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# The Plasmid of *Escherichia coli* Strain S88 (O45:K1:H7) That Causes Neonatal Meningitis Is Closely Related to Avian Pathogenic *E. coli* Plasmids and Is Associated with High-Level Bacteremia in a Neonatal Rat Meningitis Model<sup>∇</sup>

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A new *Escherichia coli* virulent clonal group, O45:K1, belonging to the highly virulent subgroup B<sub>2</sub>, was recently identified in France, where it accounts for one-third of *E. coli* neonatal meningitis cases. Here we describe the sequence, epidemiology and function of the large plasmid harbored by strain S88, which is representative of the O45:K1 clonal group. Plasmid pS88 is 133,853 bp long and contains 144 protein-coding genes. It harbors three different iron uptake systems (aerobactin, salmochelin, and the *sitABCD* genes) and other putative virulence genes (*iss*, *etsABC*, *ompT<sub>p</sub>*, and *hlyF*). The pS88 sequence is composed of several gene blocks homologous to avian pathogenic *E. coli* plasmids pAPEC-O2-ColV and pAPEC-O1-ColBM. PCR amplification of 11 open reading frames scattered throughout the plasmid was used to investigate the distribution of pS88 and showed that a pS88-like plasmid is present in other meningitis clonal groups such as O18:K1, O1:K1, and O83:K1. A pS88-like plasmid was also found in avian pathogenic strains and human urosepsis strains belonging to subgroup B<sub>2</sub>. A variant of S88 cured of its plasmid displayed a marked loss of virulence relative to the wild-type strain in a neonatal rat model, with bacteremia more than 2 log CFU/ml lower. The salmochelin siderophore, a known meningovirulence factor, could not alone explain the plasmid's contribution to virulence, as a salmochelin mutant displayed only a minor fall in bacteremia (0.9 log CFU/ml). Thus, pS88 is a major virulence determinant related to avian pathogenic plasmids that has spread not only through meningitis clonal groups but also human urosepsis and avian pathogenic strains.

*Escherichia coli* is the second cause of neonatal bacterial meningitis in industrialized countries, after group B streptococci. Despite advances in neonatal care, *E. coli* neonatal meningitis (ECNM) remains a potentially devastating illness, with a mortality rate of 10 to 15% and neurological sequelae in 30 to 50% of cases (8, 20, 28, 29, 62). Like most extraintestinal pathogenic *E. coli* (ExPEC) strains, ECNM strains mainly belong to phylogenetic group B2 and compose a small number of serotypes (O18:K1:H7, O1:K1, O83:K1, and O7:K1) (2, 11, 12, 33, 41, 49). Virulence genes in ECNM strains are involved in two major pathological steps, namely, intravascular multiplication (leading to high-level bacteremia) and blood-brain barrier penetration (38). A new virulent clone, O45:K1:H7, was recently identified in France, where it now accounts for one-third of ECNM isolates (13). The K1 capsular antigen and the salmochelin siderophore are the only two specific virulence traits

known to be shared by this clone and the archetypal strains of clonal group O18:K1:H7, even though the two clonal groups belong to the same highly virulent subgroup B<sub>2</sub>, defined by a specific sequence type (ST) designated ST29<sub>Whittam</sub> (www.shigatox.net), ST95<sub>Achtmann</sub> (www.mlst.net), or B2-IX (in Denamur's scheme) (10, 40). Hence, this new clone may serve to identify new virulence determinants and other genetic factors involved in the pathogenesis of ECNM. Strain S88, representative of clone O45:K1:H7, has been sequenced as part of a whole-genome sequencing project (ColiScope [www.genoscope.cns.fr]) at the Evry Genoscope in France. This strain harbors a large plasmid, designated pS88, that encodes the salmochelin and aerobactin siderophores and may be involved in the virulence of S88 (13).

Here we describe the complete DNA sequence, epidemiology, and function of pS88.

## MATERIALS AND METHODS

**Bacteria.** Strain S88, a representative of the French clonal group (O45:K1:H7) which accounts for one-third of ECNM isolates in this country, was isolated in 1989 from the cerebrospinal fluid of a newborn (Robert Debré Hospital, Paris, France). This strain is known to harbor a large plasmid (>100 kb) encoding at

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TABLE 1. Molecular epidemiology of plasmid pS88 by multiplex PCR of pS88 ORFs performed on 81 K1 *E. coli* strains representative of extraintestinal pathogenic clones causing meningitis and strains of other origins belonging to the highly virulent ST29/B2<sub>1</sub> subgroup B2

Source	Strain(s) <sup>a</sup>	n	Phylogroup	Sequence O type	Pathotype	Presence of <sup>b</sup> :													
						<i>cia</i>	<i>cvaA</i>	<i>etsC</i>	<i>iss</i>	ORF 143	<i>traJ<sup>c</sup></i>	<i>ompT<sub>P</sub></i>	<i>hlyF</i>	<i>sitA</i>	<i>iroN</i>	<i>iucC</i>	<i>tsh</i>	<i>eitB</i>	
Meningitis strains	S10, S38, S50, S78, S132, S145, S161, S169, S206, S267, S205, S272	12	B2	ST29 <sup>O45</sup>	NMEC	+	+	+	+	+	+	+	+	+	+	+	-	-	
	S2, S4, S26 <sup>U</sup> , S133, S153, S157, S181, S208, S147, S175, S179	11	B2	ST29 <sup>O18</sup>	NMEC	+	+	+	+	+	+	+	+	+	+	+	-	-	
	S168	1	B2	ST29 <sup>O18</sup>	NMEC	+	+	+	+	+	+	+	+	+	+	+	+	-	
	S3, S6, S35, S68	4	B2	ST29 <sup>O18</sup>	NMEC	-	+	+	+	+	+	+	+	+	+	+	ND	ND	
	S69, S131, S203, S46, S96	5	B2	ST29 <sup>O18</sup>	NMEC	-	+	+	+	+	-	+	+	+	+	+	+	ND	ND
	C5 <sup>U</sup> , RS218 <sup>U</sup> , S209	3	B2	ST29 <sup>O18</sup>	NMEC	-	-	-	-	-	-	-	-	+	+	-	ND	ND	
	S124	1	B2	ST29 <sup>O1</sup>	NMEC	-	-	-	-	-	-	+	-	+	+	+	ND	ND	
	S158, S136	2	B2	ST29 <sup>O1</sup>	NMEC	+	+	+	+	+	+	+	+	+	+	+	-	-	
	S166	1	B2	ST29 <sup>O1</sup>	NMEC	-	-	+	+	+	-	+	-	+	+	+	+	ND	ND
	S172	1	B2	ST29 <sup>O1</sup>	NMEC	-	+	-	-	-	-	-	+	+	+	+	+	ND	ND
	S21 <sup>U</sup> , S108 <sup>N</sup> , S109 <sup>U</sup> , S116 <sup>U</sup> , S121 <sup>U</sup> , S191	6	B2	ST304 <sup>O16</sup>	NMEC	-	-	-	-	-	-	-	-	+	-	+	ND	ND	
	S130	1	B2	ST692 <sup>O83</sup>	NMEC	+	+	+	+	+	+	+	+	+	+	+	-	-	
	S149	1	B2	ST696 <sup>O83</sup>	NMEC	-	-	-	-	-	-	-	-	+	-	-	ND	ND	
	S15 <sup>D</sup> , S57 <sup>U</sup> , S156, S159, S194 <sup>N</sup> , S195 <sup>N</sup> , S196 <sup>N</sup>	7	B2	ST697 <sup>O83</sup>	NMEC	-	+	+	+	+	-	+	+	+	+	+	+	ND	ND
	S94	1	B2	ST697 <sup>O83</sup>	NMEC	+	+	+	+	+	+	+	+	+	+	+	-	-	
	S151	1	B2	ST697 <sup>O83</sup>	NMEC	+	+	+	+	+	+	+	+	+	+	+	+	-	
	S13 <sup>D</sup> , S18 <sup>U</sup> , S210, S76, S79, S197	6	D	ST301 <sup>O7</sup>	NMEC	-	-	-	-	-	-	-	-	-	+	-	+	ND	ND
	S63 <sup>U</sup>	1	D	ST100 <sup>O1</sup>	NMEC	-	+	-	-	-	-	-	-	-	+	+	-	ND	ND
	S58	1	D	ST100 <sup>O1</sup>	NMEC	-	-	-	-	-	-	-	-	-	+	-	+	ND	ND
	Non meningitis strains of ST29/B2 <sub>1</sub>	HN50, HN113	2	B2	ST29 <sup>O2</sup>	UPEC	+	+	+	+	+	+	+	+	+	+	+	-	-
HN30		1	B2	ST29 <sup>O2</sup>	UPEC	+	+	+	+	+	+	+	+	+	+	+	-	-	
HN7		1	B2	ST29 <sup>O2</sup>	UPEC	-	+	+	+	+	-	+	+	+	+	+	ND	ND	
NC59, NC28		2	B2	ST29 <sup>O2</sup>	Commensal	+	+	+	+	+	+	+	+	+	+	+	-	-	
ECOR62 <sup>S</sup>		1	B2	ST29 <sup>O2</sup>	UPEC	+	+	+	+	+	+	+	+	+	+	+	-	-	
ECOR61 <sup>S</sup>		1	B2	ST29 <sup>O2</sup>	Commensal	-	-	-	-	-	-	-	-	+	+	-	ND	ND	
BEN1068 <sup>E</sup> , BEN1082 <sup>E</sup> , BEN1090 <sup>E</sup> , BEN1354 <sup>E</sup>		4	B2	ST29 <sup>O45</sup>	APEC	+	+	+	+	+	+	+	+	+	+	+	-	-	
LDA5067912, LDA5063391		2	B2	ST29 <sup>O2</sup>	APEC	-	+	+	+	+	-	+	+	+	+	+	+	+	
LDA6042253		1	B2	ST29 <sup>O1</sup>	APEC	+	+	+	+	+	+	+	+	+	+	+	-	-	

