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Chromosomal Replication Origins (*oriC*) of *Enterobacter aerogenes* and *Klebsiella pneumoniae* Are Functional in *Escherichia coli*

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The chromosomal DNA replication origins (*oriC*) from two members of the family *Enterobacteriaceae*, *Enterobacter aerogenes* and *Klebsiella pneumoniae*, have been isolated as functional replication origins in *Escherichia coli*. The origins in the *Sal*I restriction fragments of 17.5 and 10.2 kilobase pairs, cloned from *E. aerogenes* and *K. pneumoniae*, respectively, were found to be between the *asnA* and *uncB* genes, as are the origins of the *E. coli* and *Salmonella typhimurium* chromosomes. Plasmids containing *oriC* from *E. aerogenes*, *K. pneumoniae*, and *S. typhimurium* replicate in the *E. coli* cell-free enzyme system (Fuller, et al., Proc. Natl. Acad. Sci. U.S.A. **78**:7370-7374, 1981), and this replication is dependent on *dnaA* protein activity. These *Sal*I fragments from *E. aerogenes* and *K. pneumoniae* carry a region which is lethal to *E. coli* when many copies are present. We show that this region is also carried on the *E. coli* 9.0-kilobase-pair *Eco*RI restriction fragment containing *oriC*. The F_0 genes of the *atp* or *unc* operon, when linked to the *unc* operon promoter, are apparently responsible for the lethality.

Replication of the chromosomes of *Escherichia coli* and *Salmonella typhimurium* begins at a unique site or region (*oriC*) between the genes *asnA* and *uncB* (39, 43, 45), and plasmids have been isolated that contain as their only origin either the *oriC* of *E. coli* (23, 26, 41) or the *oriC* of *S. typhimurium* (43). The successful propagation of *E. coli* and *S. typhimurium oriC*-containing plasmids in strains of *Enterobacter aerogenes* and *Klebsiella pneumoniae* (42) suggested that the *E. aerogenes* and *K. pneumoniae* origins would in turn function in *E. coli*. These two organisms are both members of the tribe *Klebsielleae* in the family *Enterobacteriaceae* (8) and are more distantly related to *E. coli* and *S. typhimurium* (32), which are classified in the tribe *Escherichieae* of the same family. The *oriC* region of *Erwinia carotovora*, a member of the tribe *Erwineae* of the same family, has also been isolated as a functional origin in *E. coli*, and the nucleotide sequence of this *oriC* has been determined (36).

The approach used with *E. carotovora* (36) was also used here. Restriction fragments were selected that would allow the cloning vector, pMK2004 (20), which requires DNA polymerase I, the product of the *polA* gene, for replication (22), to replicate in an *E. coli polA* mutant. Plasmids were isolated that contained the chromosomal replication origins of *E. aerogenes* and *K. pneumoniae* as well as a *polA*-like gene from *E. aerogenes*.

The chimeric plasmids constructed in this study contain both *oriC* and ColE1 *rep* origins and can be used to detect sequences which are lethal when multiple copies are present. In this study, we localized such a high-copy lethal (HCL) region to the *unc* operon in each of these four bacterial species. Our results strongly suggest that such HCL regions would not be found in most clone banks constructed to date.

MATERIALS AND METHODS

Bacterial strains, plasmids, media, and genetic procedures. The bacterial strains and plasmids used are listed in Table 1. *E. coli* cells were grown in either YT medium (28) or M9 minimal medium (20) with 0.2% glucose and other supplements as required. We used 50 μg of kanamycin (CalBiochem) per ml, 15 μg of tetracycline (CalBiochem) per ml, or 10 μg of ampicillin (Bristol Laboratories) per ml. Complementation of *E. coli asnA*, *uncA*, and *uncB* mutants, transforma-

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TABLE 1. Bacterial strains and plasmids

Designation	Genotype[a] and phenotype[b]	Source
E. coli strain		
C600	F⁻ *thr-1 leu-6 thi-1 supE44 lacY1 tonA21*	Our laboratory
C2368	F⁻ *polA1 thy his rha hsdR hsdM*	D. Helinski
MC1024	F⁻ *araD139* Δ(*ara-leu*)*7697* Δ(*lacZ*)*M15 galU galK rpsL srl*::Tn*10*(Tc^r) *recA56*	M. Casadaban (6)
AI214	F⁻ *thi pyrE argE his proA leu mtl xyl rpsL hsdR srl*::Tn*10*(Tc^r) *recA56*	Our laboratory
JZ294	F⁻ *polA1 argH hsdR rpsL thyA36*	Footnote *c*
JZ279	F⁻ *recA56 hsdR lacY galK2 galT22 metB1 trpR55 supE44 supF58*	Footnote *d*
AN382	F⁻ *uncB401 argE3 thi-1 mtl-1 xyl-5 galK2 rpsL thr-3? supE44?*	B. Bachmann, *E. coli* Genetic Stock Center
ER	F⁺ *asnA asnB thi-1*	B. Bachmann
K. pneumoniae M5a1	Wild type	D. Helinski (24)
E. aerogenes SD1	Wild type	W. Yost
P. aerugenosa PA103	Wild type	E. Ziegler
Plasmid		
pMK2004	Tc^r Ap^r Km^r	M. Kahn (20)
pJZ1	*oriC*⁺(Sty) *asnA*⁺(Sty) *uncB*⁺(Sty) Km^r	Our laboratory (43)
pJZ19	*oriC*⁺(Sty) *asnA*⁺(Sty) Km^r Tc^r	Our laboratory (43)
pJZ25	*oriC*⁺(Sty) Km^r Ap^r	Our laboratory (43)
pOC15	*oriC*⁺(Eco) *asnA*⁺(Eco) Ap^r	W. Messer (27)
pAL1	*oriC*⁺(Eco) *asnA*⁺(Eco) *uncB*⁺(Eco) Km^r	A. Leonard (23)
pJM1	*oriC*⁺(Eco) *asnA*⁺(Eco) *uncB*⁺(Eco) Tc^r Ap^r Km^r	Footnote *e*

[a] Abbreviations used are those of Bachmann and Low (1). Bacterial and genetic elements on plasmids derived from *S. typhimurium* (Sty) and *E. coli* (Eco) are shown.

