bars) were used to probe an ordered library of *E. coli* K-12 chromosomal sequences (Kohara library). The genome of χ7122 is represented in the upper portion of the diagram, with cosmid clones carrying a unique DNA sequence shown beneath. The *E. coli* K-12 genome is shown in the lower portion of the diagram. A representation of the ordered Kohara library clones designated 1–7 is shown beneath the K-12 genome. Hybridization (solid circles) to the clones (*Lower*) indicates the position of the unique DNA present in the χ7122 genome with respect to the *E. coli* K-12 genome.

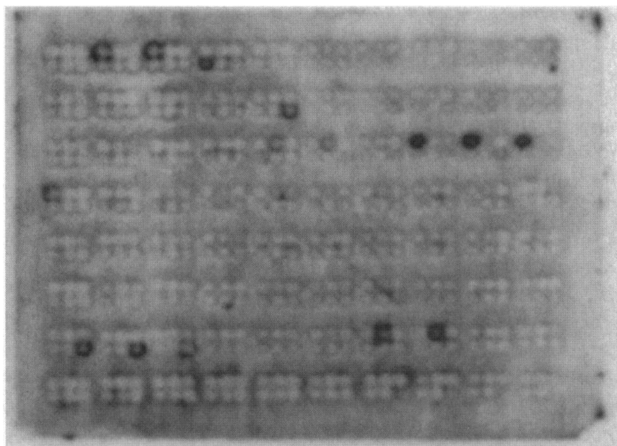

FIG. 3. An example of the result obtained when the insert DNA from a pool of 15 cosmid clones, each identified to contain DNA unique to χ7122, was radioactively labeled by random priming, and used to probe the entire Kohara miniset.

Replacement of Unique Regions with *E. coli* K-12 DNA. To assess the role of the unique regions in virulence, individual unique regions of χ7122 were replaced with the corresponding region from *E. coli* K-12. A total of four replacement mutants of strain χ7122 have so far been constructed. These included the unique regions at positions 0.0 min, 6.1 min, 45.0 min, and 66.6 min. The unique DNA sequences at positions 0.0 min and 6.1 min of χ7122 were replaced with the corresponding region of the *E. coli* K-12 genome to give strains χ7146 and χ7148, respectively (see Table 1). Strain χ7145 carries a replacement of the χ7122-unique DNA at position 45.0 min, which corresponds to the *rfb* gene cluster, encoding the O-antigen. This strain does not agglutinate with anti-O78 antibody, and no ladder pattern is detectable following SDS/PAGE gel electrophoresis and silver staining of cell extracts (data not shown), indicating that χ7145 does not produce an O-antigen. Replacement of the unique DNA at position 66.6 min of χ7122 with the corresponding region of *E. coli* K-12 gave strain χ7147. In all cases, the replacement derivatives exhibited the same growth rate as χ7122 in minimal media. All strains were shown to carry the ColV plasmid, indicating that this plasmid was not lost during strain construction.

Virulence of Replacement Mutants. To assess whether each unique region plays a role in virulence, the LD_{50} of each mutant construct was determined by injection into the right caudal air sac of 2-week-old white Leghorn chickens. Of the four mutants tested, χ7146 and χ7145, with replacements of unique DNA at 0.0 min near *thr* and *carA*, and the *rfb* region at 45 min, respectively, displayed a 100-fold reduction in virulence ($LD_{50} > 10^8$). The remaining two replacement mutants were as virulent as the wild type via this route of inoculation ($LD_{50} < 10^6$).

Isolation of Clones That Complement Replacement Mutations. Strain χ7145 is an *rfb* mutant that is unable to synthesize an O78 antigen. To complement mutations in χ7145, a single cosmid was isolated in strain χ2819 by immunoblotting with O78 antisera. The resulting clone, designated pYA3255, confers on χ2819 the ability to synthesize a long-chain O78 O-antigen as determined by slide agglutination with O78 antisera, and silver staining of cell extracts following SDS/PAGE. This clone was tranferred to strain SM10λ*pir* from which it was mobilized into strain χ7145 by conjugal transfer. The resulting strain gave a positive slide agglutination test with O78 antisera, indicating that pYA3255 was able to complement the *rfb* mutation in χ7145.