<sup>a</sup> All strains are from France except when specified by superscript letters (U, United States; D, Germany; N, The Netherlands; S, Sweden; E, Spain).  
<sup>b</sup> Strains positive for all 11 screened ORFs belonging to pS88 and negative for two genes (*tsh* and *eitB*) found in APEC O2 were considered to harbor a pS88-like plasmid (boldface). ND, not done.  
<sup>c</sup> *traJ* of F-like plasmid (as opposed to R-like plasmids).

least two iron uptake systems, namely, salmochelin and aerobactin (13). Other chromosomally encoded ExPEC traits are K1 antigen, P fimbriae with the adhesin PapGII, and yersiniabactin (13). Strain K-12, MG1655, was used as a colicin-sensitive strain. Strain J53, with natural rifampin (rifampicin) resistance, was used for conjugal transfer of plasmids as previously described (51).

To investigate the epidemiology of plasmid pS88, we also used 66 strains representative of the major meningitis clonal groups defined by the combination of their ST ([www.shigaetox.net](http://www.shigaetox.net)) and their serogroup (sequence O types) as previously described (9). Other human and avian *E. coli* strains (*n* = 15) belonging to the highly virulent subgroup B2<sub>1</sub>, characterized by ST29 (10), were also studied (Table 1).

**Sequencing and annotation of the pS88 plasmid.** The genome and plasmid of strain S88 were sequenced as part of a whole-genome sequencing project (ColiScope [[www.genoscope.cns.fr](http://www.genoscope.cns.fr)]) at the Evry Genoscope in France. Sequencing and assembly of pS88 were performed as previously described (7, 50). MaGe (Magnifying Genomes) software was used for gene annotation and comparative analysis of the S88 genome as described elsewhere (50, 63).

**Phylogenetic analysis.** To determine the genetic relatedness of the *tra* gene clusters in plasmid S88 by comparison with plasmids of other strains, the nucleotide sequences of *traJ*, *traM*, *traS*, *traT*, and *traY* from several *E. coli* conjugative plasmids were extracted from GenBank. The Clustal W program was used to align the

sequences (59). Phylogenetic and molecular evolutionary relationships were examined by using the neighbor-joining method implemented with MEGA 3.1 software (39). Bootstrap confidence values for each node of the trees were calculated over 100 replicate trees.

**Epidemiology of pS88-like plasmids by multiplex PCR and pulsed-field gel electrophoresis (PFGE).** In order to investigate the epidemiology of pS88 among human and avian *E. coli* strains, PCR was used to screen for 11 open reading frames (ORFs) or genes scattered throughout the plasmid. The PCR targets and primers are listed in Table 2. The aerobactin and salmochelin genes were PCR amplified as previously described (14). The other nine ORFs or genes were detected with two new multiplex PCR methods (one hexaplex and one triplex). PCR was carried out in a 50- $\mu$ l volume with 25  $\mu$ l of 2 $\times$  Qiagen Multiple PCR Master Mix (Qiagen, Courtaboeuf, France), 5  $\mu$ l of 5 $\times$  Q-solution, 5  $\mu$ l of a primer mix (with final concentrations in the hexaplex PCR of 0.2  $\mu$ M for *cia*, *traJ*, and *ompT<sub>P</sub>*; 0.1  $\mu$ M for *etsC* and ORF143; and 0.05  $\mu$ M for *iss* and in the triplex PCR of 0.2  $\mu$ M for *hlyF*, *cvaA*, and *sitA*), 10  $\mu$ l of distilled water, and 5  $\mu$ l of bacterial lysate, using an iCycler thermal cycler (Bio-Rad, Marnes la Coquette, France) under the following conditions: DNA denaturation and polymerase activation for 15 min at 95°C; 30 cycles of 30 s at 94°C, 90 s at 55°C, and 90 s at 72°C; and a final extension step for 10 min at 72°C. Samples were electrophoresed in 3% Resophor gels (Eurobio, France) and then stained with ethidium

TABLE 2. Oligonucleotides used for mutagenesis and PCR assay<sup>a</sup>

Name	Target	Sequence (5' to 3')	PCR product size (bp)	Reference
iroN.P0	<i>iroN</i>	A <b>ACTGTGCTCCTGGTTGGGTTGAATAGCCAGGTATCAGTATGTGTA</b> <b>GGCTGGAGCTGCTT</b>		This study
iroN.P2	<i>iroN</i>	AAGCCCGCCTGGCTCGTTATAGGTATTCGCCCTTCAGACATATGA <b>ATATCCTCCTTAG</b>		This study
iut P0	<i>iutA</i>	GCGCCAGCAGTCGCTCAACAAACCGATGATGAAACGTTCTGTGTGA <b>GGCTGGAGCTGCTT</b>		This study
iut.P2	<i>iutA</i>	CCTGCCTTTGTAGTCGTACAGTGACGCTGGGCCGTAACCCCATATGA <b>ATATCCTCCTTAG</b>		This study
sit P0	<i>sit</i> cluster	GGAACACCCGAGCAGGTACGTAAGGTTGTTGATATAGTTATGTGTGA <b>GGCTGGAGCTGCTT</b>		This study
sit P2	<i>sit</i> cluster	TGGATTATATCGCCGATGGTTATCCCGAGCATGTCGCCGACATATGA <b>ATATCCTCCTTAG</b>		This study
inter P0		AGAGCGCGTAGCATTCTTATTTTCATGAGGAAATTTACCCGTGTGTA <b>GGCTGGAGCTGCTT</b>		This study
inter P2		CTTGCCCTCTCGCCAGTAATGATTAATGGGAATGCTTCCTCATATGA <b>ATATCCTCCTTAG</b>		This study
C1	<i>cat</i>	TTATACGCAAGGCGACAAGG		This study
C2	<i>cat</i>	GATCTTCCGTACAGGTAGG		This study
iroN.FR1	<i>iroN</i>	TATCTGGCAAGGATGTGAGCTTAACGATCA		This study
iroN.FR2	<i>iroN</i>	CGCAAATTACACAGCAGGTTGTCATGAGTT		This study
iut.FR1	<i>iutA</i>	TTAACTCGCTACACAGCATCTTTGGGCTGA		This study
iut.FR2	<i>iutA</i>	TCAGAACAGCACAGAGTAGTTCAGACCAAA		This study
sit FR1p	<i>sit</i> cluster	GTGCCTAGTCCTCACCTGCTCGATAGCATT		This study
sit FR1c	<i>sit</i> cluster	TAACGCGTCGCCAGCGGAAACAACCTAAT		This study
sit FR2	<i>sit</i> cluster	GCTAATAACAAGTGTAACCAGTCCGGCAAT		This study
inter FR1	Intergenic	AGGCCATCAGCAAAACACTGATAGTCTGAA		This study
inter FR2	Intergenic	AGCTGGTAAATCAGATTTTGCATGGACTGA		This study
p-omp.1	<i>ompT<sub>p</sub></i>	GGAAAATACAGTAATGCGCCAAT	189	This study
p-omp.2	<i>ompT<sub>p</sub></i>	TTAGACGGGTATTCGGATGTTT	189	This study
traJ.1	<i>traJ<sub>F-plasmid</sub></i>	GAACCTCCCTGCATCGACTGT	230	This study
traJ.2	<i>traJ<sub>F-plasmid</sub></i>	CTCTGCGACAGAAGCCATTT	230	This study
ORF143.1	ORF143	CTATCGGCTTCCCTCTTCT	285	This study
ORF143.2	ORF143	CTGCATCTCCAGGACTTTGA	285	This study
etsC.1	<i>etsC</i>	ATCCCATCAACTGGACCAAG	359	This study
etsC.2	<i>etsC</i>	TTCTCACTGGCATGGACTG	359	This study
cia.1	<i>cia</i>	CGTGGGGTATGATTCAGAT	456	This study
cia.2	<i>cia</i>	AGAGCATCCGGCTCTCTGTA	456	This study
cva.F	<i>cvaA</i>	ATCCGGGCGTTGTCTGACGGGAAAGTTG	319	35
cva.R	<i>cvaA</i>	ACCAGGGAAACAGAGGCCACCCGGCGTATT	319	
issF	<i>iss</i>	CAGCAACCCGAACCACTTGATG	323	35
issR	<i>iss</i>	AGCATTGCCAGAGCGGCAGAA	323	
hlyF.R	<i>hlyF</i>	GGCGATTTAGGCATTCCGATACTC	599	35
hlyF.R	<i>hlyF</i>	ACGGGGTTCGCTAGTTAAGGAG	599	
tsh.F	<i>tsh</i>	GGGAAATGACCTGAATGCTGG	420	35
tsh.R	<i>tsh</i>	CCGCTCATCAGTCAGTACCAC	420	35
eitB-F	<i>eit</i>	CAGCAGCGCTTCGGACAAAATCTCCT	380	35
eitB-R	<i>eit</i>	TTCCCAACCACTCTCCGTTCTCAAAC	380	35
sitA.F	<i>sitA</i>	CGCAGGGGGCACAACCTGAT	663	52
sitA.R	<i>sitA</i>	CCCTGTACCAGCGTACTGG	663	
iroN.1	<i>iroN</i>	GAAAGCTCTGGTGGACGGTA	126	13
iroN.2	<i>iroN</i>	CGACAGAGGATTACCGGTGT	126	13
aer.1	<i>iucC</i>	AAACCTGGTTTACGCAACTGT	269	13
aer.2	<i>iucC</i>	ACCCGTCTGCAAATCATGGAT	269	13

