APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Feb. 1997, p. 703–709 Vol. 63, No. 2
0099-2240/97/$04.00+0

Detection System for *Escherichia coli*-Specific Virulence Genes: Absence of Virulence Determinants in B and C Strains

PETER KUHNERT,[1]* JÖRG HACKER,[2] INGE MÜHLDORFER,[2] ANDRÉ P. BURNENS,[1] JACQUES NICOLET,[1] AND JOACHIM FREY[1]

Institute of Veterinary Bacteriology, University of Bern, CH-3012 Bern, Switzerland,[1] *and Institut für Molekulare Infektionsbiologie, D-97070 Würzburg, Germany*[2]

Received 19 August 1996/Accepted 24 October 1996

We describe a rational approach to simultaneously test *Escherichia coli* strains for the presence of known virulence genes in a reverse dot blot procedure. Specific segments of virulence genes of *E. coli* designed to have similar hybridization parameters were subcloned on plasmids and subsequently amplified by PCR as unlabeled probes in amounts sufficient to be bound to nylon membranes. Various pathogenic isolates and laboratory strains of *E. coli* were probed for the presence of virulence genes by labeling the genomic DNA of these strains with digoxigenin and then hybridizing them to the prepared nylon membranes. These hybridization results demonstrated that besides the *E. coli* K-12 safety strain derivatives, *E. coli* B and C strains are also devoid of genes encoding any of the investigated virulence factors. In contrast, pathogenic *E. coli* control strains, used to evaluate the method, showed typical hybridization patterns. The described probes and their easy application on a single filter were shown to provide a useful tool for the safety assessment of *E. coli* strains to be used as hosts in biotechnological processes. This approach might also be used for the identification and characterization of clinically significant *E. coli* isolates from human and animal species.

The species *Escherichia coli* includes a broad variety of different types, ranging from highly pathogenic strains causing widespread outbreaks of severe disease (4) to avirulent isolates which belong to the normal intestinal flora or which are well-known and safe laboratory strains (28, 29). The pathogenicity of a given strain is determined by specific virulence factors which include adhesins, invasins, toxins, capsule, and others. As in the case of the *E. coli* alpha-hemolysin (5), they are often organized in large genetic blocks, called pathogenicity islands (6, 27), which are either located on the chromosome or large plasmids or are transmitted by bacteriophages. Genes encoding virulence factors are vigorously regulated by a variety of factors (11), and their expression is often repressed under laboratory conditions. This makes phenotypic characterization of virulence often difficult and unreliable for the determination of the virulence pattern needed for safety assessment (31). Virulence assessment with gene probes which is independent of gene expression has therefore become the method of choice for strain characterization for both biotechnological and medical applications. Gene probes specific for *E. coli* virulence factors showed the absence of virulence genes in nonpathogenic *E. coli* strains (29). This has been demonstrated in particular for the genetically well determined derivatives of *E. coli* K-12 which have been known to be devoid of any known *E. coli* virulence genes (2, 28, 29). K-12 derivatives are therefore considered to be biological containment strains in all major international regulations and are recommended as safe hosts for propagation and expression of cloned genes (31). However, the knowledge about virulence factors of many *E. coli* strains, including B and C strains, which possess a high potential for biotechnological applications, is not well documented. Such strains therefore do not fulfill the criteria for biological containment and are consequently classified in higher risk classes than *E. coli* in many guidelines (31). This necessitates high security precautions, which makes them unattractive for many practical applications, especially large-scale fermentations in industrial productions.

Hence, an easy applicable tool is needed to characterize such *E. coli* strains that have the potential to be used in biotechnological applications. For this purpose, we have developed a system which allows the screening for *E. coli*-specific virulence genes in a single working process by using a reverse dot blot approach.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The following laboratory *E. coli* strains were used in this study: XL1-Blue {K-12 derivative; *recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F′ *proAB lacI*^{q}*ZΔM15* Tn*10*(Tet^r)]; Stratagene, La Jolla, Calif.}, C600 [K-12 derivative; *e14-*(*mcrA*) *supE44 thi-1 thr-1 leuB6 lacY1 rfbD1 tonA21* F$^-$], BL21 (B strain; *ompT* $r_B^-m_B^-$ F$^-$ [45]), JF1504 (B strain; LC46, prototroph, T6^r), JF1506 (B/R strain; LC122, prototroph), JF1508 [C strain; LC430, *arg his trpAM suIII* (Str^r)], JF1509 (C strain; LC431, prototroph), TOPP2 (*rif* [F′ *proAB lacI*^{q}*ZΔM15* Tn*10*(Tet^r)]; Stratagene). The following clinical isolates were used: uropathogenic *E. coli* (UPEC) J96 (O4:K6; *fhuA*$^+$ *cnf1*$^+$ F1C$^+$ *fim*$^+$ *pap*$^+$ *hly*$^+$), 536 (O6:K15:H31; *fhuA*$^+$ *fim*$^+$ *pap*$^+$ *sfa*$^+$ *hly*$^+$), and RZ475 (O6:K5; *fhuA*$^+$ *cnf1*$^+$ F1C$^+$ *fim*$^+$ *iucC*$^+$ K5$^+$ *pap*$^+$ *hly*$^+$), isolated from patients with urinary tract infections and suffering from pyelonephritis; *E. coli* IHE3034 (O18:K1; *fhuA*$^+$ *fim*$^+$ K1$^+$ *sfa*$^+$ *hly*$^+$), causing newborn meningitis (NBM); *E. coli* IMI100 (*eltI*$^+$ *fhuA*$^+$ *cfa/I*$^+$ *fim*$^+$ ST$^+$); the enteropathogenic *E. coli* (EPEC) strain NZ1743-95 (*fhuA*$^+$ *bfp*$^+$ *eae*$^+$ *fim*$^+$); the enterotoxigenic *E. coli* (ETEC) strains NZ3211-94 (*eltI*$^+$ *fhuA*$^+$ *cfa/II*$^+$ ST$^+$), isolated from a 2-year-old girl hospitalized for dehydrating diarrhea, 34344f (O6:K15:H$^-$; *eltI*$^+$ *fhuA*$^+$ *cfa/II*$^+$ *fim*$^+$ ST$^+$), and C9221a (O6:K15:H16; *fhuA*$^+$ *cfa/II*$^+$); the enterohemorrhagic *E. coli* (EHEC) strain NZ4253-92 (O157:H7; SLT$^+$ *fhuA*$^+$ *eae*$^+$ *fim*$^+$ *ely*$^+$), isolated from stools of a 6-year-old boy with bloody diarrhea; the enteroinvasive *Shigella flexneri* NZ1679-94 (*fhuA*$^+$ *fim*$^+$ *ipa*$^+$ *iucC*$^+$); and the enteroaggregative *E. coli* (EAggEC) strain NZ1470-95 (reference strain O44, kindly provided by H. Karch; *aaf/I*$^+$ *fhuA*$^+$). For subsequent DNA isolation, all strains were grown on Luria-Bertani agar.

Preparation of genomic DNA and cloning procedures. Genomic DNA was prepared by the method of Pitcher et al. (36) or with the QIAamp tissue kit (QIAGEN, Hilden, Germany).