[b] Abbreviations for drug resistance: Ap, ampicillin; Km, kanamycin; Tc, tetracycline.

[c] P1 transduction from strain CM5280 *polA1* Tn*10* (Tc^r) *metE70 thyA desC2 lacZ lacY14*(Am) *rha-5 rpsL* (from N. Grindley) into strain JA228 *thyA36 argH hsdR rpsL* (M. Ares) followed by Tc^s mutant selection on Bochner plates (3).

[d] P1 transduction from strain AI214 (44) into strain LE392 *hsdR lacY galK2 galT22 metB1 trpR55 supE44 supF58* (from N. Grindley), followed by Tc^s mutant selection on Bochner plates (3).

[e] Described in the text.

tions, and P1 transductions were as described previously (43). To measure plasmid loss, cells growing exponentially in antibiotic-containing media were transferred after centrifugation to media lacking an antibiotic and grown further. Samples were periodically plated on media containing or lacking an antibiotic. Loss of plasmids when cells were grown without selection was exponential, permitting calculation of the data presented.

Preparation of chromosomal and plasmid DNAs. Bacterial chromosomal DNA was prepared as described previously (36). Total lysates were subjected to isopycnic ethidium bromide-cesium chloride gradient centrifugation. No plasmid DNA band was observed in the ethidium bromide-cesium chloride gradients of chromosomal DNAs of *E. coli* C600, *K. pneumoniae* M5a1, *E. aerogenes* SD1, or *Pseudomonas aeruginosa* PA103. Plasmid DNA was isolated as previously described (43).

Enzymes. Restriction endonucleases *Ava*I, *Ava*II, *Bam*HI, *Bgl*II, *Eco*RI, *Pst*I, *Sal*I, *Hae*III, *Hha*I, *Hin*dIII, *Xho*I (all from Bethesda Research Laboratories), and *Sma*I (New England Biolabs) were used as recommended by the suppliers. For DNA ligation, 0.25 to 1.0 U of T4 DNA ligase (Bethesda Research Laboratories) was used under conditions recommended by the manufacturer, with 5 to 40 μg of DNA per ml for intermolecular ligations and less than 2 μg of DNA per ml for intramolecular self-ligation.

Gel electrophoresis. The molecular weights of plasmids and restriction fragments were determined in 0.8 to 1.5% vertical agarose (SeaKem) slab gels in Tris-acetate-EDTA buffer (12) or in 5 to 10% polyacrylamide gels (25).

DNA-DNA hybridization. DNA was transferred from 0.8% agarose gels to nitrocellulose filter strips by the procedure of Southern (34). Preincubation (24 to 48 h) and hybridization (60 to 75 h) were performed in 2× Denhardt solution (9) at 55°C. Filters were exposed to Kodak XR-5 film for 5 to 12 days. Radioactive probes were made by two procedures. In the first procedure, pJZ19 (45) was radioactively labeled with [^{32}P]dCTP (400 Ci/mmol, Amersham Corp.) by nick translation with DNase I and *E. coli* DNA polymerase I (Bethesda