<sup>a</sup> Oligonucleotide primers used for gene recombination are designated by the suffixes P0 and P2; bold characters in the primer sequences indicate the 20 nucleotides homologous to the *cat* gene sequence. The primers used to control the correct introduction of the *cat* gene are designated by the suffixes FR1 and FR2 and flank the DNA target segment. Oligonucleotides designated by sit FR1p and sit FR1c were used to check for the correct introduction of the *cat* gene in the plasmid and chromosomal *sit* loci, respectively. Oligonucleotides designated by inter P0 and inter P2 amplify a noncoding plasmid region located between ORFs 131 and 132. Oligonucleotide primers from p-omp.1 to the end of the table were used to study the molecular epidemiology of the plasmid. *TraJ<sub>F-plasmid</sub>* corresponds to the *traJ* gene of F-type plasmids.

bromide and photographed with UV transillumination. In each PCR run, a lysate of strain S88 was used as a positive control. Finally, all strains harboring the 11 pS88 genes were also screened for *eitB* and *tsh* genes using the primers described by Johnson et al. (35).

Plasmid sizes were determined by PFGE of undigested DNAs of strains harboring the 11 pS88 genes. PFGE followed by Southern blot hybridization with an *iroN* probe were performed as previously described (13).

**Cure of the pS88 plasmid.** Strain S88 was grown for 18 h in Luria-Bertani (LB) medium at 37°C with shaking. The culture was then diluted to 10<sup>5</sup> CFU/ml, and serial concentrations (2.5, 5, and 10%) of sodium dodecyl sulfate were added. After 18 h of growth with shaking, the cultures were plated on LB agar. After overnight incubation, 200 colonies were screened for colicin production by pricking them out on LB agar plates overlaid with a suspension of *E. coli* strain K-12, which is sensitive to colicin. Test strains were assumed to have lost the ColV

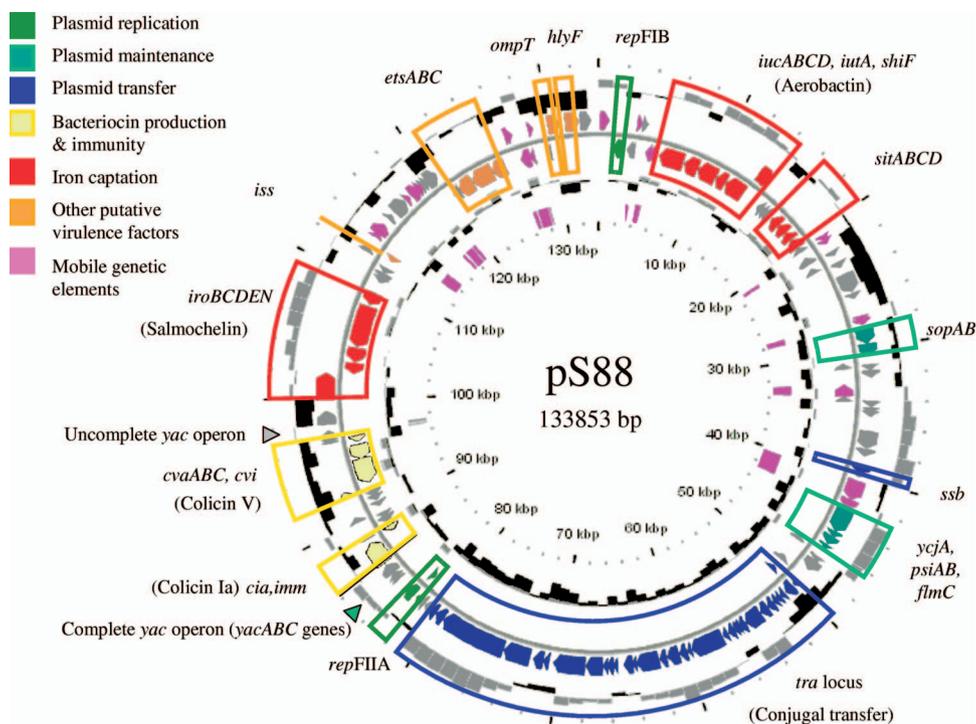


FIG. 1. Circular representation of the *Escherichia coli* strain S88 plasmid (pS88). Circles display (from the outside) (i) GC percent deviation (GC window – mean GC) in a 1,000-bp window, (ii) predicted ORFs transcribed in the clockwise direction, (iii) predicted ORFs transcribed in the counterclockwise direction, (iv) GC skew ( $G + C/G - C$ ) in a 1,000-bp window, (v) transposable elements (pink), and (vi) coordinates in kilobase pairs (kbp) from the origin of replication. Genes displayed in circles ii and iii are categorized by color as follows: red, iron uptake systems; orange, other putative virulence factors; yellow, bacteriocin production and immunity; pink, mobile genetic elements; dark blue, plasmid transfer; green, plasmid replication; teal, plasmid maintenance; gray, unknown.

plasmid if they were unable to inhibit the growth of strain K-12. This was confirmed, using the PCRs described above, by the loss of the 11 plasmid-related ORFs or genes and by the disappearance of extrachromosomal DNA on agarose gel electrophoresis after plasmid preparation with the relevant colonies (Qiaprep Minispin, Qiagen). For each variant thus obtained, we checked the expression of the K1 capsule antigen and the presence of chromosomal virulence genes, using multiplex PCR as previously described (14).

**Mutant construction.** S88 mutants were obtained with the PCR-based method of Datsenko and Wanner (19) as previously described, with plasmids kindly provided by Lionello and Nara Bossi (Centre de Génétique Moléculaire, CNRS, Gif sur Yvette, France) (47, 50). The primers used for insertional mutation are listed in Table 2. Correct introduction of the *cat* gene into the target was controlled by PCR using primers homologous to the *cat* gene and the flanking region of the target, as previously described (47, 50). For each mutant thus obtained, we checked the expression of the K1 capsule antigen, bacteriocin production, the presence of nondeleted plasmid genes, and chromosomal virulence genes, using multiplex PCRs.

**Conjugal plasmid transfer.** The *cat* gene was introduced into a noncoding region of pS88 between ORFs 131 and 132, using the method of Datsenko and Wanner (19), so that the plasmid harbored a chloramphenicol resistance determinant (plasmid pS88*cat*) for the selection of transconjugant clones after conjugal transfer. The cured S88 variant was made resistant to nalidixic acid in order to select transconjugants for pS88*cat* reintroduction.