Clones containing parts of characterized virulence genes were produced by PCR on either plasmid or genomic DNA harboring corresponding genes. Details concerning primers and their locations as well as fragment lengths are given below and in Table 1. Restriction sites were included in the primers to allow easy cloning and subsequent precise excision of cloned fragments, so that no plasmid

* Corresponding author. Mailing address: Institute of Veterinary Bacteriology, Laenggass-Str. 122, CH-3012 Bern, Switzerland. Phone: 41 31 6312369. Fax: 41 31 6312634. E-mail: Kuhnert@vbi.unibe.ch.

TABLE 1. Plasmids and primer pairs generated in this study and used for probe preparation by PCR

Plasmid	Gene(s)	Position on sequence of accession no.	Primers	Sequence	Length (nucleotides)	Reference
pJFFEC1	*sltI*	278–801 on L04539	EC1/2-L	5′ GC**TCTAGA**TTGAACGAAATAATTTATATG 3′	530	34
			EC1-R	5′ GC**TCTAGA**TGATGATGACAATTCAGTAT 3′		
pJDFFEC2	*sltII*	278–795 on M12534	EC2-R	5′ GC**GGATCC**ATGATGGCAATTCAGTAT 3′	520	48
			EC1/2-L	5′ GC**TCTAGA**TTGAACGAAATAATTTATATG 3′		
pJFFEC3	*eltIA*	70–750 on V00275	EC3-L	5′ AC**GGATCC**TACCGTGCTGACTCTAGACC 3′	680	44
			ETEC-LT-R	5′ TTCATCCC**GAATTC**TGTTAT 3′		
pJFFEC4	*eltIIA*	48–711 on M17894	EC4-L	5′ TC**GAATTC**AGCAAACGATTTCTTTAGAG 3′	665	35
			EC4-R	5′ AA**GGTACC**CCTGCGTTTTAAGAGTTTTT 3′		
pJFFECAAF	*aaf/I*	53–644 on X81423	ECAAF-L	5′ TCT**GAATTC**GACACAGACTCTGGCGAAAG 3′	730	40
			ECAAF-R	5′ TGT**GAATTC**TGGGATTGCACTCTCAGGA 3′		
pJFFECAER	*fhuA*	1039–2036 on M12486	ECAER-L	5′ ATG**GAATTC**CCGGTTTCCGTGCTTTA 3′	1,000	12
			ECAER-R	5′ CGG**GAATTC**CGGCAACGCGGTTAA 3′		
pJFFECBFP	*bfpA*	32–599 on Z12295	ECBFP-L	5′ CCT**GAATTC**ACGGGGGTTTTATAAGGAAA 3′	570	14
			ECBFP-R	5′ TCA**GAATTC**TTACATGCAGTTGCCGCTTC 3′		
pJFFECCFA	*cfa/I*	9–661 on M55661	ECCFA-L	5′ AAT**ATCGAT**GATAACTGTGTAAAAA 3′	650	20
			ECCFA-R	5′ GTTTC**CTGCAG**TTGGGGCGGTAC 3′		
pJFFECCNF	*cnf-1*	1321–2229 on X70670	ECCNF-L	5′ TTT**AAGCTT**TTACTAAAAAATTATTA 3′	910	15
			ECCNF2-R	5′ ACT**AAGCTT**TACAATATTGACATG 3′		
pJFFECCS3	*cfa/II*	11–556 on M35657	ECCS3-L	5′ GTA**GAATTC**AGGTACGTATACTGTTGG 3′	540	8
			ECCS3-R	5′ TAT**GAATTC**ACGGTAATTACCTGAAACT 3′		
pJFFECEAE	*eae*	2565–3241 on M58154	ECEAE-L	5′ GGC**GAATTC**CGCATGAGCGGCTG 3′	680	19
			ECEAE-R	5′ ATT**GAATTC**ATAGGCGCGAGCCGTCAC 3′		
pJFFECF1C	F1C gene	185–704 on M13053	ECF1C-L	5′ GC**GAATTC**ATCTCCATGGCTGTA 3′	520	46
			ECF1C-R	5′ GC**GAATTC**ACTTTAAAGGTGGCGTCG 3′		
pJFFECFIM	*fimA*	625–1132 on X00981	ECFIM-L	5′ GGC**GAATTC**TGTTCTGTCGGCTCTGTC 3′	510	21
			ECFIM-R	5′ TTG**GAATTC**AACCTTGAAGGTCGCATC 3′		
pJFFECIPA	*ipaH*	991–1676 on M76445	ECIPA-L	5′ TCC**GAATTC**CTTGACCGCCTTT 3′	690	17
			ECIPA-R	5′ TTC**GAATTC**ACGCATCACCTGTGCA 3′		
pJFFECIUC	*iucC*	2829–3635 on X76100	ECIUC-L	5′ GC**GAATTC**GGCGATGACCGCTACTG 3′	810	26
			ECIUC-5	5′ GC**GAATTC**CAGCGTGAAGCCAGTG 3′		
pJFFECK1	*neuA*, *neuC*	From 786 on J05023	ECK1B-L	5′ GCG**GAATTC**ATTGGACACTCGCTGTTTG 3′	840	50
		to 499 on M84026	ECK1B-R	5′ GCG**GAATTC**GCATTGATGCTGCGATAG 3′		49
pJJECK5	*kfiB*	5120–5719 on X77617	ECK5-L	5′ TCT**GAATTC**GACTACCTCCCATAATG 3′	600	Unpublished data
			ECK5-R	5′ CGC**GAATTC**CGGGTGGGCAGATCCATCT 3′		
pJFFECPAP	*papA*	1775–2300 on X61239	ECPAP-L	5′ ATT**GAATTC**GTTATTGCCGGTGCGGTA 3′	530	25
			ECPAP-R	5′ TCA**GAATTC**AATTCGCAACTGCTGAGA 3′		
pJFFECSFS	*sfaS*	626–1195 on X16664	ECSFS-L	5′ GC**GAATTC**TTATATTGGCCACCGGTC 3′	570	42
			ECSFS-R	5′ GC**GAATTC**AACAATGCAAACGATGGC 3′		
pFFECST	*stIA* or *stIB*	Part of pKAD008	ECST-L	5′ ATC**TCTAGA**GATCGAATTCCCG 3′	620	1
			ECST-R	5′ ATG**TCTAGA**CCCAGAATCTGAGCACA 3′		
pJFFECSFA	*sfaA*	176–705 on X17420	ECSFA-L	5′ TTA**GAATTC**ATCTCCATGGCTGTA 3′	530	41
			ECSFA-R	5′ TGA**GAATTC**TGGTACTGAACTTTAAAGGT 3′		
pJFFRTX6II	*hylA*	3420–3696 on M14107	RTX6II-L	5′ TAT**GAATTC**ACTCATATCAATGG 3′	680	18
			RTX6II-R	5′ TCT**GAATTC**TGATTAGAGATATCACCTGACTC 3′		
pJFFRTX7	*elyA*	2140–2922 on X79839	RTX7-L	5′ GAT**GAATTC**AAAGGCGGTAA 3′	780	39
			RTX7-R	5′ TAA**GAATTC**ATCACCTGAATCGAAC 3′		

sequences remained. Wherever possible, *Eco*RI was included as a restriction site to generalize the procedure. PCR fragments were purified with a QIAquick PCR purification kit (QIAGEN, Basel, Switzerland). Plasmid pBluescriptII-SK$^-$ (Stratagene) and purified PCR fragments were digested with the corresponding restriction enzyme(s) (Boehringer GmbH, Mannheim, Germany), subjected to agarose gel electrophoresis (Sigma, Buchs, Switzerland), purified by Jet-Sorb (Genomed, Bad Oeynhausen, Germany), and ligated for 2 h at room temperature before transformation of DH5-α by the calcium chloride method (38). All clones obtained were analyzed by sequencing.