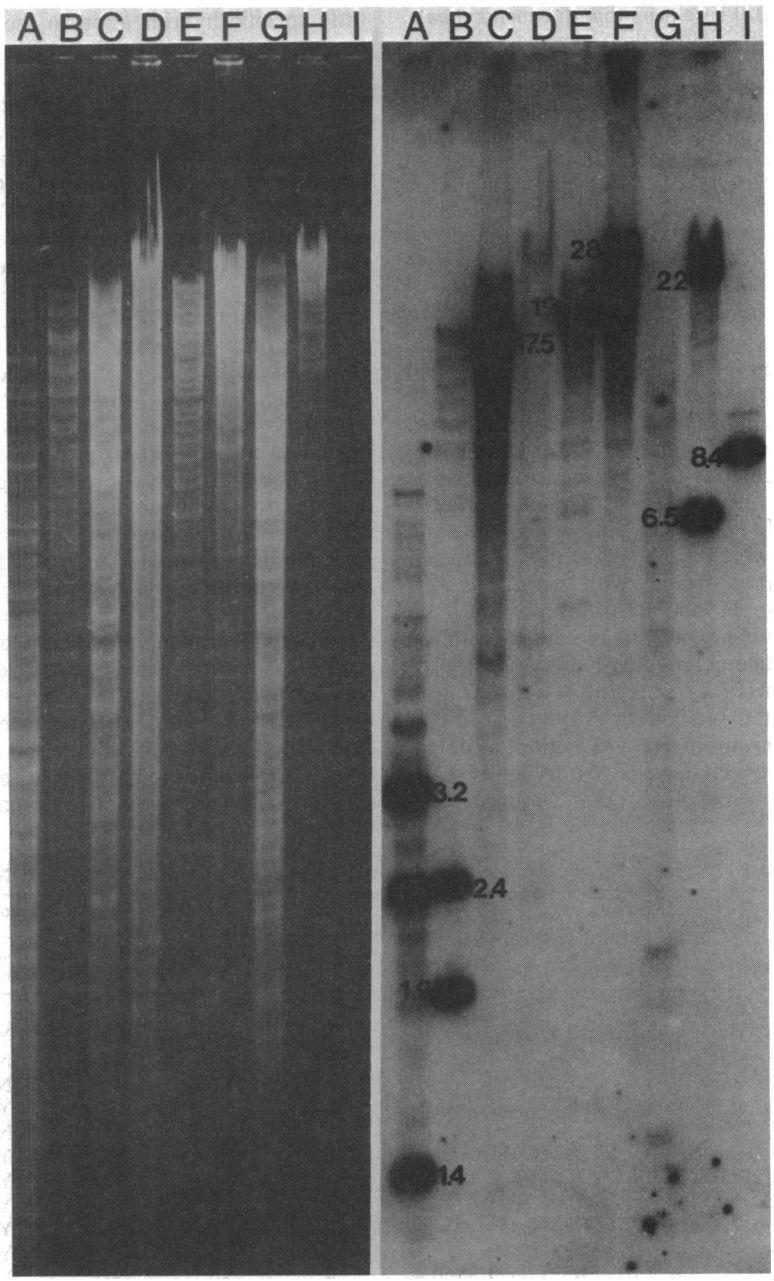

FIG. 1. Hybridization of chromosomal restriction enzyme fragments to the *S. typhimurium* origin region. Chromosomal DNAs from *E. aerogenes*, *P. aerugenosa*, and *E. coli* were separately digested with *Pst*I, *Sal*I, and *Xho*I. The restriction fragments (10 μg total DNA) were separated on a 0.8% horizontal agarose gel, transferred to a nitrocellulose filter, and hybridized to the pJZ19 *S. typhimurium oriC* probe (see text). Hybridization was for 69 h at 55°C and autoradiographic exposure was for 5 days. Lane A, *Pst*I digest of *E. aerogenes* DNA; lane B, *Pst*I digest of *E. coli* DNA; lane C, *Sal*I digest of *E. aerogenes* DNA; lane D, *Sal*I digest of *P. aeroginosa* DNA; lane E, *Sal*I digest of *E. coli* DNA; lane F, *Xho*I digest of *E. aerogenes* DNA; lane G, *Xho*I digest of *P. aeruginosa* DNA; lane H, *Xho*I digest of *E. coli* DNA; and lane I, *Pst*I digest of pJZ1 (total DNA, 4 ng).

Research Laboratories). After digestion with *Pst*I, the 8.4-kilobase-pair (kb) insert containing the *S. typhimurium* origin was purified by electrophoresis on a 1% agarose gel, and the fragment was extracted from the agarose with a saturated potassium iodide solution at 37°C and then subjected to hydroxyapatite chromatography as described previously (33). In the second procedure, pJZ25, containing three *Bam*HI fragments

of 91, 106, and 220 base pairs (bp) (fragments *Bam*D, *Bam*E, and *Bam*F; 45) from the *S. typhimurium* origin, was digested with *Bam*HI. The three fragments were purified by polyacrylamide gel electrophoresis, and their 5'-ends were labeled with ^{32}P by using polynucleotide kinase (Bethesda Research Laboratories) and [γ-^{32}P]ATP as described by Maxam and Gilbert (25).

In vitro protein synthesis. DNA-dependent protein synthesis was conducted by using the in vitro coupled transcription-translation system previously described (18). Sodium dodecyl sulfate-polyacrylamide gels were run as described previously (4).

RESULTS

Hybridization of *E. aerogenes* chromosomal DNA to the *S. typhimurium oriC* region. We used Southern filter hybridization to determine if *E. aerogenes* chromosomal DNA contains sequences homologous to the origin region of *S. typhimurium* (Fig. 1). The *S. typhimurium Pst*I fragment of pJZ19 (45) carrying *oriC*, *asnA*, and part of *uncB*, which was used as the ^{32}P-labeled probe, hybridized strongly with two *E. coli Pst*I fragments (Fig. 1, lane B) of 2.45 and 1.9 kb (possibly a doublet band), in agreement with the size of *Pst*I fragments which map within the *E. coli oriC* region (27). The degree of hybridization reflects the extensive homology between *E. coli* and *S. typhimurium* in the *oriC* region, as determined by sequence analysis (45). In contrast, *P. aeruginosa* chromosomal DNA showed very little specific hybridization to the *S. typhimurium* probe (Fig. 1, lanes D and G). However, the *S. typhimurium* probe hybridized equally well with three *E. aerogenes Pst*I fragments of 3.2, 2.35, and 1.4 kb (Fig. 1, lane A). Single *E. coli Sal*I fragments of 19 kb (lane E) and a 17.5-kb fragment from *E. aerogenes* (lane C) hybridized to the probe. A single 28-kb *Xho*I fragment from *E. aerogenes* (lane F) and two *E. coli Xho*I fragments of 22 and 6.5 kb (lane H) showed hybridization. These results, together with restriction analysis of *oriC* plasmids, indicate that there is only one copy of *oriC* per bacterial chromosome in each of these three organisms.

Construction of *E. aerogenes oriC* plasmids. A partial *Sal*I digest of *E. aerogenes* DNA was ligated to pMK2004 (20) previously cleaved at the unique *Sal*I site within the *tet* gene, and the ligation products were used to transform *E. coli* C2368 *polA1 hsdR* followed by selection of Km^r Ap^r colonies. Plasmid DNA from 11 transformants was purified and hybridized by Southern transfer to a ^{32}P-labeled probe consisting of the three *Bam*HI fragments containing the *oriC* region of *S. typhimurium* (see above). The three contiguous *Bam*HI fragments have a total length of 417 bp. Two plasmid types were obtained. One plasmid type, consisting of plasmids pNH2, pNH3, and pNH5, hybridized to the *oriC* probe (Table 2; data not shown). The remaining eight

TABLE 2. Properties of plasmids cloned from *E. aerogenes* and *K. pneumoniae* DNAs