Donor and recipient cells were grown overnight at 37°C in LB broth. A  $10^{-2}$  dilution was prepared with fresh LB medium. After incubation at 37°C for 2.5 h without shaking, 2.5-ml aliquots of each culture were mixed. After overnight incubation, the mating mixture was harvested and bacteria were plated on selective medium. The transconjugants thus obtained were checked for colicin production and the presence of plasmid-related genes by PCR and by gel electrophoresis of a plasmid extract (Qiaprep minispin; Qiagen).

**Experimental models.** We assessed the ability of the wild-type strain and mutants to induce high-level bacteremia in newborn rats, as previously described (30). Briefly, pathogen-free Sprague-Dawley rats were obtained from Charles River Laboratories at 4 days of age, with their mothers. At 5 days of age, the pups

were inoculated intraperitoneally with a normal saline suspension containing ~500 CFU of the test strain. A tail incision was made 18 h after inoculation, and 5  $\mu$ l of blood was sampled. Serial dilutions were plated for CFU counting. Comparisons of bacterial counts in the animal model were based on a two-sample unpaired *t* test. Data are expressed as mean  $\pm$  standard deviation. *P* values below 0.05 were considered to denote statistically significant differences.

Serum bactericidal activity against *E. coli* strains was determined using pooled sera of healthy volunteers as previously described (30). To measure bactericidal serum activity, 20  $\mu$ l of a bacterial inoculum of  $10^8$  CFU/ml in physiological serum was added to 180  $\mu$ l of freshly thawed pure serum. Quantitative cultures were done 5 h later. Experiments were repeated five times.

**Nucleotide sequence accession number.** The DNA sequence of pS88 has been deposited in GenBank under accession number CU928146.

## RESULTS

**Sequencing of pS88. (i) Overview.** A complete circularized DNA sequence of plasmid pS88 was obtained. It was 133,853 bp long, with an overall G+C content of 49.28% (Fig. 1). A total of 133 protein-coding genes plus 11 fragments of protein-coding genes corresponding to six pseudogenes were identified in the sequence (Table 3). Globally, based on sequence homologies, the plasmid could be separated into two halves. The first, from base 20,000 to base 86,000, is mostly composed of the genes involved in plasmid-related functions (conjugal transfer, maintenance, and partition); all are almost exclusively transcribed in the clockwise direction, with an average G+C content of 51%. The other half contains most of the putative or known virulence-associated genes, such as those encoding iron uptake systems and factors involved in resistance to the innate

TABLE 3. Predicted ORFs identified on the DNA sequence of plasmid pS88 with their homologs on plasmids pAPEC-O2-ColV and pAPEC-O1-ColBM

Position		Gene	ORF	Product	pAPEC-O2-ColV homolog		pAPEC-O1-ColBM homolog	
Beginning	End				Name	Identity (%)	Name	Identity (%)
132	872	<i>int</i>	0001	Putative site-specific recombinase	O2ColV23	100	O1CoBM187	99.59
1157	2134	<i>repA</i>	0002	RepFIB replication protein RepA	O2ColV22	100	O1CoBM186	100
2543	2839		0003	Conserved hypothetical protein	O2ColV21	100	O1CoBM185	100
2808	3029		0004	Conserved hypothetical protein				
3244	3309		0005	Putative transposase (fragment) IS27 family				
3494	3883		0006	Putative fragment of ImpB UV protection protein	O2ColV19	100	O1CoBM183	100
4063	4566		0007	Transposase InsB (ORF2) IS1	O2ColV18	100	O1CoBM2	100
4485	4709		0008	Transposase InsA (ORF1) IS1	O2ColV17	100	O1CoBM159	100
5285	7486	<i>intA</i>	0009	Ferric aerobactin receptor precursor IntA	O2ColV15	99.9	O1CoBM168	100
7568	8845	<i>intD</i>	0010	L-Lysine 6-monooxygenase LucD	O2ColV14	99.8	O1CoBM169	99.29
8842	10584	<i>intC</i>	0011	Aerobactin siderophore biosynthesis protein LucC	O2ColV13	100	O1CoBM170	100
10584	11531	<i>intB</i>	0012	N(6)-Hydroxylase acetylase LucB	O2ColV12	99.7	O1CoBM171	100
11532	13319	<i>intA</i>	0013	Aerobactin siderophore biosynthesis protein LucA	O2ColV11	99.7	O1CoBM172	100
13392	14585		0014	Putative membrane transport protein ShiF	O2ColV9	92	O1CoBM173	100
14965	15345	<i>shf</i>	0015	Putative membrane protein; CreB-like protein	O2ColV8	97.7	O1CoBM174	100
15417	15689		0016	Conserved hypothetical protein	O2ColV7	100	O1CoBM175	100
15686	16120		0017	Putative enolase	O2ColV6	100	O1CoBM176	100
16588	17445	<i>sitD</i>	0019	SitD protein, iron transport protein, inner membrane component	O2ColV4	100	O1CoBM177	100
17442	18299	<i>sitC</i>	0020	SitC protein, iron transport protein, inner membrane component	O2ColV3	99.2	O1CoBM178	99.3
18296	19123	<i>sitB</i>	0021	SitB protein, iron transport protein, ATP-binding component	O2ColV2	100	O1CoBM179	99.64
19123	20037	<i>sitA</i>	0022	SitA protein, iron transport protein, periplasmic-binding protein	O2ColV1	99	O1CoBM180	100
20160	20351		0023	Hypothetical protein				
20393	20668	<i>insA</i>	0024	IS7 repressor protein InsA	O2ColV182	100		
20695	21090	<i>insB</i>	0025	Fragment of transposase (partial)	O2ColV181	100	O1CoBM164	100
21101	21775		0026	Hypothetical protein				
21933	22127		0027	Hypothetical protein; putative exported protein				
22203	22682		0028	Conserved hypothetical protein				
22800	23249		0029	Conserved hypothetical protein				
23866	25368		0030	Conserved hypothetical protein				
25594	25785		0031	Hypothetical protein	O2ColV197	98.4		
27444	27668	<i>insB</i>	0035	Transposase InsA (ORF1) IS1	O2ColV198	97.8		
27587	28090		0036	Transposase	O2ColV199	99		
28381	29556	<i>sopA</i>	0037	SopA protein (plasmid partition protein A)	O2ColV161	26.5	O1CoBM3	100
29556	30527	<i>sopB</i>	0038	SopB protein (plasmid partition protein B)	O2ColV160	25.3	O1CoBM4	100
31055	31216		0039	Hypothetical protein				
31244	31591		0040	Conserved hypothetical protein				
31654	32235		0041	Hypothetical protein	O2ColV185	25		
32721	33692		0042	Transposase IS1/0 family	O2ColV83	28.9		
33844	34014		0043	Hypothetical protein				
34073	34519	<i>yubI</i>	0044	Putative antirestriction protein			O1CoBM7	100
34609	34980		0045	Conserved hypothetical protein	O2ColV152	25	O1CoBM8	100
34980	35162		0046	Conserved hypothetical protein				
36438	37799	<i>yubA</i>	0047	Conserved hypothetical protein				
37847	38410	<i>ydcA</i>	0048	Putative adenine-specific DNA methylase			O1CoBM11	99.56
39216	39848	<i>ssb</i>	0050	Single-stranded DNA-binding protein	O2ColV148	51.7	O1CoBM12	100
39784	40044		0051	Conserved hypothetical protein			O1CoBM14	89.3
40381	41922	<i>yubL</i>	0052	Putative transposase ORF1, IS27 family	O2ColV72	46.8	O1CoBM15	84.88
41934	42683		0053	Putative transposase ORF2, IS27 family	O2ColV75	45.3	O1CoBM77	100
42799	46652	<i>ycjA</i>	0054	Putative DNA-binding protein involved in plasmid partitioning (ParB-like partition protein)			O1CoBM76	100
				Plasmid SOS inhibition protein B	O2ColV145	97.2	O1CoBM16	96.11
44704	45141	<i>psiB</i>	0055	Plasmid SOS inhibition protein A			O1CoBM17	99.31
45138	45857	<i>psiA</i>	0056	Plasmid SOS inhibition protein A	O2ColV144	97.5	O1CoBM18	97.49
45991	46290	<i>fimC</i>	0057	Putative F-plasmid maintenance protein C			O1CoBM20	48.57
46532	46849	<i>yubN</i>	0059	Conserved hypothetical protein			O1CoBM9	42.86
47193	47480	<i>yubO</i>	0060	Conserved hypothetical protein	O2ColV143	94.7	O1CoBM23	94.74
47518	48420	<i>yubP</i>	0061	Conserved hypothetical protein	O2ColV142	93	O1CoBM24	94.98
48716	49225	<i>yubQ</i>	0062	X polypeptide (P19 protein); putative transglycosylase	O2ColV141	94.1	O1CoBM25	98.08
49640	50023	<i>traM</i>	0063	Protein TraM (conjugal transfer protein M)	O2ColV140	85	O1CoBM26	100
50129	50899	<i>traJ</i>	0064	Protein TraJ (positive regulator of conjugal transfer operon)	O2ColV139	25.5	O1CoBM27	100