Sequencing. Sequencing was done with the AmpliTaq FS dye terminator cycle sequencing kit (Perkin-Elmer, Norwalk, Conn.) as described in the manufacturer's recommendations with the vectors T3 and T7 matching primers flanking the cloned inserts in pBluescriptII-SK$^-$, and the sequencing reactions were run on an ABI PRISM 310 genetic analyzer.

Probe preparation. To get pure, plasmid contaminant-free probes, cloned fragments were excised with the appropriate restriction enzyme(s) as described in the supplier's recommendations, purified twice over agarose gels with the Jet-Sorb kit (Genomed), and then used as a template for PCR with internal primers. This yielded enough pure template to be used for the dot blot assay.

PCR conditions. PCR was performed with either a model PE9600 or PE2400 automated thermocycler with MicroAm tubes (Perkin-Elmer Cetus, Norwalk, Conn.). The reaction was carried out in a 50-μl volume containing 5 μl of 10× buffer (supplied with *Taq*), 20 pmol of primer (each), 1 mM deoxynucleoside triphosphate, 2.5 U of *Taq* DNA polymerase (Boehringer), and 1 μl of bacterial lysate or 100 ng of genomic DNA. About 1 to 10 ng of plasmid DNA or insert DNA was used. PCR conditions were as follows: 35 cycles at 94°C for 30 s, 52°C for 30 s, and 72°C for 1 min. A final extension step for 7 min at 72°C was included.

Blotting and hybridization. Dot blot hybridizations were performed in a reverse mode with unlabeled specific PCR fragments on the filter, which was hybridized with labeled total genomic DNA of various *E. coli* strains. Briefly, dot blots were carried out with a Bio-dot apparatus (Bio-Rad Laboratories, Herkules, Calif.) on positively charged nylon membranes (Boehringer). Approximately 200 or 20 ng of PCR product was solubilized in 100 μl of TE (10 mM Tris, 1 mM EDTA [pH 8.0]), denatured by adding 50 μl of 1 M NaOH for 20 min at room temperature, and neutralized by adding 50 μl of 1 M HCl. Blotting was then performed as described in the manufacturer's instructions with 100 μl of DNA solution per well. Alternatively, 1 μl of PCR product was directly spotted on nylon membranes and, after drying, denatured for 5 min in 1.5 M NaCl–0.5 M NaOH and neutralized for 5 min in 1.5 M NaCl–0.5 M Tris (pH 8.0). After blotting, the filters were baked at 80°C under vacuum for 30 min. After at least 1 h of prehybridization, hybridization was carried out in a mixture of 5× SSC, 2% blocking reagent (Boehringer), 0.1% *N*-lauroylsarcosine–Na salt, and 0.02% sodium dodecyl sulfate (SDS) at 68°C in rotating glass tubes overnight (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). Total genomic DNA labeled by

random priming with the digoxigenin (DIG)-High Prime DNA labeling kit (Boehringer) was included in the hybridization solution. The washing of filters was performed as follows: two times, 5 min each, with 50 ml of 2× SSC–0.1% SDS per 100 cm^2 at room temperature, followed by two times, 15 min each, with 50 ml of 0.2× SSC–0.1% SDS per 100 cm^2 at room temperature. The filter was then processed with phosphatase-labeled anti-DIG antibody as described in the producer's protocol. Signals were produced with the chemiluminescent substrate CDP-Star (Boehringer). Signal detection was done on X-ray films.

RESULTS

Construction of probes. In an attempt to establish a method which allows the simultaneous screening of *E. coli* strains for the presence of known major virulence factors, we constructed several gene probes, all of which hybridize with comparable melting temperatures and washing conditions. Moreover, it was necessary to be able to produce substantial amounts of these probes at a high purity. We therefore cloned specific segments 500 to 1,000 bp in length from the different described virulence genes which served as templates in PCR for their amplification and subsequent cloning as summarized in Table 1.

In detail, two clones were constructed for the detection of Shiga-like toxins found in EHEC strains. Plasmid pJFFEC1, which contains the probe based on the *sltIA* gene, detects the genes encoding Shiga-like toxin I as well as Shiga toxin and was constructed by PCR with pJN37-19 (32) as a template and the primers EC1/2-L and EC1-R. The resulting PCR product was digested with *Xba*I and ligated into the corresponding site of pBluescript. For gene probe preparation, the 530-bp insert of pJFFEC1 was cut with *Xba*I and a second PCR amplification was done with the purified insert as a template and the same primers, EC1/2-L and EC1-R. Plasmid pJFFEC2, which is based on the *sltIIA* gene, is able to detect genes encoding either Shiga-like toxin II or its variants and was constructed with pNN111-19 (32) as a template and the primers EC1/2-L and EC2-R for PCR amplification. The PCR product was double digested with *Bam*HI-*Xba*I and subsequently ligated into pBluescript digested with the same enzymes. The 520-bp gene probe was amplified with the purified insert of pJFFEC2, which had been excised with *Bam*HI-*Xba*I, as a template and the primers EC1/2-L and EC2-R by PCR.

The heat-labile enterotoxins LT-I and LT-IIa are found in ETEC strains from humans or cattle, respectively. Two clones were generated for the corresponding virulence genes. Plasmid pJFFEC3, based on *eltIA*, was constructed with pEWD299 (13) as a template and the primers EC3-L and ETEC-LT-R by PCR amplification. The resulting 680-bp fragment was then simultaneously digested with *Bam*HI and *Eco*RI and ligated into the matching sites of pBluescript. Afterwards, the gene probe was amplified with the same primers, EC3-L and ETEC-LT-R, and the purified *Bam*HI-*Eco*RI insert of pJFFEC3 as a template. Plasmid pJFFEC4, containing part of *eltIIA*, was constructed by PCR amplification with pTC200 (10) as a template and the primers EC4-L and EC4-R. The PCR product was digested with *Eco*RI-*Kpn*I and subsequently ligated into the equivalent sites of pBluescript. For gene probe production, the 665-bp insert was excised with *Eco*RI and *Kpn*I, and a second PCR was performed on the purified insert with the same primer pair, EC4-L–EC4-R.