In contrast to χ7145, strain χ7146 does not have an *in vitro* phenotype with which to screen for complementing clones. However, following infection of birds with ≈10^8 cfu of χ7146, bacteria could not be detected in the blood 3 days later. We therefore used an approach in which cosmid clones that complement the deletion in χ7146 were selected based on the ability to restore virulence. To select clones that complement the defect in χ7146, six pools of 15 cosmid clones carrying unique sequence were transferred to χ7146 by conjugal transfer from strain SM10λ*pir*. Approximately 10^8 cfu of each pool was used to infect individual 2-week-old chickens by air sac injection. After 3 days, heart blood from each bird was plated on selective media to isolate surviving clones. From a total of six chickens, ampicillin-resistant bacteria were isolated from the blood of one bird (and thus one pool) at a concentration of 12 cfu/ml. Restriction enzyme digestion of cosmid DNA from 12 colonies indicated that each carried the same cosmid clone, which we have designated pYA3394. However, when restriction enzyme digests of chromosomal DNA from χ7122, χ7146, and χ289 were probed with radioactively labeled pYA3394, the genomes of χ7122 and χ7146 gave identical banding patterns, while no similarity with these strains could be observed in the banding pattern produced by χ289. This indicates that the DNA carried by pYA3394 does not correspond to the replaced region at 0.0 min.

To confirm that pYA3255 and pYA3394 were able to restore virulence to χ7145 and χ7146, respectively, groups of four birds were inoculated with ≈10^8 cfu of χ7145 (pYA3255) and χ7146 (pYA3394). After 7 days, two of four birds infected with χ7146 (pYA3394), and three of four birds infected with χ7145 (pYA3255) had severe airsacculitis, pericarditis, and serositis of the liver. Bacteria could be isolated from the air sacs, pericardium, blood, and liver of these birds, indicating that pYA3255 and pYA3394 could restore virulence to the respective mutants. Chickens infected with 10^8 cfu of χ7145 or χ7146 showed no signs of disease following necropsy, and no bacteria could be isolated from blood or internal organs. The inability of pYA3255 and pYA3394 to restore full virulence to χ7145 and χ7146, respectively, may be due to cosmid instability in the absence of ampicillin selection in the animal.

DISCUSSION

We describe the use of a subtractive hybridization procedure to enrich for sequences that are present in the APEC strain χ7122 but not in the avirulent *E. coli* K-12 strain χ289. The unique DNA sequences generated by PCR of the subtractive hybridization product hybridized to ≈8% of clones (not including those carrying ColV sequence) from a genomic cosmid library of χ7122, indicating that the chromosome contains a substantial amount of strain-specific DNA. Although it is not clear whether *E. coli* K-12 has an equivalent amount of unique DNA not present in the χ7122 genome, recent studies by Bergthorsson and Ochman (27) indicate that the *E. coli* K-12 genome is most related to natural isolates with genomes of about 4.8 Mb in size. This is smaller than other *E. coli* genomes that have been tested by as much as 600 kb. Our estimate of ≈8% (300–400 kb) of genome difference falls well within this value. Since some of the DNA isolated by subtractive hybridization may not be associated with virulence, we established the position of unique DNA on the chromosome to facilitate a relatively rapid means of deleting these sequences by replacement with the corresponding portion of the *E. coli* K-12 chromosome. This method of replacement results in an exact deletion of the unique region without disruption of adjacent genes, which may be important for virulence (28).