50992	<i>traY</i>	51393	0065	Protein TraY (conjugative transfer: <i>oriT</i> nicking)	O2ColV138	53.2	O1CoBM28	99.25
51426	<i>traA</i>	51791	0066	Fimbrial protein precursor TraA (Pilin)	O2ColV137	96.7	O1CoBM29	100
51806	<i>traL</i>	52117	0067	F pilus assembly protein TraL	O2ColV136	100	O1CoBM30	100
52139	<i>traE</i>	52705	0068	F pilus assembly protein TraE	O2ColV135	98.4	O1CoBM31	99.47
52692	<i>traK</i>	53420	0069	F pilus assembly protein TraK	O2ColV134	100	O1CoBM32	100
53420	<i>traB</i>	54847	0070	F pilus assembly protein TraB	O2ColV133	84.4	O1CoBM33	99.79
54837	<i>traP</i>	55427	0071	Putative conjugal transfer protein TraP	O2ColV132	90.2	O1CoBM34	100
55381	<i>trbD</i>	55611	0072	Conserved hypothetical protein TrbD	O2ColV131	98.3	O1CoBM35	100
55623	<i>trbG</i>	55874	0073	Conserved hypothetical protein TrbG	O2ColV130	97.3	O1CoBM36	100
55871	<i>trav</i>	56386	0074	F pilus assembly protein TraV	O2ColV127	98.3	O1CoBM37	100
56521	<i>traR</i>	56742	0075	Putative conjugal transfer protein TraR	O2ColV126	98.4	O1CoBM38	100
56902	<i>traC</i>	59529	0077	F pilus assembly protein TraC	O2ColV125	98.3	O1CoBM39	99.66
59526	<i>trbI</i>	59912	0078	F pilus extension/retraction protein TrbI	O2ColV124	98.4	O1CoBM40	92.97
59909	<i>traW</i>	60541	0079	F pilus assembly protein TraW	O2ColV123	98	O1CoBM41	97.98
60538	<i>traU</i>	61530	0080	Conjugal transfer protein TraU	O2ColV122	99.4	O1CoBM42	98.79
61536	<i>trbC</i>	62177	0081	F pilus assembly protein TrbC	O2ColV121	99.1	O1CoBM43	98.59
62174	<i>traN</i>	63982	0082	Mating contact stabilization protein TraN	O2ColV120	98.5	O1CoBM44	99
64006	<i>trbE</i>	64266	0083	Conjugal transfer protein TrbE	O2ColV118	90.7	O1CoBM45	95.35
64229	<i>traF</i>	65002	0084	F pilus assembly protein TraF	O2ColV117	98.4	O1CoBM46	98.05
65484	<i>traQ</i>	65768	0085	F pilin synthesis protein TraQ	O2ColV116	99	O1CoBM49	96.15
65755	<i>trbB</i>	66300	0086	Putative disulfide bond isomerase TrbB	O2ColV115	99.4	O1CoBM50	93.92
66230	<i>trbJ</i>	66592	0087	Conjugal transfer protein TrbJ	O2ColV114	99.2	O1CoBM51	97.94
66589	<i>traH</i>	67965	0088	F pilus assembly protein TraH	O2ColV113	100	O1CoBM53	96.39
67962	<i>traG</i>	70472	0089	Mating contact stabilization protein TraG	O2ColV112	99.5	O1CoBM26	100
70791	<i>traS</i>	71306	0091	Surface exclusion inner membrane protein TraS	O2ColV111	100	O1CoBM55	99.18
71239	<i>traT</i>	72069	0092	Complement resistance and surface exclusion outer membrane protein TraT	O2ColV110	97.7	O1CoBM56	97.35
72321	<i>traD</i>	74474	0093	Conjugal transfer coupling protein TraD	O2ColV109	98	O1CoBM58	97.55
74474	<i>traI</i>	79744	0094	<i>oriT</i> nicking and unwinding protein TraI (DNA helicase I)	O2ColV107	95.6	O1CoBM59	91.53
79764	<i>traX</i>	80510	0095	F pilin acylase TraX	O2ColV105	97.9	O1CoBM61	94.62
80514	<i>finO</i>	81125	0096	Fertility inhibition protein FinO (conjugal transfer repressor)	O2ColV102	95.5	O1CoBM62	92.86
81263	<i>yigA</i>	81475	0097	Conserved hypothetical protein YigA	O2ColV101	97.7	O1CoBM64	49.38
81658	<i>yigB</i>	82119	0098	Putative nuclease YigB	O2ColV98	99.3	O1CoBM67	96.54
82164	<i>repA2</i>	82424	0099	Replication regulatory protein RepA2 (protein CopB)	O2ColV88	100	O1CoBM128	100
82705	<i>repA1</i>	83574	0100	Replication initiation protein RepA1	O2ColV67	26.7	O1CoBM81	98.75
84490	<i>yacA</i>	84759	0101	Conserved hypothetical protein YacA, possible repressor	O2ColV66	100	O1CoBM82	100
84756	<i>yacB</i>	85037	0102	Putative plasmid stabilization system protein YacB	O2ColV65	97.5	O1CoBM84	98.51
85083	<i>yacC</i>	85931	0103	Putative exoribonuclease YacC	O2ColV64	100	O1CoBM124	99.76
86117	<i>cia</i>	87997	0104	Colicin-Ia	O2ColV63	100	O1CoBM127	98.55
88019	<i>imm</i>	88354	0105	Colicin-Ia immunity protein	O2ColV98	100	O1CoBM131	100
88483	<i>ybaA</i>	88740	0106	Conserved hypothetical protein YbaA	O2ColV61	100	O1CoBM134	100
88760	<i>ydeA</i>	89350	0108	Conserved hypothetical protein YdeA	O2ColV59	99.6	O1CoBM137	99.17
89347	<i>ydfA</i>	89607	0109	Conserved hypothetical protein YdfA	O2ColV57	99.2	O1CoBM138	99.69
89900	<i>cvi</i>	91761	0110	Putative acetyltransferase	O2ColV56	100	O1CoBM139	99.76
90408	<i>yacA</i>	90352	0111	Predicted dehydrogenase	O2ColV55	99.8	O1CoBM140	99.84
90674	<i>yacB</i>	90674	0111	Predicted dehydrogenase	O2ColV54	100	O1CoBM141	100
90942	<i>ydfA</i>	91346	0112	Predicted dehydrogenase	O2ColV53	100		
91318	<i>cvi</i>	91761	0113	Predicted dehydrogenase				
91880	<i>cvaC</i>	92116	0114	Colicin V immunity protein				
92094	<i>cvaC</i>	92405	0115	Colicin V precursor (microcin V bacteriocin)				
92575	<i>cvaB</i>	94671	0116	Colicin V secretion/processing ATP-binding protein CvaB				
94664	<i>cvaA</i>	95938	0117	Colicin V secretion protein CvaA				
96346	<i>yacA</i>	96555	0118	Conserved hypothetical protein				
96549	<i>yacB</i>	96818	0119	Conserved hypothetical protein				
96815	<i>ybaA</i>	96997	0120	Fragment of putative plasmid stabilization protein YacB				
97096	<i>ybaA</i>	97404	0121	Fragment of conserved hypothetical protein YbaA				
97964	<i>iroN</i>	102013	0123	Putative phospho-2-dehydro-3-deoxyheptonatealdolase				
99851	<i>iroE</i>	102013	0124	IroN, salmochelin siderophore receptor				
102058	<i>iroE</i>	103014	0125	IroE, putative hydrolase				
103099	<i>iroD</i>	104328	0126	IroD, putative ferric enterochelin esterase				
104432	<i>iroC</i>	108091	0127	IroC, ATP binding cassette (ABC) transporter homolog				
108231	<i>iroB</i>	109346	0128	IroB, Putative glucosyltransferase				