Plasmids harboring parts of the hemolysin gene clusters found in hemolytic UPEC and of the enterohemolysin gene of EHEC strains were also constructed. pJFFRTX6II was assembled by using pHLY152 (33) as a template and the primers RTX6II-L and RTX6II-R for PCR amplification. The resulting *hlyA*-specific fragment was digested with *Eco*RI and cloned into the corresponding site of vector pBluescript. The 680-bp fragment was afterwards excised with *Eco*RI, purified, and applied as a template in a second PCR with the same primer pair, RTX6II-L–RTX6II-R. Plasmid pRTX7 was created with pCVD419 (24) as a template and the primers RTX7-L and RTX7-R by PCR amplification, subsequent digestion of the product with *Eco*RI, and cloning into the same site of pBluescript. Preparation of this *elyA*-specific 780-bp gene probe was done by using the purified insert excised with *Eco*RI as a template and the primer pair RTX7-L and RTX7-R for PCR amplification.

pJFFECAAF is the plasmid specific for the *aaf/I* gene found in EAggEC and was constructed by PCR amplification with pCVD432 (3) as a template and the primers ECAAF-L and ECAAF-R. The 730-bp product was digested with *Eco*RI and cloned into the matching site of pBluescript. The gene probe was then synthesized by PCR with the purified *Eco*RI fragment from pJFFECAAF as a template and the primers ECAAF-L and ECAAF-R.

pJFFECBFP was constructed by PCR amplification with pJN16 (30) as a template and the primer pair ECBFP-L–ECBFP-R. The resulting fragment was restricted with *Eco*RI and cloned accordingly into pBluescript. The gene probe was produced by PCR with the purified *Eco*RI fragment from pJFFECBFP as a template and the primer pair ECBFP-L–ECBFP-R, resulting in a 570-bp probe specific for *bfpA* of invasive EPEC.

Clones containing gene fragments of colonization factor antigens found in ETEC were based on the *cfa/I* gene and the gene encoding CS3 (*cfa/II*). Plasmid pJFFECCFA specific for *cfa/I* was produced with pTZCFAI (20) as a template and the primers ECCFA-L and ECCFA-R by PCR amplification. Digestion of the 650-bp PCR product with *Eco*RI and its cloning into the corresponding site in pBluescript resulted in plasmid pJFFECCFA. The PCR probe was generated by using the purified *Eco*RI insert of pJFFECCFA as a template and the primers ECCFA-L and ECCFA-R. Plasmid pJFFECCS3 was generated by performing PCR on pCS100 (7) with the primers ECCS3-L and ECCS3-R. The resulting 540-bp fragment was then cloned analogously to pJFFECCFA. The CS3-specific gene probe was amplified with the purified *Eco*RI insert of pJFFECCS3 as a template and the primer pair ECCS3-L–ECCS3-R.

A 910-bp *Hin*dIII fragment from plasmid pUC8G (15) was cloned into the matching site of pBluescript, resulting in plasmid pJFFECCNF. It covers part of the chromosomally located *cnf-1* gene found in UPEC strains. The probe was prepared by using the purified *Hin*dIII insert of pJFFECCNF as a template and the primers ECCNF-L and ECCNF-R for PCR amplification.

The pJFFECEAE plasmid was generated by amplifying a 680-bp fragment from pCVD434 (19) with the primers ECEAE-L and ECEAE-R. Digestion of the PCR fragment with *Eco*RI and subsequent cloning into pBluescript resulted in a plasmid containing part of the *eae* gene, which is specific for the locus of enterocyte effacement present in some EPEC and EHEC strains. The gene probe was then amplified from the purified *Eco*RI insert of pJFFECEAE by using the primers ECEAE-L and ECEAE-R.

Plasmid pJFFECSFA, used for detection of the S-fimbrial gene (*sfa*), was generated by PCR amplification of a part of the *sfaA* (41) gene from DNA of UPEC 536 with the primers ECSFA-L and ECSFA-R. The 530-bp PCR product was digested with *Eco*RI and cloned into pBluescript accordingly. The gene probe was created by PCR of the purified *Eco*RI insert of pJFFECSFA again by use of the primer pair ECSFA-L–ECSFA-R. Since the *sfaA* gene may vary in different strains, the *sfaS* (42)-specific construct pJFFECSFS was developed in

addition by performing PCR with DNA of strain 536 by use of the primer set ECSFS-L–ECSFS-R. The PCR fragment was cloned analogously to pJFFECSFA. The 570-bp *sfaS*-specific gene probe was then amplified from the *Eco*RI insert of pJFFECSFS with the primers ECSFS-L and ECSFS-R.

An amplification product of genomic DNA from UPEC J96 is pJFFECF1C, which resulted from a PCR with primers ECF1C-L and ECF1C-R. The PCR fragment was digested with *Eco*RI and cloned into the matching site in pBluescript. It covers part of the F1C fimbrial subunit gene (46) which shows some similarity to *sfaA* (41) but was included as a separate probe for the reasons mentioned above. The 520-bp probe was produced by use of the purified insert of pJFFECF1C and the primers ECF1C-L and ECF1C-R.

Plasmid pJFFECPAP, which specifically detects P-fimbrial genes, was produced by amplification of a 530-bp fragment from the UPEC strain-specific *papA* gene from *E. coli* J96, with primers ECPAP-L and ECPAP-R, and subsequent cloning of the fragment into the *Eco*RI site of pBluescript. The gene probe was then amplified with the purified *Eco*RI insert of pJFFECPAP as a template and the primers ECPAP-L and ECPAP-R.

Plasmid pJFFECIPA was developed for the detection of the so-called invasion plasmid which is found in enteroinvasive *E. coli*. It was cloned by *Eco*RI digestion of the 690-bp fragment resulting from PCR amplification of plasmid pWR393 (47) with the primers ECIPA-L and ECIPA-R and cloning into the corresponding site in pBluescript. pJJFECIPA is based on the *ipaH* gene since it is the most stable *ipa* gene and might also integrate into the chromosome (17).

Another virulence factor which is frequently found in septicemic *E. coli* and UPEC strains is the iron uptake system named aerobactin. One of the genes of the aerobactin cluster is the so-called *iucC* gene (26). pJFFECIUC contains part of the *iucC* gene amplified from plasmid pRG12 (16) with the primers ECIUC-L and ECIUC-R and cloned into the *Eco*RI site of pBluescript. The 810-bp probe was generated by PCR with the purified *Eco*RI insert pJFFECIUC as a template and the primers ECIUC-L and ECIUC-R.

Moreover, plasmids specific for the two capsule antigens K1 (pJFFECK1) and K5 (pJFFECK5), frequently found in isolates from patients suffering from urinary tract infection and NBM, were constructed. For pJFFECK1, an 840-bp part of region 2 (overlapping parts of *neuA* and *neuC* genes) (49, 50) contained in plasmid pKT274 (37) was amplified with the primers ECK1-L and ECK1B-R and subsequently cloned into the *Eco*RI site of pBluescript. For pJFFECK5, a 600-bp part from region 2 contained in plasmid pGB110 (37) was amplified with primers ECK5-L and ECK5-R and cloned analogously to pJFFECK1. The gene probes were then produced by PCR from purified *Eco*RI inserts of pJFFECK1 and pJFFECK5 with the primer pairs ECK1-L–ECK1B-R and ECK5-L–ECK5-R, respectively.