Plasmid	Size of cloned *Sal*I fragments (kb)	MMS[a]	Hybridization[b]	Plasmid loss[c]	Transforms *E. coli polA*$^+$ strains	Tc
E. aerogenes						
pNH1	3.05, 1.75, 1.35, 0.45	R	No	ND[d]	Yes	S
pNH2	17.50, 8.90	S	Yes	2.5	No	S
pNH3	17.5	S	Yes	3.8	No	S
pNH4	3.60, 3.05, 2.45, 1.75, 1.35, 0.45	ND	No	ND	Yes	R[e]
pNH5	17.5	S	Yes	3.0	No	S
pNH6	7.3, 5.1, 3.05, 1.75, (1.35, 0.45)[f]	R	No	ND	Yes	S
pNH7	7.3, 3.6, 3.05, 2.45, 1.75, 1.35, (0.45)[f]	R	No	ND	Yes	S
pNH8	3.05, 1.75, 1.35, 0.45	R	No	8.2	Yes	S
pNH9	7.3, 3.05, 1.75, 1.35, 0.45	R	No	ND	Yes	R[e]
pNH10	7.3, 3.05, 1.75, 1.35, (0.45)[f]	ND	No	ND	Yes	S
pNH11	6.7, 3.6, 3.05, 2.45, 1.75, 1.35, 0.45	R	No	ND	Yes	S
K. pneumoniae						
pNH62	10.2	S	Yes	3.8	No	S
pNH64	10.2	S	Yes	5.8	No	S

[a] R, Resistant; S, sensitive.

[b] Hybridization to the *S. typhimurium oriC* region, as shown by Southern blotting analysis of plasmid DNA with the three *Bam*HI fragments (total, 417 bp) from the *S. typhimurium* origin as the probe.

[c] Plasmid loss is given as the number of generations required for 50% of the cells to lose the plasmid when grown in the absence of the antibiotic.

[d] ND, Not determined.

[e] More than one copy of pMK2004 was present per insert.

[f] Presumed to be present by comparison with restriction maps of plasmids pNH1, pNH4, and pNH6 through pNH11.

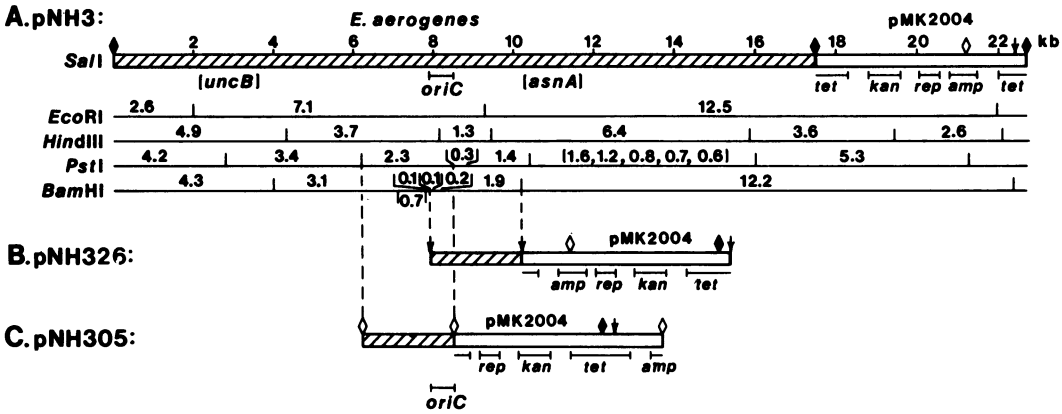

FIG. 2. Physical and genetic map of pNH3 and derivative plasmids containing the origin of *E. aerogenes*. (A) pNH3. (B) pNH326 (pNH3 *Bam*HI fragments inserted into pMK2004). (C) pNH305 (pNH3 *Pst*I fragment inserted into pMK2004). Hatched regions designate the *E. aerogenes* chromosomal DNA, and open regions indicate the cloning vehicle pMK2004. The ColE1 origin of pMK2004 is indicated by *rep*. The scale is in kb. Symbols: ◆, *Sal*I sites; ◇, *Pst*I sites; ↓, *Bam*HI sites.

isolates did not exhibit any detectable homology.

Endonuclease restriction analysis of these plasmids with *Sal*I demonstrated that pNH2, pNH3, and pNH5 contained a 17.5-kb fragment in common which was not present in any of the other plasmids (Table 2). This fragment was identical in size to the unique *E. aerogenes* chromosomal DNA *Sal*I fragment which hybridized to the *S. typhimurium* origin probe (Fig. 1). A detailed physical map of pNH3 is shown in Fig. 2. The remaining eight plasmids contained a number of smaller *Sal*I fragments, four of which were found in common (3.05, 1.75, 1.35, and 0.45 kb). *E. coli polA1* cells containing this class of plasmids were no longer sensitive to methyl methanesulfonate (MMS), suggesting that these plasmids possess a *polA*-like gene from *E. aerogenes* which allows replication of the pMK2004 plasmid vector in the *E. coli polA* mutant. Cells carrying a *polA1* mutation are incapable of normal DNA repair and as a result are more sensitive to MMS than are *polA*$^+$ cells (17). Preparations of pNH6 or pNH7 also contained small plasmid derivatives which were not digested with *Sal*I, and the preparations of pNH4 and pNH9 contained multiple copies of pMK2004 (data not shown). Replication of pMK2004 and the smaller derivative plasmids was most likely supported by a gene product supplied in trans from the larger plasmid containing *E. aerogenes* chromosomal DNA.

Cloning of the *K. pneumoniae* origin region. Using the same procedure as that described above with a total *Sal*I digest of *K. pneumoniae* chromosomal DNA, we found that DNAs from two of five Km^r Ap^r MMS^s plasmids hybridized to the *S. typhimurium oriC* probe (Table 2; data not shown). *Sal*I restriction analysis of pNH62 and pNH64 showed that each plasmid contained a single 10.2-kb insert. The relevant properties of these two plasmids are shown in Table 2. A physical map of pNH62 is shown in Fig. 3.