Continued on following page

TABLE 3—Continued

Position	Gene	ORF	Product	pAPEC-O2-CoIV homolog		pAPEC-O1-ColBM homolog	
				Name	Identity (%)	Name	Identity (%)
110582		0130	Conserved hypothetical protein	O2CoIV51	95.6	O1CoBM143	100
111217		0131	Conserved hypothetical protein	O2CoIV50	99	O1CoBM144	100
111652		0132	Hypothetical protein	O2CoIV49.2	100		
112378		0133	Iss (Increased serum survival)	O2CoIV49	100		
112492	<i>iss</i>	0134	Transposase ORF A, IS2	O2CoIV48.2	99.6	O1CoBM146	100
113412	<i>yqiG</i>	0135	Transposase ORF 2, IS2	O2CoIV48	97.3	O1CoBM126	100
113735	<i>yqiG</i>	0136	Hypothetical protein			O1CoBM149	100
115306		0137	Conserved hypothetical protein; putative GTPase	O2CoIV47	100	O1CoBM150	100
117057		0138	Putative transposase ORF B (fragment), IS2	O2CoIV46	100	O1CoBM151	99.75
117190		0139	Putative transposase (fragment)	O2CoIV45	95.8	O1CoBM152	100
117694		0140	Putative transposase (fragment)	O2CoIV45	83.9	O1CoBM153	100
118538		0141	Putative transposase (fragment)	O2CoIV45	93		
118697		0142	Conserved hypothetical protein			O1CoBM154	100
119120		0143	Conserved hypothetical protein			O1CoBM155	100
120259		0146	Putative type I secretion outer membrane protein EisC	O2CoIV35	100	O1CoBM156	100
121056	<i>etsC</i>	0147	Putative type I secretion ATP-binding protein EisB	O2CoIV34	99.9	O1CoBM199	100
122430	<i>etsB</i>	0148	Putative type I secretion membrane-fusion protein EisA	O2CoIV33	98.7	O1CoBM198	99.85
124370	<i>etsA</i>	0149	Putative transposase ORF B (fragment), IS3 family			O1CoBM197	99.75
125575		0150	Putative transposase ORF B (fragment), IS3 family			O1CoBM196	100
126448		0151	Transposase ORF A, IS3 family, IS51 group	O2CoIV95	99.2	O1CoBM71	100
126687		0152	Transposase ORF B, IS3 family, IS51 group	O2CoIV96	93.5	O1CoBM194	100
127008		0153	Putative transposase ORF B (fragment), IS3 family, IS407 group	O2CoIV29	98.1	O1CoBM192	100
127895		0154	Putative transposase ORF B (fragment), IS3 family, IS407 group	O2CoIV27	96.9	O1CoBM154	100
128405		0155	Hypothetical protein	O2CoIV26	100	O1CoBM155	100
129343		0156	Outer membrane protease (ompTn)				
129836	<i>ompT<sub>p</sub></i>	0157	Hemolysin HlyF	O2CoIV24	100	O1CoBM189	100
131222	<i>hlyF</i>		Conserved hypothetical protein; putative Mfg-14 protein			O1CoBM188	99.67
132331							
133302							

immune system (see below); they were transcribed in both directions and showed sharp deviations from the average G+C content (29.9% to 63.6%), suggesting a foreign mosaic origin. Thirty-five ORFs of unknown function and 14 insertion sequence-like genes were scattered throughout the plasmid. Two different colicins, colicin Ia and colicin V, along with their immunity protein, were encoded by pS88.

#### (ii) Replication, transfer, and maintenance regions of pS88.

Plasmid pS88 harbors two replicons. The first replicon region, RepFIB, contains the typical replication gene *repA* and the site-specific integrase *int* (55). The second, RepFIIA, encodes the CopB repressor and the RepA1 replication proteins. Thus, pS88 appears to belong to both the IncFI and IncFII incompatibility groups. The complete F-like transfer region of pS88 spans 31,485 bp and contains 32 genes (from *traM* to *finO*). The DNA sequences of the three regulator genes *traM*, *traJ*, and *traY*, relative to publicly available databases, appear to be characteristic of an F-type plasmid rather than an R1-type plasmid (15) (Fig. 2). None of the other *tra* genes were discriminated (as exemplified by *traT*), except for *traS*, which was closer to that of R1-type plasmids (Fig. 2). Upstream of the transfer locus lie genes involved in single-stranded DNA transfer; *ssb*, the gene encoding single-stranded binding protein; and *psiA* and *psiB*, plasmid SOS inhibition genes (6). Two loci putatively involved in plasmid maintenance were identified. One, close to the transfer region, was composed of the *sopA* and *sopB* genes, coding for the plasmid partition proteins (45). Located downstream of the RepFIIA replicon, the *yacABC* operon may represent a toxin-antitoxin plasmid stability system of which a second copy, albeit truncated, lies between the colicin V and salmochelin operons.

(iii) Putative virulence region of S88. The putative virulence region of pS88 harbored three different iron uptake systems, namely, aerobactin (*iucABCD* and *iutA*), salmochelin (*iroBCDEN*), and the *sitABCD* genes (35, 53). The *sitABCD* genes were also chromosomally integrated. The other putative virulence genes found on pS88 were the increased serum survival gene *iss*, involved in complement resistance (17); the *etsABC* genes, encoding a putative type 1 secretion system (35); *ompT*, encoding an outer membrane protease (58); and *hlyF*, encoding a hemolysin (44). The S88 *ompT* gene is 100% homologous to the APEC O2 and O1 plasmid orthologs (34, 35) but differs significantly from the common *E. coli* chromosomal *ompT* gene. Therefore, we designate this gene *ompT<sub>p</sub>* (*ompT* of plasmids).

(iv) Comparison with other plasmids. BLAST comparisons of the overall pS88 sequence with other sequences revealed that pS88 is closely related to pAPEC-O2-CoIV and pAPEC-O1-ColBM (Table 3). These plasmids (184,501 and 174,240 bp, respectively) came from avian pathogenic *E. coli* (APEC) O2:K1 and O1:K1 strains causing colibacillosis in chickens (34, 35). Alignment of the three plasmid sequences using the online comparison tool WebACT Artemis (1) is shown in Fig. 3. The line plot revealed several large blocks of highly homologous DNA between pS88 and the two APEC plasmids. Depending on the region examined, the genes and their organization were more similar to pAPEC-O1-ColBM or to pAPEC-O2-CoIV. The virulence region, from the locus *iroBCDEN* to the locus *sitABCD*, appears to be highly homologous to the virulence region of pAPEC-O2-CoIV (the “conserved” virulence region



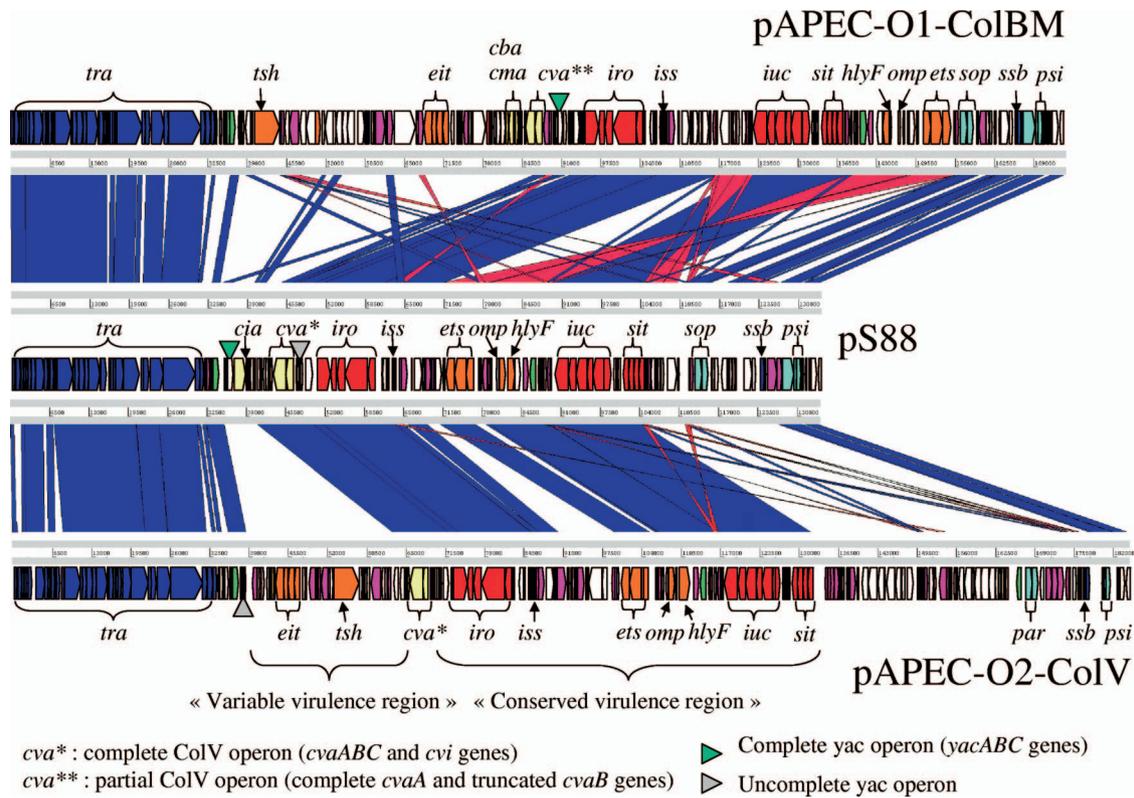


FIG. 3. Comparison of plasmid pS88 (133,853 bp) with plasmids pAPEC-O1-ColBM (174,241 bp) and pAPEC-O2-CoIV (184,501 bp) using the line-plot representation of homologous regions. For convenience, the *traM* start codon was chosen as the beginning of the three sequences. Strand conservations are indicated in blue and strand inversions in red. Genes displayed are categorized using the color scheme described in Fig. 1. Variable and conserved virulence regions are defined as previously described by Johnson et al. (35).