We determined the approximate position of 12 sites on the chromosome of χ7122 at which unique DNA was inserted. The insertion sites are distributed around the chromosome, and 5 of the 12 sites correspond to the position of previously reported virulence attributes. These include the *tsh* gene, the pathoge-

Table 2. Positions of unique DNA sequence of χ7122 relative to the *E. coli* K-12 genome

Position (min)* of unique sequence	Kohara clone numbers	Flanking genes*
20 kb (0.0)	9E4, 6H3, 22B12, 2F7	*thr, carA*
285 kb (6.1)	5A5, 3C7, 21C1, 5E5	*proAB, bet*
1065 kb (22.8)	2F1, 5A12	*appA, agpA*
1480 kb (31.7)	1A7, 7F12	*trkG, cybB*
2115 kb (45.0)	6D9, 21H10	*his, alkA*
2420 kb (51.8)	9C2	*menD, ackA*
3110 kb (66.6)	23G4, 12C6	*metK, nupG*
3460 kb (74.0)	18C4, 5F12	*aroE, rplN*
3610 kb (77.2)	10F5	*asd, ugp*
3750 kb (80.8)	6F2, 10C8, 7C3, 9B3	*glyS, selB*
4430 kb (94.8)	3H6, 3A1, 6G4, 1G10	*ampC, purA*
4530 kb (96.8)	E1F5, 5C4, E4D8, 16H7S, 7G7	*pyrB, fecA*

The position of unique DNA sequences were determined by hybridization of cosmid pools, each identified to contain DNA uniques to χ7122, to the Kohara miniset.
*Based on the *E. coli* K-12 map (18).

nicity islands PAI I (and LEE) and PAI II, the group II capsule genes, and the *rfb* gene cluster.

We previously reported the sequence of the *tsh* gene from strain χ7122 (19). Sequence immediately downstream of the *tsh* gene exhibits 100% identity with that of the *fms* gene of *E. coli* K-12, indicating that the *tsh* gene lies at the edge of the unique region at 74.0 min. The pathogenicity island PAI I of urinary tract pathogenic strain 536, and the LEE of enteropathogenic strains lies at 82.4 min on the *E. coli* chromosome (12, 13), and strain χ7122 carries a unique region at 80.8 min, which is within 80 kb of the insertion position of PAI I and LEE (the *selC* gene). PAI I is 70 kb long and includes the gene encoding hemolysin I (11, 12), while the LEE locus is 35 kb long and carries the genes for attachment and effacement of epithelial cells (13). In addition, strain χ7122 carries a unique region at 96.8 min, which is the insertion position of PAI II (near *leuX*) of UPEC strains (11). The PAI II region is 190 kb long in UPEC strains, and carries the genes for Pap-related fimbriae and hemolysin II (12). However, strain χ7122 does not exhibit hemolysis of erythrocytes or attachment and effacement of epithelial cells (unpublished data), and it is therefore likely that the unique regions at 80.8 min and 96.8 min in this strain encode proteins different to those encoded by PAI I, LEE, and PAI II. We are at present constructing replacements in these regions to test whether they also play a role in the virulence of APEC strains.

The region between *nupG* and *metK* at 66.6 min has been shown to carry the genes for synthesis and export of group II capsules in *E. coli* isolates (29). However, strain χ7122 produces a K80 capsule that is not classified as group II, but believed to be an O antigen capsule (30). The K80 capsule is thought to be involved in prevention of phagocytosis by polymorphonuclear leukocytes (31), and while it is not known whether the unique region at 66.6 min contributes to K80 capsule synthesis, replacement of this region with DNA from *E. coli* K-12 did not reduce virulence. This indicates that the unique DNA at 66.6 min is not necessary for virulence of this strain following air-sac inoculation, or that it encodes a virulence attribute which is duplicated in function by some other sequences.

In contrast, the genes necessary for O-antigen synthesis are essential for virulence of χ7122. The O-antigen is encoded by the *rfb* gene cluster at 45.0 min, and this region was identified as carrying unique sequences in χ7122. The *rfb* region of *E. coli* K-12 carries an insertion element, resulting in a rough phenotype (10). Replacement of the O78 *rfb* region of strain χ7122 with that from *E. coli* K-12 results in a rough strain, the LD_{50} of which is at least 100-fold higher than the wild type. A cosmid expressing the O78 O-antigen was able to restore partial virulence to χ7145, indicating that an O-antigen is required for virulence. Strains with an O78 O-antigen are one of the most predominant serotypes associated with colisepticemia of poultry (1), and virulent O78 isolates belong to a limited number of clonal groups (30, 32). It is not clear, however, whether the O78 serotype predominates due to the clonal nature of avian *E. coli*, or because this O-antigen possesses inherent structural properties that facilitate success of strains expressing this serotype in colisepticemia.