Subcloning the *oriC* region from *E. aerogenes* and *K. pneumoniae*. A characteristic of the plasmids containing the large *Sal*I *oriC* chromosomal inserts from *E. aerogenes* and *K. pneumoniae* was their inability to transform *E. coli polA*$^+$ strains (Table 2). In *polA* mutants, these plasmids were maintained at a low copy number (about one per cell; data not shown). In *E. coli polA*$^+$ strains, the replication origin of pMK2004 would be used with a resulting copy number of about 40 (20). Thus, the chromosomal inserts presumably contain DNA sequences that are lethal when many copies are present (HCL sequences). Smaller derivative plasmids which were able to transform *E. coli polA*$^+$ strains were therefore constructed (Fig. 2 and 3; Table 3).

Derivative plasmids containing a functional *E. aerogenes* origin were constructed from the initial isolated plasmid, pNH3, by either complete *Pst*I or partial *Bam*HI digestion. Restriction fragments were ligated to excess pMK2004 vector DNA digested with the appropriate enzyme, and *E. coli* C2368 *polA1* cells were transformed to select for kanamycin resistance. Plasmid DNA from several resulting isolates was used to transform *E. coli polA*$^+$ cells, and Km^r isolates were analyzed further. Plasmid DNA from these cells still retained the ability to replicate in *E. coli* C2368 *polA1* cells. All *E. aerogenes Pst*I-derived *oriC*$^+$ plasmids contained in common a 2.35-kb *Pst*I fragment from within the *Sal*I insert of pNH3 (see Fig. 2). Although plasmids with

both orientations of the 2.35-kb *Pst*I fragment were initially obtained, one orientation was unstable, resulting in large spontaneous deletion derivatives which had lost one of the *Pst*I sites (data not shown). A comparison of the stable *Pst*I-derived plasmid, pNH305, with the *Bam*HI-derived plasmid, pNH326, showed that the cloned *E. aerogenes* DNA inserts overlap only in a small region (Fig. 2), limiting the size of the required origin DNA sequence to approximately 640 bp of the initial 17.5-kb insert.

Derivative plasmids containing the *K. pneumoniae* origin were constructed from plasmid pNH62 with a complete *Pst*I digest of the parent plasmid as described above. Plasmids isolated from five *E. coli* C2368 *polA1* Km^r clones were able to transform *E. coli polA*$^+$ cells, and restriction analysis of these derivative plasmids showed that each contained a single *Pst*I insert of 4.8 kb. One isolate, pJZ70, was analyzed extensively, and its physical map is presented in Fig. 3.

The *Bam*HI fragments from the *Pst*I insert of pJZ70 were subcloned into the *Bam*HI site of pMK2004, selecting for Km resistance in *E. coli* AI214 *polA*$^+$. Only Km^r plasmids which contained a common 310-bp *Bam*HI fragment were able to replicate in *E. coli* C2368 *polA1*. One plasmid, pJC132 (Fig. 3), contained only the 310-bp *Bam*HI *K. pneumoniae* fragment. It should be noted that repeated attempts to reclone this fragment into pMK2004 in the reverse orientation were unsuccessful, although head-to-tail dimers of the 310-bp *oriC*$^+$-containing fragment in its original orientation were obtained. Thus, the *K. pneumoniae* origin is limited to a 310-bp region within the original 10.2-kb insert, which is close to the minimum size of the *E. coli* origin (between 237 and 245 bp) (31). Because *oriC* is totally contained on this single small *Bam*HI fragment from *K. pneumoniae*, transfer of *oriC* to new vectors is simplified.

***E. coli oriC*-containing *Eco*RI fragment: lethal at high copy number.** The purified 9.0-kb *E. coli* *Eco*RI fragment of pAL1 (23) was ligated to *Eco*RI-digested pMK2004 (20). pJM1 was isolated from a Km^r Ap^r Tc^r clone obtained after transformation of *E. coli* JZ294 *polA1*. This plasmid consisted of pMK2004 plus the *Eco*RI fragment containing the *E. coli oriC* region including *uncB*. No colonies were obtained after transforming *E. coli polA*$^+$ strains AI214 or JZ259 with this plasmid. This *E. coli* *Eco*RI fragment thus also contains HCL sequences.

Mapping of the *E. aerogenes* and *K. pneumoniae* genes carried on the origin plasmids. None of the *oriC*-containing plasmids carrying the *E. aerogenes* or the *K. pneumoniae* *Sal*I fragments cloned into pMK2004 transformed *E. coli polA*$^+$ strains (Table 2). When pMK2004 was eliminated from the *E. aerogenes oriC* plasmid pNH3 by religation of the *Sal*I-digested plasmid, however, the resulting self-ligated 17.5-kb fragment complemented both the *asnA* and *uncB* mutations of