A plasmid containing specific parts of the genes for the heat-stable enterotoxins was constructed from pKAD008 (1) with the primers ECST-L and ECST-R in a PCR amplification and by cloning into the *Xba*I site of pBluescript, thereby obtaining the divalent plasmid pJFFECST. This plasmid contains parts of the *stIA* and *stIB* genes but not the LT-I-specific part also present in pKAD008. The 620-bp gene probe was generated by amplification of the purified *Xba*I insert with the primers ECST-L and ECST-R.

We included three positive control gene probes in our probe set. One was pJFFECFIM, based on the *fimA* gene (21), of which a 510-bp fragment was amplified out of genomic DNA from strain J96 with primers ECFIM-L and ECFIM-R and cloned into the *Eco*RI site of pBluescript. The specific probe was generated by PCR amplification with the purified *Eco*RI insert of pJFFECFIM as a template and the primers ECFIM-L and ECFIM-R. Since only about 70% of *E. coli* strains elaborate type I fimbriae, we included another widespread gene, the gene for ferrichrome-iron receptor (*fhuA*), contained in pJFFECAER (12). This plasmid was created by amplification of a part of pRG12 (16) with the primers ECAER-L and ECAER-R and cloning the fragment into the *Eco*RI site of pBluescript. As a control, plasmid pBluescript, used for cloning, was included in the hybridization experiments. Due to common sequences it hybridized with all *E. coli* strains tested. Table 1 summarizes all plasmids and primers generated in this study.

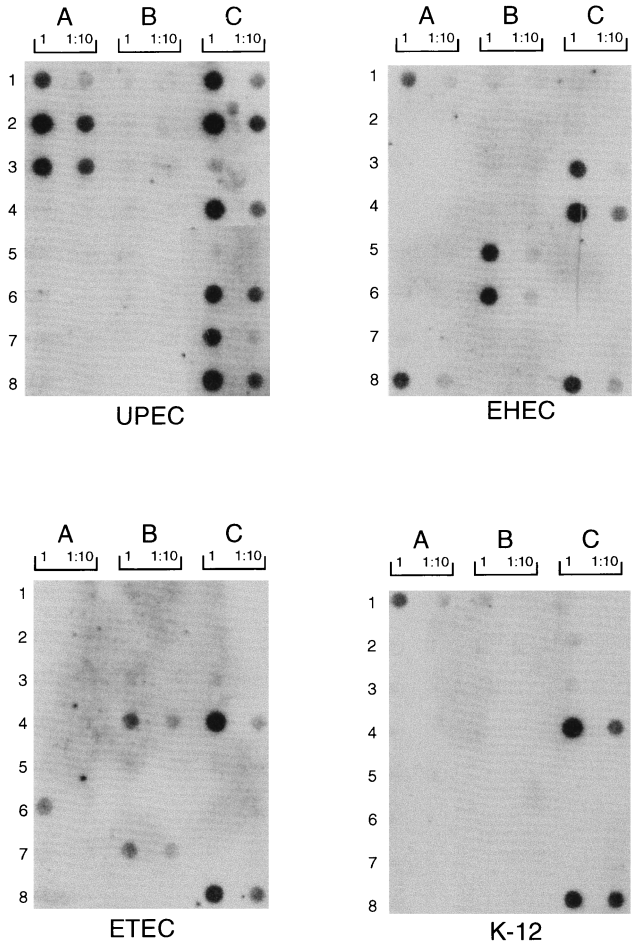

FIG. 1. Dot blot hybridizations of filters covered with unlabeled PCR probes and hybridized with labeled genomic bacterial DNA. About 100- and 10-ng amounts of PCR product amplified with internal primers from twice-purified fragments were bound to nylon membranes. Hybridization was then carried out with total genomic DNA labeled with DIG by random priming. The strains used as representatives of the different *E. coli*, indicated at the bottom of the blots, are as follows: UPEC, J96; ETEC, NZ3211-94; EHEC, NZ4253-92; K-12, XL1-Blue. The probes used were as follows: A1, ECFIM; A2, ECPAP; A3, ECSFA; A4, ECK1; A5, ECK5; A6, ECCS3; A7, ECBFP; A8, ECEAE; B1, ECIPA; B2, ECAAF; B3, ECCFA; B4, ECST; B5, EC1; B6, EC2; B7, EC3; B8, EC4; C1, ECCNF; C2, RTX6II; C3, RTX7; C4, ECAER; C5, ECIUC; C6, ECSFS; C7, ECF1C; C8, pBluescript KS.

Hybridization with specific strains. For probe preparation, the inserts were first excised from plasmids with the appropriate restriction enzyme(s) and the fragment was then purified twice over agarose gels prior to PCR. This purification step is

TABLE 2. Hybridization results of probes with labeled genomic DNA from pathogenic *E. coli* strains used as positive controls

Probe	Gene	Hybridization with strain											
		UPEC			*E. coli* IHE3034[a]	EAggEC NZ1470-95	Enteroinvasive NZ1679-94[b]	EPEC NZ1743-95	*E. coli* IMI100	ETEC			EHEC NZ4253-92
		536	J96	RZ475						34344f	C9221a	NZ3211-94	
EC1	*sltIA*	−	−	−	−	−	−	−	−	−	−	−	+
EC2	*sltIIA*	−	−	−	−	−	−	−	−	−	−	−	+
EC3	*eltIA*	−	−	−	−	−	−	−	+	+	−	+	−
EC4	*eltIIA*	−	−	−	−	−	−	−	−	−	−	−	−
ECAAF	*aaf/I*	−	−	−	−	+	−	−	−	−	−	−	−
ECAER	*fhuA*	+	+	+	+	+	+	+	+	+	+	+	+
ECBFP	*bfpA*	−	−	−	−	−	−	+	−	−	−	−	−
ECCFA	*cfa/I*	−	−	−	−	−	−	−	+	−	−	−	−
ECCNF	*cnfI*	−	+	+	−	−	−	−	−	−	−	−	−
ECCS3	*cfa/II*	−	−	−	−	−	−	−	−	+	+	+	−
ECEAE	*eae*	−	−	−	−	−	−	+	−	−	−	−	+
ECF1C	F1C gene	+	+	+	+	−	−	−	−	−	−	−	−
ECFIM	*fimA*	+	+	+	+	−	+	+	+	+	−	−	+
ECIPA	*ipaH*	−	−	−	−	−	+	−	−	−	−	−	−
ECIUC	*iucC*	−	−	+	−	−	+	−	−	−	−	−	−
ECK1	*neuAC*	−	−	−	+	−	−	−	−	−	−	−	−
ECK5	*kfiB*	−	−	+	−	−	−	−	−	−	−	−	−
ECPAP	*papA*	+	+	+	−	−	−	−	−	−	−	−	−
ECSFS	*sfaS*	+	+	+	+	−	−	−	−	−	−	−	−
ECST	*stIA* or *-B*	−	−	−	−	−	−	−	+	+	−	+	−
EXSFA	*sfaA*	+	+	+	+	−	−	−	−	−	−	−	−
RTX6II	*hylA*	+	+	+	−	−	−	−	−	−	−	−	−
RTX7	*elyA*	−	−	−	−	−	−	−	−	−	−	−	+
pBluescript		+	+	+	+	+	+	+	+	+	+	+	+

[a] The strain causing NBM.
[b] *S. flexneri*.

most important because plasmid sequences, which are unspecifically coamplified if contained in the template, hybridize with *E. coli* (Fig. 1). Approximately 100 and 10 ng of PCR product were then bound to nylon membranes with a dot blot apparatus and afterwards hybridized with labeled bacterial DNA. Since the applied probe fragments all have about the same lengths and melting temperatures, the same stringency conditions could be used for the whole filter. The new approach was tested with known pathogens to validate the technique and all the probes. It was then applied to well-known and recently developed laboratory strains. Figure 1 shows the results of four such dot blot experiments in which three pathogenic *E. coli* strains as well as the safety strain *E. coli* K-12 were included. The latter reacted only with the positive controls, whereas the pathogenic strains hybridized to several probes depending on the type of strain. Tables 2 and 3 list the strains and results obtained by this kind of hybridization experiment.