A second unique region that is involved in virulence lies at 0.0 min near the *thr* and *carA* loci. Replacement of this region of the chromosome of χ7122 with the corresponding region of *E. coli* K-12 increases the LD_{50} by at least 100-fold. No known virulence factor gene has been described in this position, emphasizing the ability of the method described above to detect novel regions on the chromosome associated with virulence. Using an *in vivo* selection procedure, we isolated a cosmid clone which increases the ability of χ7146 to persist in the bird, but does not correspond to the replaced region of χ7146. We are at present working to determine how extra copies of this unique DNA can compensate for the replacement at 0.0 min. We are also continuing attempts to isolate clones that correspond to the replaced region, and that complement the virulence defect of strain χ7146.

The remaining seven regions that carry unique DNA do not correspond to positions of genes for known virulence factors. Replacement of the unique DNA of χ7122 between *proAB* and *betA* at 6.1 min does not reduce virulence following air-sac inoculation. However, inoculation by air-sac injection will not identify mutants attenuated in the early stages of respiratory tract colonization, and replacement mutants showing wild-type levels of virulence by this route of infection may exhibit attenuation when administered by a different route. In addition, replacement of a single unique DNA region may not be sufficient to reduce virulence owing to the existence of multiple mechanisms by which avian *E. coli* can cause disease. We have shown previously that a knockout mutation of the *tsh* gene does not eliminate hemagglutination in χ7122 (19), indicating that other adhesins are expressed in this strain, illustrating the existence of back-up mechanisms in avian *E. coli*. In addition, virulence attributes that are also present in *E. coli* K-12 will not be deleted using a replacement approach, as is the case with type 1 fimbriae, which are found in many *E. coli* isolates including avian pathogens and *E. coli* K-12 (33). In avian strains, type 1 fimbriae can mediate adhesion to avian respiratory tissue (34), and expression of these structures can be detected in the air sacs and trachea of birds experimentally infected with avian *E. coli*, suggesting that type 1 fimbriae play a role in colonization of the avian respiratory tract (35). Therefore, replacement of unique regions that encode adhesins involved in colonization of the respiratory tract may not

exhibit a measurable phenotype in the presence of type 1 fimbriae. We are currently constructing single and multiple replacements of the remaining sites in χ7122, and combining these with mutations in the genes for type 1 fimbriae and curli to determine which unique DNA regions contribute to the virulence of APEC strains. We are also, in collaboration with others, evaluating the virulence of all the replacement mutants when administered to chickens by an aerosol route.

The ability to identify and replace unique DNA on the genome of *E. coli* is an effective means of rapidly determining regions of virulence on the chromosome. Knowledge of the location and function of unique DNA in different *E. coli* isolates should lead to an understanding of the interaction between specific bacteria and their hosts, and to how an *E. coli* pathogen evolves by acquiring DNA that facilitates adaption to a particular niche.