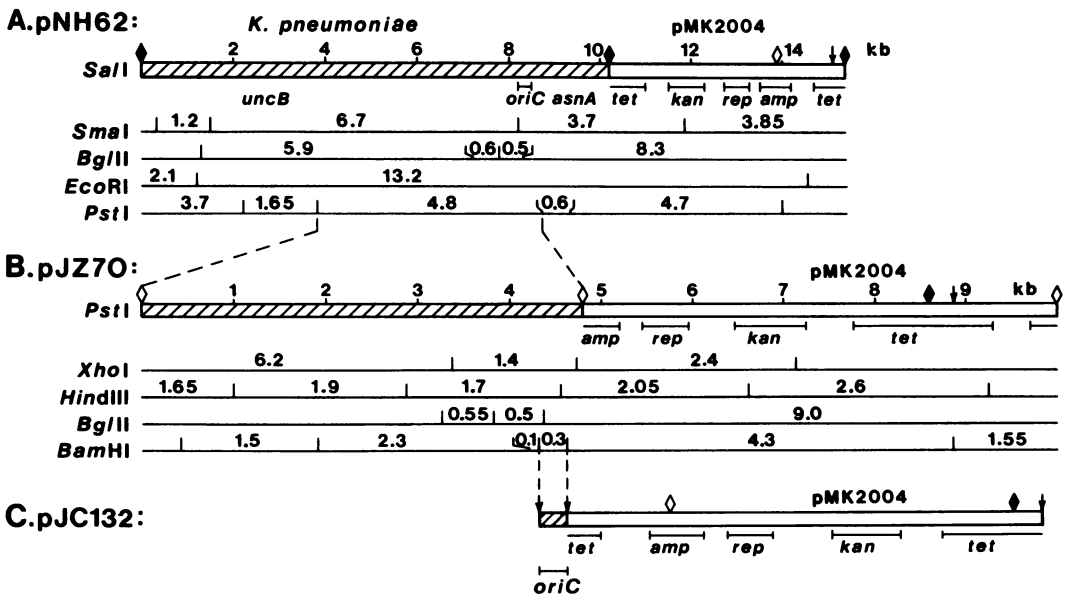

FIG. 3. Physical and genetic map of pNH62 and derivative plasmids containing the origin of *K. pneumoniae*. (A) pNH62. (B) pJZ70 (pNH62 *Pst*I fragment inserted into pMK2004). (C) pJC132 (pJZ70 *Bam*HI fragment inserted into pMK2004). Other symbols and nomenclature are given in the legend to Fig. 2.

E. coli ER and AN382, respectively. Further, a nonreplicating 5.6-kb *Sal*I fragment containing the *kan* gene from the plasmid pDF11 (20) was ligated to the *oriC*-containing *Sal*I fragment from *K. pneumoniae*. The resulting plasmid, pJC62, which contained the 10.2-kb *Sal*I fragment from *K. pneumoniae*, replicated in both *polA*$^+$ and *polA E. coli* strains. Complementation experiments showed that this plasmid carried both the *asnA* and *uncB* genes. The genes *asnA* and *uncB* flank *oriC* in both *S. typhimurium* and *E. coli*. When the 8.3-kb *Bgl*II fragment of pNH62 (Fig. 3) was cloned as a plasmid, this plasmid, pJC627, complemented *asnA* but not *uncB* and did not have the *oriC* function. This result shows the positions of all three markers with respect to one another and is in agreement with the locations of these loci in *E. coli* and *S. typhimurium*.

The 13.2-kb *Eco*RI fragment of pNH62 was self-ligated to yield plasmid pJC629. This smaller version of pNH62 did not transform *E. coli polA*$^+$ strains. Since pJC627 and pJZ70 did transform *polA*$^+$ strains and pJC629 did not, the HCL region was delimited on the pNH62 restriction map by the *Eco*RI site at 1.2 kb and by the *Pst*I site at 3.9 kb (Fig. 3).

K. pneumoniae plasmids pNH62 and pJC629 directed the in vitro synthesis of polypeptides (Fig. 4) similar in size to the a, c, b, and δ polypeptide products of the *uncB*, *-E*, *-F*, and *-H* genes, respectively (16, 18; Fig. 5). The large pNH62-coded polypeptide of M_r 54,000 (54K) (Fig. 4, lane B) could be a hybrid fusion product of part of the *uncA* gene and the cloning vector. This plasmid does not complement the *uncA* mutation in a *polA6* derivative of *E. coli* AN120 (5). Plasmids pNH62 and pJC629 also directed the synthesis of polypeptides similar in size to the *E. coli gid* (38) and *asnA* (38) gene products, although the *K. pneumoniae* (lanes B and C) and *S. typhimurium* (lane E) *asnA* polypeptides are slightly larger than the *E. coli asnA* polypeptide (lane A). A polypeptide similar in size to the *E. coli* 26K M_r polypeptide (lane A) does not appear to be encoded by *K. pneumoniae* plasmid pNH62 or pJC629 (lanes B and C) or by *S. typhimurium* plasmid pJZ19 (lane E).

Enzymatic replication of plasmids containing the origins of the *E. aerogenes* and *K. pneumoniae* chromosomes. The *E. aerogenes* plasmid pNH305 and the two *K. pneumoniae* plasmids pJZ70 and pJC132 were active as templates (R. S. Fuller, J. M. Kaguni, and A. Kornberg, personal communication) in the soluble enzyme system that specifically recognizes and replicates plasmids containing the *E. coli* chromosomal origin (13). The cloning vehicle pMK2004 was inert as a template, indicating that the *oriC* origins in the plasmids pNH305, pJZ70, and pJC132 were responsible for this activity. When the *dnaA*-complementing fraction was omitted from the reaction, little activity was detected. Fuller et al. also found that the *S. typhimurium oriC*-containing plasmids pJZ19 and pJZ34 (45) were both active as templates in this system and that replication was dependent upon the presence of *dnaA* protein.