DISCUSSION

E. coli has a long-standing tradition to be used as a target organism of basic research and for gene cloning and expression. Whereas K-12 derivatives are declared safety strains and are easily discernible from different *E. coli* strains by a specific PCR (22), very little is known about other and newly developed *E. coli* hosts which might be of great value for research and industrial applications. However, due to the lack of knowledge on potential virulence, they are normally classified at higher security levels (31). The work presented here provides a tool for the genetic characterization of *E. coli* strains with regard to their harboring of potential virulence factor genes. The detection of genes encoding virulence factors is a straightforward means of differentiating between a nonpathogenic isolate and a potentially pathogenic strain, provided that accurate gene probes for their detection are available. All the virulence genes which lay the basis for the development of specific gene probes are well characterized. The probes include all known genes for major virulence factors which have recently been reviewed (23, 43) and are summarized in Table 1.

To simultaneously probe for all virulence genes, it was necessary to subclone parts of these plasmids or genes to obtain specific probes of approximately equal lengths to be used with the same hybridization conditions. To simplify the whole procedure of probe preparation, the same restriction sites were used to extract the fragments serving as probes, and the same annealing temperatures of primers to amplify probes were applied. For processing of the plasmids containing *E. coli*-specific virulence gene probes, we used *E. coli* itself. Even though *E. coli* K-12 is considered a biological containment for many foreign genes, it presents some biological hazard if parental virulence genes are introduced into it. Therefore, due to biosafety reasons, we decided to choose the primers in a way that only incomplete gene parts were amplified to avoid the generation of strains harboring functional virulence genes. The system is based on individual probes, thus allowing the addition of new virulence genes that might still be detected.

The main problem in using our approach was to produce sufficiently pure templates for the PCR amplification. When the whole plasmids containing the probes were used as PCR templates, minute amounts of plasmid sequences were unspecifically amplified and caused a high background reaction. This was shown in the control experiments using the empty cloning vector pBluescript, which hybridized to all strains tested (Fig. 1 and Table 2). Most likely, this is due to the presence of common sequences such as parts of the *lac* operon on this vector. This problem was resolved by subcloning and subse-

TABLE 3. Hybridization results of *E. coli* probes with labeled genomic DNA from laboratory and production strains

Probe	Gene	Hybridization with strain							
		Type B			TOPP JF1500	Type C		K-12	
		JF1504	JF1506	BL21		JF1508	JF1509	C600	XL1-Blue
EC1	*sltIA*	−	−	−	−	−	−	−	−
EC2	*sltIIA*	−	−	−	−	−	−	−	−
EC3	*eltIA*	−	−	−	−	−	−	−	−
EC4	*eltIIA*	−	−	−	−	−	−	−	−
ECAAF	*aaf/I*	−	−	−	−	−	−	−	−
ECAER	*fhuA*	+	+	+	+	+	+	+	+
ECBFP	*bfpA*	−	−	−	−	−	−	−	−
ECCFA	*cfa/I*	−	−	−	−	−	−	−	−
ECCNF	*cnf1*	−	−	−	−	−	−	−	−
ECCS3	*cfa/II*	−	−	−	−	−	−	−	−
ECEAE	*eae*	−	−	−	−	−	−	−	−
ECF1C	*F1C*	−	−	−	−	−	−	−	−
ECFIM	*fimA*	+	+	+	+	−	−	+	+
ECIPA	*ipaH*	−	−	−	−	−	−	−	−
ECIUC	*iucC*	−	−	−	−	−	−	−	−
ECK1	*neuAC*	−	−	−	−	−	−	−	−
ECK5	*kfiB*	−	−	−	−	−	−	−	−
ECPAP	*papA*	−	−	−	−	−	−	−	−
ECSFS	*sfaS*	−	−	−	−	−	−	−	−
ECST	*stla* or *-b*	−	−	−	−	−	−	−	−
EXSFA	*sfaA*	−	−	−	−	−	−	−	−
RTX6II	*hlyA*	−	−	−	−	−	−	−	−
RTX7	*elyA*	−	−	−	−	−	−	−	−
pBluescript		+	+	+	+	+	+	+	+

quent purification of inserts containing the specific probes prior to PCR amplification. In addition, a negative control strain (i.e., *E. coli* K-12) was included in the investigation to verify the quality of the probe preparations and to evaluate the hybridization signal intensities (Table 3 and Fig. 1). To assess the specificity of our test, we hybridized various characterized pathogenic *E. coli* strains as well as nonpathogenic *E. coli* K-12 strains. It became obvious that the probes specifically detected the virulence-associated gene(s) in genomic DNA of the corresponding pathogenic strains. The discrepancy between the genotypes of UPEC strains 536, IHE3034, J96, and RZ475 and the hybridization results is due to high similarity between *sfa* and *foc* genes. Cross-hybridization as observed in this case is an advantage for biosafety assessment of a certain strain, since it shows that not only homologous but also similar genes can be detected, which broadens the range of the detection system. The signal intensities of the probes varied, as can be seen in Fig. 1, but the hybridization signals were clearly positive and distinguished from the negative control strain K-12. The hybridization patterns of the pathogenic strains corresponded with the virulence factors expected from the particular clinical isolates. This suggests that the technique is also valuable for precise characterization of the virulence gene pattern of any clinical *E. coli* isolate, thereby providing a useful additional diagnostic tool for the characterization and epidemiology of clinically significant *E. coli* isolates.

To provide a safety assessment of *E. coli* strains with useful features for gene expression and protein secretion, we investigated a few strains currently utilized in many research and biotechnology laboratories. The results in Table 3 present strong evidence for the absence of virulence genes in the strains investigated. The three B strains showed a hybridization pattern identical to that of K-12 derivatives, reacting with all three positive probes but with none of the probes for specific virulence factors, although B strains are distinct from K-12 derivatives. The same results were found for the commercial strain *E. coli* TOPP, which belongs neither to *E. coli* K-12 nor to *E. coli* type B or C. The two C strains differed from K-12 only by not reacting with the probe specific for type 1 fimbriae which are present in both nonpathogenic and pathogenic *E. coli* strains, about 70% of which express this kind of fimbriae (21). The results of our hybridization experiments with *E. coli* strains of types B, C, and TOPP indicate that these strains do not have any of the known major virulence genes and therefore can be considered, like K-12 derivatives, nonpathogenic. This supports the findings of Claassen (9), who reported that B and C strains are rough, do not contain plasmids, are very sensitive to complement, are supposed to contain no colonization factors, and hence are assumed to be nonpathogenic.