ECOR61; the two ST29<sup>O2</sup> APEC strains harbored a pS88-close plasmid that was PCR negative for *cia* and *traJ*<sub>F-plasmid</sub>, suggesting the presence of a pAPEC-O2-CoIV-like plasmid.

PFGE of undigested DNAs of several strains representative of different sequence O types positive for the 11 pS88 genes showed that all harbored a plasmid of about 130 kb hybridizing with the *iroN* probe (Fig. 4).

Overall, our molecular epidemiology studies indicate that the pS88-like plasmid is not restricted to France, as such plasmids can be found in strains from the United States, Spain, or Sweden, and not only to meningitis strains (Table 1).

**Functional analysis. (i) Plasmid curation and experimental virulence.** Exposure to sodium dodecyl sulfate yielded S88 variants at a high rate (99%). One of these variants (CH7) was markedly less virulent than the wild-type strain in the animal model, with a level of bacteremia at least 2 log CFU/ml lower in two separate experiments (Table 4). To confirm that this was due to the loss of pS88, we complemented the S88 variant with pS88<sub>cat</sub>, using a double-conjugal plasmid transfer method. Virulence was fully restored with the complemented strain, designated S88E (Table 4). Comparative experiments with growth in serum showed that S88 and S88E were more resistant to serum bactericidal activity than CH7, with a mean difference of 1.1 log CFU/ml ( $P < 0.05$ ) after 5 hours of incubation (data not shown).

**(ii) Mutagenesis.** As we have previously shown that *iroN* has a role in the virulence of strain C5 (O18:K1:H7) (47), we

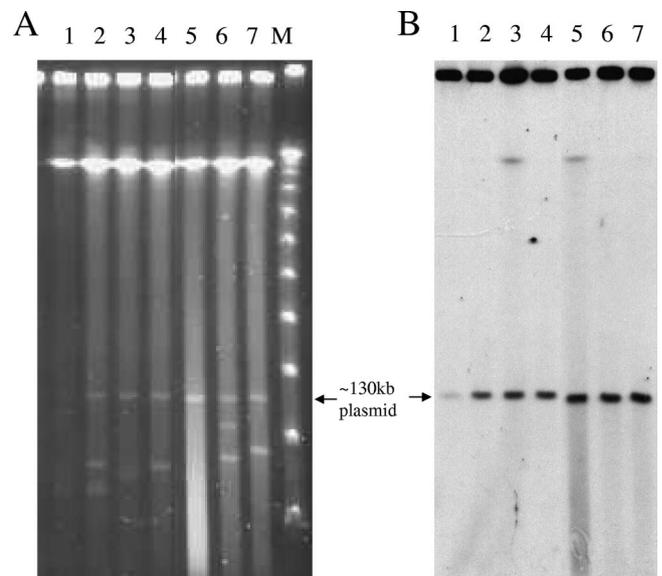


FIG. 4. (A) PFGE of undigested DNAs from seven representative strains harboring the 11 ORFs characteristics of pS88. Lane 1, S145; lane 2, Ben1068; lane 3, S4; lane 4, S133; lane 5, S94; lane 6, HN30; lane 7, ECOR62; lane M, molecular size marker (50-kb DNA ladder). (B) Hybridization with *iroN*-specific probe on a Southern blot.

TABLE 4. Mean bacteremia values in a neonatal rat model challenged with S88 and its mutants, variants, and transconjugants

Expt and strain	Strain description	No. of infected animals	Mean level of bacteremia, log CFU/ml (SD)
1	S88 S88 wild type	10	6.02 (1.52)
	CH7 S88 $\Delta$ p	10	3.54 (0.71) <sup>a</sup>
2	S88 S88 wild type	10	5.26 (1.9)
	S88E S88 $\Delta$ p (pS88 $\Delta$ cat)	10	5.95 (1.65)
3	S88 S88 wild type	13	6.50 (1.67)
	CH1 S88 ( <i>iroN::cat</i> )	13	5.67 (1.87) <sup>b</sup>
4	S88 S88 wild type	9	6.17 (0.81)
	CH23 S88 ( <i>iut::cat</i> )	9	5.85 (1.56)
5	S88 S88 wild type	14	5.71 (1.20)
	CH19 S88 ( <i>sit::cat</i> )	14	5.54 (1.35)

<sup>a</sup>  $P < 0.05$  versus S88 bacterial count in experiment 1.

<sup>b</sup>  $P < 0.05$  versus S88 bacterial count in experiment 3.

examined its involvement, as well as that of the other two iron uptake systems (aerobactin and the *sit* operon), in the virulence linked to pS88. Only *sitABCD* was also present on the chromosome. A CH1 mutant with the salmochelin receptor gene (*iroN*) disrupted was slightly less virulent than the wild-type strain, with a decrease in bacteremia of 0.9 log CFU/ml (Table 4). Two other mutants were also constructed: CH19, which lacks both the chromosome- and plasmid-encoded copies of the *sit* operon (double deletions were obtained in one step of mutagenesis), and CH23, which lacks the aerobactin receptor. The virulence of these two mutants was similar to that of the wild-type strain (Table 4).

## DISCUSSION

S88, the representative strain of the recently described French meningitis clone O45:K1:H7, harbors only a few of the specific virulence factors, such as the K1 capsule antigen and salmochelin, known to influence the onset of meningitis in newborns (13). The *E. coli* genomic project ColiScope, which include the sequencing of S88, provides the opportunity to discover new genetic determinants involved in the virulence of this clone. Indeed, we recently demonstrated the major role of the O-antigen gene cluster, which, surprisingly, encodes a new O antigen slightly different from the O45 reference antigen (50). Sequencing of S88 revealed the presence of a large plasmid encoding two bacteriocins: colicin V and colicin Ia. Although ColV plasmids have long been known to contribute to the virulence of ExPEC strains (3, 4, 54, 57, 64, 66), pS88 is the first ColV plasmid of a human ExPEC strain to be entirely sequenced. However, ColV plasmids have also been linked to APEC virulence, and recently an APEC ColV plasmid (pAPEC-O2-ColV) and a related plasmid (pAPEC-O1-ColBM) have been sequenced (34, 35).

Comparison of pS88 with other sequenced plasmids re-

vealed strong homologies with pAPEC-O2-ColV and pAPEC-O1-ColBM. One of the most striking homologies involves a “conserved” virulence region in APEC plasmids. This region contains numerous known or putative virulence determinants (*iroBCDEN*, *iss*, *etsABC*, *ompT<sub>P</sub>*, *hlyF*, *iucABCD/iutA*, and *sitABCD*) but also ORFs of unknown function, which are all present in the three plasmids in a DNA block less than 60 kb long. Several of these virulence genes have been described as chromosomally encoded in pathogenicity islands, including the salmochelin locus in PAI III<sub>536</sub> (16), the aerobactin locus in PAI I<sub>CF7073</sub> (65), and the *sitABCD* locus in *Salmonella* pathogenicity island 1 (31), or on phage-related DNA, such as *iss* in several wholly sequenced uropathogenic *E. coli* (UPEC) genomes (37). In contrast, the three contiguous putative virulence determinants *etsABC*, *ompT<sub>P</sub>*, and *hlyF* have never been described on the chromosomes of *E. coli* or related species, including those examined for the ColiScope project. These genes may therefore be plasmid specific and represent a signature for the presence of a virulence plasmid in a given strain. The absence of these three genes in *E. coli* chromosomes suggests that they were acquired more recently than other virulence traits that have been integrated in either the chromosome or the plasmid. Their marked %GC differences (48%, 43%, and 38%, respectively) indicate that this nucleus was constructed step by step rather than being acquired “en bloc.” This is supported by the fact that *hlyF* can be found alone in plasmid p1658-97 (accession number AF550679) and associated with *ompT<sub>P</sub>* (but without *ets*) in plasmid pSMS-3-5 (accession number CP000971).