DISCUSSION

The following evidence strongly supports the conclusion that the plasmids isolated in this study contain chromosomal rather than endogenous plasmid or prophage origins. (i) An *S. typhimurium* origin probe hybridized to both the

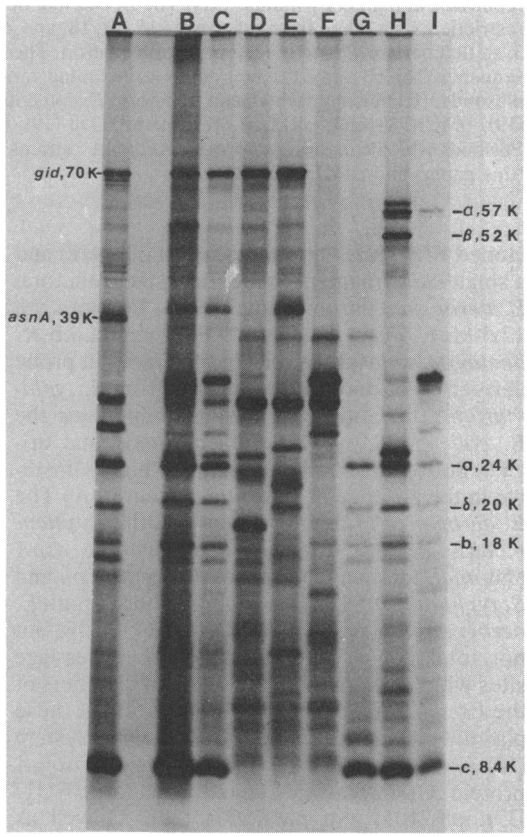

FIG. 4. In vitro protein synthesis products of plasmids containing the *oriC* regions of *E. coli*, *K. pneumoniae*, and *S. typhimurium*. In vitro transcription-translation, followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography, was performed. The locations and molecular weights of the *unc* polypeptides α, β, a, δ, b, and c (18), as well as the *asn*A (38) and the *gid* (38) proteins, are indicated. Lane A, pAL1 (23); lane B, pNH62; lane C, pJC629 (self-ligated 13.2-kb *Eco*RI fragment of pNH62); lane D, pJZ70; lane E, pJZ19 (46); lane F, pMK2004 (20); lane G, pRPG45 (18); lane H, pRPG54 (18); and I, pRPG23 (18).

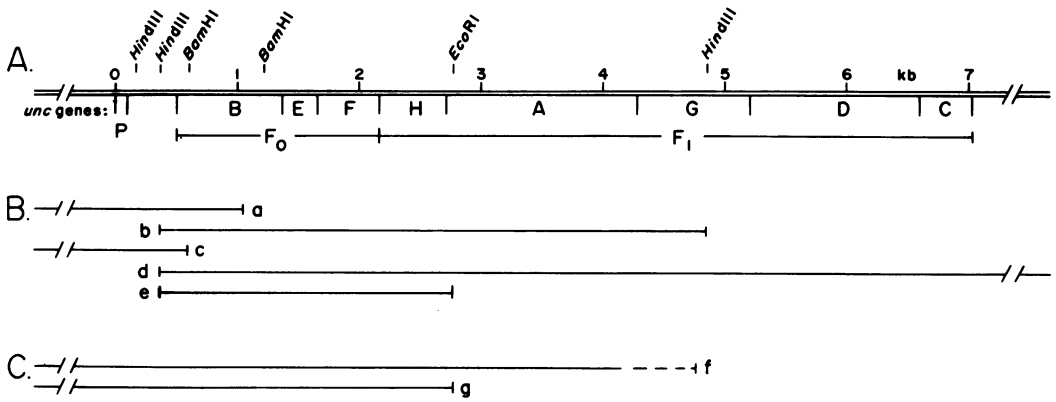

FIG. 5. Bacterial chromosomal fragments carrying parts of the *unc* operon which can (B) and cannot (C) be replicated at high copy number. (A) The *E. coli unc* operon. Capital letters designate *unc* genes. Gene order and restriction sites taken from references 15, 16, 18, and 29 are in the opposite orientation to the maps in Fig. 2 and 3, so that here *oriC* is to the left of the *unc* operon. The location of the promoter (P) is derived from the nucleotide sequence (16). (B and C) Bacterial DNA, including various parts of the *unc* operon cloned into high-copy-number plasmids. (B) Plasmids which can transform *E. coli polA*$^+$ strains include (a) pJZ19 (45); (b) pAN51 (11), pFH167 (19), pMCR533 (21), pRPG23 (18); (c) pFH350 (19); (d) pAN45 (11), pRPG54 (18); and (e) pRPG45 (18). (C) Plasmids which cannot transform *E. coli polA*$^+$ strains include (f) pNH3 and pNH62 (this paper); and (g) pJC629 (this paper) and pJM1 (this paper).

cloned 17.5-kb *E. aerogenes* insert of pNH3 and a single *Sal*I fragment of identical size from total *E. aerogenes* chromosomal DNA. (ii) Both the 17.5-kb *E. aerogenes* insert and the 10.2-kb *K. pneumoniae* insert hybridized to an origin probe derived from only 417 bp of *oriC* from *S. typhimurium*. (iii) Similar to plasmids containing the *E. coli* and *S. typhimurium* chromosomal origins, both pNH3 and pNH62 were highly unstable in the absence of selective pressure. (iv) The *E. aerogenes* 17.5-kb fragment and the *K. pneumoniae* 10.2-kb fragment contained the *asnA* and *uncB* loci, which bracket *oriC* in *E. coli* and *S. typhimurium*. (v) The origin regions of both *E. aerogenes* and *K. pneumoniae* had extensive but not total conservation of restriction cleavage sites which map in the *oriC* region of members of the *Escherichieae* tribe. (vi) Replication of these plasmids in the *E. coli* cell-free enzyme system (13) was dependent on the presence of *dnaA* protein. Other plasmids tested, such as pBEU17 (37), pSC101, and pMK2004 (20), as well as ϕX174 replicative-form DNA, are inert as templates (13).