The oligonucleotide primers described in Table 1 can also be used for a direct amplification of the corresponding virulence gene fragments from lysates or genomic DNA of *E. coli* isolates, thereby providing a rapid PCR-based detection method. For a safety assessment of a given strain, however, the described hybridization method is of broader value, since it allows the detection of genetic variants of the different virulence genes which might remain undiscovered in PCR. The hybridization method, which is designed for the simultaneous analysis of known virulence factors of *E. coli*, also bears the potential for automation, thus allowing the screening of a large number of strains.

Our finding that *E. coli* B and C strains as well as the commercial strain TOPP are devoid of *E. coli* virulence genes indicates that these strains can be considered nonpathogenic. The data might also serve as a basis for the classification of these strains in safety classes which allow rational and economic work in research and production.

ACKNOWLEDGMENTS

This work was supported by the Priority Program Biotechnology of the Swiss National Science Foundation (grant 5002-038920) and by a grant from the Bayrische Forschungsstiftung (FORBIOSICH, F15).

We thank I. Brodard for technical assistance.

REFERENCES

1. **Abe, A., K. Komase, A. Bangtrakulnonth, O.-A. Ratchtrachenchat, K. Kawahara, and H. Danbara.** 1990. Trivalent heat-labile- and heat-stable-enterotoxin probe conjugated with horseradish peroxidase for detection of enterotoxigenic *Escherichia coli* by hybridization. J. Clin. Microbiol. **28:**2616–2620.
2. **Bachmann, B. J.** 1996. Derivations and genotypes of some mutant derivatives of *Escherichia coli* K-12, p. 2460–2488. *In* F. C. Neidhardt, R. Curtiss III, J. L. Ingraham, E. C. C. Lin, K. B. Low, Jr., B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella*: cellular and molecular biology, 2nd ed. American Society for Microbiology, Washington, D.C.
3. **Baudry, B., S. J. Savarino, P. Vial, J. B. Kaper, and M. M. Levine.** 1990. A sensitive and specific DNA probe to identify enteroaggregative *Escherichia coli*, a recently discovered diarrheal pathogen. J. Infect. Dis. **161:**1249–1251.
4. **Bell, B. P., M. Goldoft, P. M. Griffin, M. A. Davis, D. C. Gordon, P. I. Tarr, C. A. Bartleson, J. H. Lewis, T. J. Barrett, J. G. Wells, R. Baron, and J. Kobayashi.** 1994. A multistate outbreak of *Escherichia coli* O157:H7-associated bloody diarrhea and hemolytic uremic syndrome from hamburgers—the Washington experience. JAMA **272:**1349–1353.
5. **Blum, G., V. Falbo, A. Caprioli, and J. Hacker.** 1995. Gene clusters encoding the cytotoxic necrotizing factor type 1, Prs-fimbriae and α-hemolysin from the pathogenicity island II of the uropathogenic *Escherichia coli* strain J96. FEMS Microbiol. Lett. **126:**189–196.
6. **Blum, G., M. Ott, A. Lischewski, A. Ritter, H. Imrich, H. Tschäpe, and J. Hacker.** 1994. Excision of large DNA regions termed pathogenicity islands from tRNA-specific loci in the chromosome of an *Escherichia coli* wild-type pathogen. Infect. Immun. **62:**606–614.
7. **Boylan, M., D. C. Coleman, and C. J. Smyth.** 1987. Molecular cloning and characterization of the genetic determinant encoding CS3 fimbriae of enterotoxigenic *Escherichia coli*. Microb. Pathog. **2:**195–209.
8. **Boylan, M., C. J. Smyth, and J. R. Scott.** 1988. Nucleotide sequence of the gene encoding the major subunit of CS3 fimbriae of enterotoxigenic *Escherichia coli*. Infect. Immun. **56:**3297–3300.
9. **Claassen, T. H. G.** 1995. Risico-schatting voor toepassing van de gastheren *Escherichia coli* B en *E. coli* C in het recombinant-DNA onderzoek. En vergelijkend onderzoek tussen *E. coli* B, *E. coli* C en *E. coli* K12, p. 1–10. *In* Molecular and cell biology. University of Utrecht, Utrecht, The Netherlands.
10. **Connell, T. D., and R. K. Holmes.** 1992. Characterization of hybrid toxins produced in *Escherichia coli* by assembly of A and B polypeptides from type I and type II heat-labile enterotoxins. Infect. Immun. **60:**1653–1661.
11. **Cotter, P. A., and J. F. Miller.** 1996. Triggering bacterial virulence. Science **273:**1183–1184.
12. **Coulton, J. W., P. Mason, D. R. Cameron, G. Carmel, R. Jean, and H. N. Rode.** 1986. Protein fusions of beta-galactosidase to ferrichrome-iron receptor of *Escherichia coli* K-12. J. Am. Vet. Med. Assoc. **165:**181–192.
13. **Dallas, W. S., D. M. Gill, and S. Falkow.** 1979. Cistrons encoding *Escherichia coli* heat-labile toxin. J. Bacteriol. **139:**850–858.
14. **Donnenberg, M. S., J. A. Giron, J. P. Nataro, and J. B. Kaper.** 1992. A plasmid-encoded type IV fimbrial gene of enteropathogenic *Escherichia coli* associated with localized adherence. Mol. Microbiol. **6:**3427–3437.
15. **Falbo, V., T. Pace, L. Picci, E. Pizzi, and A. Caprioli.** 1993. Isolation and nucleotide sequence of the gene encoding cytotoxic necrotizing factor 1 of *Escherichia coli*. Infect. Immun. **61:**4909–4914.
16. **Gross, R., F. Engelbrecht, and V. Braun.** 1984. Genetic and biochemical characterization of the aerobactin synthesis operon on pColV. Mol. Gen. Genet. **196:**74–80.
17. **Hartman, A. B., M. Venkatesan, E. V. Oaks, and J. M. Buysse.** 1990. Sequence and molecular characterization of a multicopy invasion plasmid antigen gene, *ipaH*, of *Shigella flexneri*. J. Bacteriol. **172:**1905–1915.
18. **Hess, J., W. Wels, M. Vogel, and W. Goebel.** 1986. Nucleotide sequence of a plasmid-encoded hemolysin determinant and its comparison with a corresponding chromosomal sequence. FEMS Microbiol. Lett. **34:**1–11.
19. **Jerse, A. E., J. Yu, B. D. Tall, and J. B. Kaper.** 1990. A genetic locus of the enterohemorrhagic *Escherichia coli* necessary for the production of attaching and effacing lesions on tissue culture cells. Proc. Natl. Acad. Sci. USA **87:**7839–7843.
20. **Karjalainen, T. M., D. G. Evans, M. So, and C.-H. Lee.** 1989. Molecular cloning and nucleotide sequence of the colonization factor antigen I gene of *Escherichia coli*. Infect. Immun. **57:**1126–1130.