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1. Gross, W. B. (1984) in *Diseases of Poultry*, eds. Hofstad, M. S., Barnes, H. J., Calnek, B. W., Reid, W. M. & Yoder, H. W. (Iowa State Univ. Press, Ames), pp. 270–277.
2. Cloud, S. S., Rosenberger, J. K., Fries, P. A., Wilson, R. A. & Odor, E. M. (1985) *Avian Dis.* **29,** 1084–1093.
3. Glantz, P. J., Narotsky, S. & Bubash, G. (1962) *Avian Dis.* **6,** 322–328.
4. Harry, E. G. (1964) *Vet. Rec.* **76,** 466–470.
5. Heller, E. D. & Perek, M. (1968) *Br. Vet. J.* **124,** 509–513.
6. Carlson, H. C. & Whenham, G. R. (1968) *Avian Dis.* **12,** 297–302.
7. Nakamura, K., Yuasa, Y., Abe, F. & Narita, M. (1990) *Avian Pathol.* **19,** 713–721.
8. Leitner, G. & Heller, E. D. (1992) *Avian Dis.* **36,** 211–220.
9. Boulnois, G. J. & Roberts, I. S. (1990) *Curr. Top. Microbiol. Immunol.* **150,** 1–18.
10. Liu, D. & Reeves, P. R. (1994) *Microbiology* **140,** 49–57.
11. Hacker, J., Bender, L., Wingender, J., Lund, B., Marre, R. & Goebel, W. (1990) *Microb. Pathog.* **8,** 213–225.
12. Blum, G., Ott, M., Lischewski, A., Ritter, A., Imrich, H., Tschape, H. & Hacker, J. (1994) *Infect. Immun.* **62,** 606–614.
13. McDaniel, T. K., Jarvis, K. G., Donnenberg, M. S. & Kaper, J. B. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 1664–1668.
14. Provence, D. L. & Curtiss, R., III (1992) *Infect. Immun.* **60,** 4460–4467.
15. Singer, M., Baker, T. A., Schnitzler, G., Deischel, S. M., Goel, M., Dove, W., Jaacks, K. J., Grossman, A. D., Erickson, J. W. & Gross, C. A. (1989) *Microbiol. Rev.* **53,** 1–24.
16. Miller, V. L. & Mekalanos, J. J. (1988) *J. Bacteriol.* **170,** 2575–2583.
17. Jacobs, W. R., Barrett, J. F., Clark-Curtiss, J. E. & Curtiss, R., III (1986) *Infect. Immun.* **52,** 101–109.
18. Miller, J. H. (1992) *A Short Course in Bacterial Genetics: A Laboratory Manual and Handbook for Escherichia coli and Related Bacteria* (Cold Spring Harbor Lab. Press, Plainview, NY).
19. Provence, D. L. & Curtiss, R., III, (1994) *Infect. Immun.* **62,** 1369–1380.
20. Hull, R. A., Gill, R. E., Hsu, P., Minshew, B. H. & Falkow, S. (1981) *Infect. Immun.* **33,** 933–938.
21. Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab. Press, Plainview, NY), 2nd Ed.
22. Kohara, Y. (1990) in *The Bacterial Chromosome*, eds. Drlica, K. & Riley, M. (Am. Soc. for Microbiol., Washington, DC), pp. 29–42.
23. Straus, D. & Ausubel, F. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 1889–1893.
24. Goldberg, R. B., Bender, R. A. & Streicher, S. L. (1974) *J. Bacteriol.* **118,** 810–814.
25. Sternberg, N. L. & Maurer, R. (1991) *Methods Enzymol.* **204,** 18–43.
26. Waters, V. L. & Crosa, J. H. (1991) *Microbiol. Rev.* **55,** 437–450.
27. Bergthorsson, U. & Ochman, H. (1995) *J. Bacteriol.* **177,** 5784–5789.
28. Ritter, A., Blum, G., Emody, L., Kerenyi, M., Bock, A., Neuhierl, B., Rabsch, W., Scheutz, F. & Hacker, J. (1995) *Mol. Microbiol.* **17,** 109–121.
29. Vimr, E. R. (1991) *J. Bacteriol.* **173,** 1335–1338.
30. Cherifi, A., Contrepois, M., Picard, B., Goullet, P., Orskov, I. & Orskov, F. (1994) *J. Clin. Microbiol.* **32,** 1197–1202.
31. Rozenberg-Arska, M., Salters, M. E. C., Van Strijp, J. A. G., Geuze, J. J. & Verhoef, J. (1985) *Infect. Immun.* **50,** 852–859.
32. White, D. G., Dho-Moulin, M., Wilson, R. A. & Whittam, T. S. (1993) *Microb. Pathog.* **14,** 399–409.
33. Dozois, C. M., Fairbrother, J. M., Harel, J. & Bosse, M. (1992) *Infect. Immun.* **60,** 2648–2656.
34. Gyimah, J. E. & Panigrahy, B. (1988) *Avian Dis.* **32,** 74–78.
35. Dozois, C. M., Chanteloup, N., Dho-Moulin, M., Bree, A., Desautels, C. & Fairbrother, J. M. (1994) *Avian Dis.* **38,** 231–239.