One feature of these newly cloned bacterial origins was that plasmids containing either the *E. aerogenes* or *K. pneumoniae oriC Sal*I fragment inserted into pMK2004 did not transform *E. coli polA*$^+$ strains, although these *oriC Sal*I fragments, when linked to a nonreplicating *Sal*I *kan* fragment, did transform *E. coli polA*$^+$ strains. In addition, the plasmid pJM1, which is composed of the *E. coli oriC*-containing *Eco*RI fragment (23, 41) and pMK2004, did not transform *polA*$^+$ strains. This property can be explained if the *E. aerogenes* and *K. pneumoniae Sal*I fragments and the *E. coli Eco*RI fragment carry a region of DNA which is lethal when many copies are present in a cell. Regions to the right of *oriC* have been cloned at high copy number with no lethal effect (35, 45). Although overlapping fragments to the left of *oriC* have been subcloned at high copy number (11, 18, 19, 21, 45), never have both the *unc* promoter and the first four genes of the *unc* operon been cloned together (Fig. 5). A comparison of restriction fragments from *E. coli* and *S. typhimurium* cloned into high-copy-number plasmids (11, 18, 19, 21, 35, 45), together with an analysis of fragments subcloned from the *E. aerogenes* and *K. pneumoniae Sal*I fragments isolated in this study (Table 3), suggests that the lethality is due to a gene dosage effect of one or all of the *unc* genes coding for the proton channel-forming subunits (Fig. 5). The *unc* (10) or *atp* (38) operon contains structural genes for the ATP synthetase complex of *E. coli*. The *uncB*, *-F*, and *-E* genes code for polypeptides a, b, and c, respectively, which together form the membrane proton-translocating channel called F_0 (14, 16), whereas the *uncA*, *-D*, *-G*, *-H*, and *-C* genes code for polypeptides α, β, γ, δ, and ε, respectively, the subunits of the soluble F_1 moiety (18). In agreement with our conclusions, Yamaguchi and Yamaguchi (submitted for publication) have isolated the *Hae*II fragment (2.5 kb) from *E. coli* which contains the *unc* operon promoter and the *uncB* and *E* genes coding for the a and c proteins and have found that this *Hae*II fragment can only be cloned at a reduced copy number. The

deleterious effect of this region was first observed by von Meyenburg et al. (40). When *asn* transducing phages carrying *oriC* and genes coding for the F_0 membrane subunits are established as self-replicating minichromosomes, the result is a threefold reduction in growth rate and an increase in minichromosome instability. von Meyenburg et al. suggested that the presence of additional copies of ATP synthetase subunit genes are responsible for the poor growth rate and increased instability of strains harboring these phages.

The differences in copy number observed for plasmids containing only *oriC* origins probably reflect whether the plasmids carry this HCL region of the *unc* operon. Plasmids containing this region, such as pNH3 (Fig. 2), have a copy number of one to three copies per cell (23, 41, 43). Plasmids missing this region have a copy number of 8 to 10 copies per cell (23, 43). One explanation for this difference in copy number is that the instability of these *oriC*-containing plasmids leads to a heterogeneity in the number of plasmids contained in each cell. When the plasmid contains an HCL gene, only cells having one to three copies of the plasmid are viable. When the HCL gene is missing, cells with more than three copies of the plasmid also survive, accounting for the observed increase in copy number. This could also explain why the copy number appears to increase with mutations in the *unc* promoter and the *uncB* gene (30). We observed only 1 to 5% viability in cultures of cells containing plasmid pNH3 or pNH62 (Fig. 2 and 3), which is consistent with this explanation of copy number differences.

TABLE 3. Plasmids which do and do not contain the HCL region

Plasmid	Source of plasmid[a]	Transforms *E. coli polA*$^+$ strains
pNH3	See Table 2 and Fig. 2	No
pNH326	See Fig. 2	Yes
pNH305	See Fig. 2	Yes
pNH62	See Table 2 and Fig. 3	No
pJC62	10.2-kb *Sal*I fragment of pNH62 ligated to the 5.6-kb nonreplicating Km^r *Sal*I fragment	Yes
pJC627	Self-ligation of the 8.3-kb *Bgl*II fragment of pNH62	Yes
pJC629	Self-ligation of the 13.2-kb *Eco*RI fragment of pNH62	No
pJZ70	See Fig. 3	Yes
pJC132	See Fig. 3	Yes
pJM1	9.0-kb *E. coli Eco*RI fragment of pAL1 (23) ligated to pMK2004	No

[a] See text for further details.

The method used in this study to isolate bacterial origins of replication has revealed that there are sequences which are lethal to *E. coli* when the copy number is high (HCL sequences). One may infer from these results that no clone bank in *E. coli* is complete if the plasmid vector contains only a high-copy-number origin. A complete clone bank in *E. coli*, however, could be obtained with a plasmid such as pJC132 (Fig. 3). This plasmid consists of the *K. pneumoniae oriC*-containing 310-bp *Bam*HI fragment inserted into the *Bam*HI site of pMK2004. Single restriction sites in pJC132 available for cloning are the *Xho*I, *Pst*I, *Sal*I, and *Sma*I sites, and these are in either of the two functional antibiotic resistance genes, *amp* and *kan*. Both *oriC* and ColE1 *rep* origins are present, and therefore, pJC132 can be maintained at a low copy number in *polA* cells and at a high copy number in *polA*$^+$ cells. Clone banks can be constructed in an *E. coli polA* strain so that the whole genome is recovered. When a particular clone of interest has been identified, the plasmid can be isolated and transformed into an *E. coli polA*$^+$ strain. After amplification (2), large amounts of plasmid DNA can be isolated. If the insert is lethal to *E. coli* at a high copy number, low but sufficient amounts of plasmid DNA can still be recovered from the *polA* cells.

The *oriC* regions of *E. aerogenes* and *K. pneumoniae* appear to be completely colinear with the *oriC* regions of *E. coli*. *S. typhimurium*, and *E. carotovora* (36). A comparison of nucleotide differences among these species has been used to define nucleotide sequences which are essential and nonessential for *oriC* function (7, 36, 44), and in the process, a consensus sequence of *oriC* has been compiled (46). The fact that these newly characterized origins function in *E. coli* argues that the mechanisms and gene products involved in initiation of DNA replication, as well as the actual site of initiation, *oriC*, are conserved among these species.

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