The other region showing strong DNA homology with APEC plasmids was, as expected, the transfer region. However, several interesting features were noted. *traY*, one of the three regulatory genes of the *tra* locus, was used initially by Boyd, and since by several other authors, to discriminate between F- and R1-type plasmids (15, 32). Phylogenetic relatedness inferred from publicly available DNA sequences of *traY* and the two adjacent genes, *traJ* and *traM*, clearly show that pS88 may be considered an F-plasmid, like pAPEC-O1-ColBM, while pAPEC-O2-ColV is closer to an R1-type plasmid. None of the other genes, except for *traS*, could discriminate, owing to their strong homology (*traT* for instance). Interestingly, *traS* was paradoxically closer to that of pAPEC-O2-ColV than to that of pAPEC-O1-ColBM. This supports the mosaic structure of the pS88 transfer locus, in keeping with the results of previous *tra* sequence analyses (15). In contrast to genetic and biochemical mechanisms, we know little of the environmental and physiological factors that affect conjugal transfer, even though they are likely to play an important role (23). The striking segregation of the three regulatory genes, and especially *traJ*, into two groups may correspond to two different regulatory systems adapted to two different environments or two different lifestyles. Of note, *traJ* of the R1-like plasmid is known to have a role in the virulence of the major neonatal meningitis clone O18:K1:H7 (represented by strain UTI89 in Fig. 2) (5). In this clone, TraJ contributes significantly to the survival of *E. coli* K1 inside professional phagocytes (27). Therefore, it would be of interest to determine whether the distantly related pS88 *traJ* gene (related to an F-like plasmid) has a similar or a different role.

One particular feature of pS88 is the cooccurrence of the

two bacteriocins ColV and ColIa. Cells producing two bacteriocins can kill single colicin producers and have fitness advantages, as bacteriocin can be produced under different conditions of stress (25). In a collection of 1,308 animal and human *E. coli* strains, Jeziorowski and Gordon showed that colicin Ia and colicin V were both present in a given strain more often than would be expected by chance and that the two bacteriocin genes were carried on a single plasmid (32). Based on sequence polymorphism analysis of the two operons and their rate of occurrence, it has been postulated that this coassociation may result from the bacteriocin V operon moving onto colicin Ia plasmids (32). The first complete sequence of a plasmid harboring both bacteriocins throws light on the coevolution of the two bacteriocins. First, we found that the two bacteriocin loci were located close together. This would facilitate their horizontal cotransfer on plasmid DNA segments. Although we observed no mobile genetic elements flanking the ColV and ColIa loci, the locus *yacABC* upstream of the ColIa-encoding genes was partially duplicated downstream of the ColV genes, possibly indicating a recombination event in which the two colicins were acquired in a single step. Therefore, pS88, which resembles pAPEC-O2-ColV and pAPEC-O1-ColBM, may have evolved by cotransfer of bacteriocins Ia and V with a recombination event between two conserved regions, the virulence region and the transfer region. This cotransfer might have provided the host strain with a fitness advantage and have favored the spread of this plasmid to different clonal groups.

Our molecular epidemiology studies of pS88 indicates that the pS88-like plasmid is not restricted to the virulent O45:K1:H7 clone but is also distributed, albeit less frequently, in other *E. coli* clonal groups such as O18:K1, O1:K1, O2:K1, and O83:K1. Intriguingly, these clones (except for the less extensively studied group O83:K1) may possess the same outer membrane protein profile, designed OMP9 (2, 48, 61, 67). OMP9 is linked to a particular protein (called PCP) encoded by a large IncFI plasmid that has not been further characterized (42). Moreover, OMP9 has never been described in clones O16:K1 and O7:K1, in which we found no pS88-like plasmid. This strongly suggests that pS88 harbors the genes encoding the PCP. We also found that all B<sub>2</sub>, O45:K1:H7 strains, both avian and human, harbor a pS88-like plasmid, further supporting the genetic relatedness of avian and human O45:K1:H7 strains (10). This is consistent with other observations suggesting that avian species may be the source of neonatal meningitis strains (22, 36, 46). The monomorphism of the plasmid content observed here in B<sub>2</sub>, O45:K1 strains and of the chromosomal determinants previously observed in these strains (13) suggests recent clonal emergence. Moreover the high prevalence of pS88-like plasmids among ST29/B21 strains (40/56, 71%) compared to other STs could represent an example of fine-tuning between a plasmid and a particular genetic background. Finally, the role of pS88 does not appear to be restricted to meningeal virulence, as some members of the O2:K1 clonal group, known to cause urosepsis but not meningitis (10), also harbor a pS88-like plasmid (Table 1). Thus, the virulence mechanisms of this plasmid may have implications that go beyond a particular clone or pathotype.

To determine whether plasmid pS88 is directly involved in the ability of strain S88 to induce high-level bacteremia, the

main step in the pathogenesis of neonatal meningitis, we obtained an S88 variant lacking pS88. The variant produced a far lower level of bacteremia in the rat model (at least 2 log units lower). In order to confirm that the fall in virulence was due to loss of pS88, we reinserted a tagged pS88 in the cured strain S88. The resulting variant was as virulent as the wild-type strain. These results demonstrate the key role of the pS88 plasmid in the virulence of strain S88. Although numerous studies have established a link between the ColV plasmid and the virulence of various avian and human ExPEC strains (56), this is the first direct evidence supporting a role of a ColV plasmid in a specific step of neonatal meningitis, namely, sustained high-level bacteremia. This property may be due in part to the contribution of pS88 in resistance to serum bactericidal activity that we observed.

In a first attempt to understand the mechanism by which the plasmid contributes to the virulence of S88, we examined the three iron uptake systems encoded by the plasmid, namely, the siderophores salmochelin and aerobactin and the Sit system. Iron is essential for bacterial growth, but its availability within the host is limited (26). We have previously shown that salmochelin is involved in the high-level bacteremia achieved by the representative O18:K1:H7 strain C5 (47), in which this factor is chromosome borne. Hence, we examined whether this siderophore was also involved in the virulence of strain S88. We also investigated the respective virulence roles of the other two plasmid-encoded iron uptake systems, namely, aerobactin and the Sit system. Aerobactin is the most frequent siderophore in neonatal meningitis *E. coli* (NMEC) strains, after yersiniabactin (18, 13). Although this iron uptake system has been implicated in virulence in several experimental models (21, 24, 43, 60) its role in neonatal meningitis remains to be determined. The *sitABCD* operon, encoding a member of the family of ATP-binding cassette (ABC) proteins initially described for *Salmonella enterica*, was first functionally characterized as a manganese and iron transporter in an APEC strain (53). This newly described system is required for full virulence of *Salmonella enterica* serovar Typhimurium and APEC (31, 52). However, little is known of its distribution in human ExPEC strains or its role in their virulence (36, 42, 52).

As previously reported with strain C5 (47), we found that the virulence of strain S88 was attenuated when *iroN* was lacking. In contrast, the absence of aerobactin and the Sit system had no discernible influence on the virulence of strain S88. Our results thus prove the key role of salmochelin in the bacteremic step of *E. coli* meningitis. However, the lower virulence of the *iro*-defective mutant (~1 log CFU/ml lower) cannot alone explain the marked loss of virulence observed with the variant lacking the whole plasmid (>2 log CFU/ml). The fact that the loss of the other two siderophores did not influence the level of bacteremia does not totally exclude a role in the virulence of S88, as the *iro* locus may compensate for their loss. Further studies with double and triple mutants are under way. Alternatively, these siderophores may be involved in steps other than sustained bacteremia, such as gut translocation or cerebrospinal fluid invasion. Other iron uptake systems such as yersiniabactin, enterobactin, and the Fec<sub>ABCD</sub> system exist on the S88 chromosome ([www.genoscope.fr](http://www.genoscope.fr)) and may be able to compensate for the loss of the plasmid-encoded systems. Finally, it is likely that the virulence of the ColV plasmid involves

other determinants. The pS88 plasmid carries two genes, *iss* and *traT*, which play a role in serum survival and resistance to macrophage phagocytosis, respectively. Several other pS88 coding sequences have no homologs and could also encode new virulence determinants.

In conclusion, pS88 shares a similar structure with avian virulence plasmids, pointing to a possible common ancestor. It is an essential meningeal virulence determinant comparable to the K1 capsule and O45<sub>S88</sub> somatic antigens and is not restricted to the French O45:K1:H7 clone. A comprehensive study of the molecular mechanisms responsible for its virulence may have important implications, possibly beyond the pathogenesis of neonatal meningitis.

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