21. **Klemm, P.** 1984. The *fimA* gene encoding the type-1 fimbrial subunit of *Escherichia coli*. Nucleotide sequence and primary structure of the protein. Eur. J. Biochem. **143:**395–399.
22. **Kuhnert, P., J. Nicolet, and J. Frey.** 1995. Rapid and accurate identification of *Escherichia coli* K-12 strains. Appl. Environ. Microbiol. **61:**4135–4139.
23. **Law, D.** 1994. Adhesion and its role in the virulence of enteropathogenic *Escherichia coli*. Clin. Microbiol. Rev. **7:**152–173.
24. **Levine, M. M., J. G. Xu, J. B. Kaper, H. Lior, V. Prado, B. Tall, J. Nataro, H. Karch, and K. Wachsmuth.** 1987. A DNA probe to identify enterohemorrhagic *Escherichia coli* of O157:H7 and other serotypes that cause hemorrhagic colitis and hemolytic uremic syndrome. J. Infect. Dis. **156:**175–182.
25. **Marklund, B.-I., J. M. Tennent, E. Garcia, A. Hamers, M. Baga, F. Lindberg, W. Gaastra, and S. Normark.** 1992. Horizontal gene transfer of the *Escherichia coli pap* and *prs* pili operons as a mechanism for the development of tissue-specific adhesive properties. Mol. Microbiol. **6:**2225–2242.
26. **Martinez, J. L., M. Herrero, and V. De Lorenzo.** 1994. The organization of intercistronic regions of the aerobactin operon of pColV-K30 may account for the differential expression of the iuc ABCD *iutA* genes. J. Mol. Biol. **238:**288–293.
27. **McDaniel, T. K., K. G. Jarvis, M. S. Donnenberg, and J. B. Kaper.** 1995. A genetic locus of enterocyte effacement conserved among diverse enterobacterial pathogens. Proc. Natl. Acad. Sci. USA **92:**1664–1668.
28. **Mühldorfer, I., G. Blum, A. Donohue-Rolfe, H. Heier, T. Ölschläger, H. Tschäpe, U. Wallner, and J. Hacker.** 1996. Characterization of *Escherichia coli* strains isolated from environmental water habitats and from stool samples of healthy volunteers. Res. Microbiol. **147:**625–635.
29. **Mühldorfer, I., and J. Hacker.** 1994. Genetic aspects of *Escherichia coli* virulence. Microb. Pathog. **16:**171–181.
30. **Nataro, J. P., M. M. Baldini, J. B. Kaper, R. E. Black, N. Bravo, and M. M. Levine.** 1985. Detection of an adherence factor of enteropathogenic *Escherichia coli* with a DNA probe. J. Infect. Dis. **152:**560–565.
31. **National Institutes of Health.** 1996. Guidelines for research involving recombinant DNA molecules. NIH guidelines, March 1996. National Institutes of Health, Bethesda, Md.
32. **Newland, J. W., and R. J. Neill.** 1988. DNA probes for Shiga-like toxins I and II and for toxin-converting bacteriophages. J. Clin. Microbiol. **26:**1292–1297.
33. **Noegel, A., U. Rdest, and W. Goebel.** 1981. Determination of the functions of hemolytic plasmid pHly152 of *Escherichia coli*. J. Bacteriol. **145:**233–247.
34. **Paton, A. W., J. C. Paton, P. N. Goldwater, M. W. Heuzenroeder, and P. A. Manning.** 1993. Sequence of a variant Shiga-like toxin type-I operon of *Escherichia coli* O111:H$^-$. Gene **129:**87–92.
35. **Pickett, C. L., D. L. Weinstein, and R. K. Holmes.** 1987. Genetics of type IIa heat-labile enterotoxin of *Escherichia coli*: operon fusions, nucleotide sequence, and hybridization studies. J. Bacteriol. **169:**5180–5187.
36. **Pitcher, D. G., N. A. Saunders, and R. J. Owen.** 1989. Rapid extraction method of bacterial genomic DNA with guanidinium thiocyanate. Lett. Appl. Microbiol. **8:**151–156.
37. **Roberts, I., R. Mountford, N. High, D. Bitter-Suermann, K. Jann, K. Timmis, and G. Boulnois.** 1986. Molecular cloning and analysis of genes for the production of K5, K7, K12, and K92 capsular polysaccharides in *Escherichia coli*. J. Bacteriol. **168:**1228–1233.
38. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
39. **Schmidt, H., L. Beutin, and H. Karch.** 1995. Molecular analysis of the plasmid-encoded hemolysin of *Escherichia coli* O157:H7 strain EDL 933. Infect. Immun. **63:**1055–1061.
40. **Schmidt, H., C. Knop, S. Franke, S. Aleksic, J. Heesemann, and H. Karch.** 1995. Development of PCR for screening of enteroaggregative *Escherichia coli*. J. Clin. Microbiol. **33:**701–705.
41. **Schmoll, T., J. Hacker, and W. Goebel.** 1987. Nucleotide sequence of the *sfaA* gene coding for the S-fimbrial protein subunit of *Escherichia coli*. FEMS Microbiol. Lett. **41:**229–235.
42. **Schmoll, T., H. Hoschützky, J. Morschhäuser, F. Lottspeich, K. Jann, and J. Hacker.** 1989. Analysis of genes coding for the sialic acid-binding adhesin and two other minor fimbrial subunits of the S-fimbrial adhesin determinant of *Escherichia coli*. Mol. Microbiol. **3:**1735–1744.
43. **Sears, C. L., and J. B. Kaper.** 1996. Enteric bacterial toxins: mechanisms of action and linkage to intestinal secretion. Microbiol. Rev. **60:**167–215.
44. **Spicer, E. K., and J. A. Noble.** 1982. *Escherichia coli* heat-labile enterotoxin. Nucleotide sequence of the A subunit gene. J. Biol. Chem. **257:**5716–5721.
45. **Studier, F. W., and B. A. Moffat.** 1986. Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. J. Mol. Biol. **189:**113–130.
46. **Van Die, I., B. van Geffen, W. Hoekstra, and H. Bergmans.** 1984. Type 1C fimbriae of a uropathogenic *Escherichia coli* strain: cloning and characterization of the genes involved in the expression of the 1C antigen and nucleotide sequence of the subunit gene. Gene **34:**187–196.
47. **Venkatesan, M. M., J. M. Buysse, and A. B. Hartman.** 1991. Sequence variation in two *ipaH* genes of *Shigella flexneri* 5 and homology to the LRG-like family of proteins. Mol. Microbiol. **5:**2435–2445.
48. **Weinstein, D. L., M. P. Jackson, J. E. Samuel, R. K. Holmes, and A. D. O'Brien.** 1988. Cloning and sequencing of a Shiga-like toxin type II variant from an *Escherichia coli* strain responsible for edema disease of swine. J. Bacteriol. **170:**4223–4230.
49. **Zapata, G., J. M. Crowley, and W. F. Vann.** 1992. Sequence and expression of the *Escherichia coli* K1 *neuC* gene product. J. Bacteriol. **174:**315–319.
50. **Zapata, G., W. F. Vann, W. Aaronson, M. S. Lewis, and M. Moos.** 1989. Sequence of the cloned *Escherichia coli* K1 CMP-*N*-acetylmuraminic acid synthetase gene. J. Biol. Chem. **264:**14769–14774.