The chromosomal origin of replication (*oriC*) of *Erwinia carotovora*

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ABSTRACT

The chromosomal DNA replication origin (*oriC*) of the plant pathogen *Erwinia carotovora* has been isolated and sequenced. The minimal *E. carotovora oriC* region functional in *Escherichia coli* is a 374 base pair region located on a 7.9 kilobase pair *Sall* fragment which also contains a functional *asnA* gene. Differences between the nucleotide sequences of the minimal origin regions of *E. carotovora* and those of *E. coli* and *Salmonella typhimurium* are clustered nucleotide substitutions, with regions of complete homology, up to 19 base pairs long, between the three origins. Nine GATC sites are found in the minimal origin, and all are conserved. In contrast, the region toward *asnA* from the minimal origin shows little clustering and the differences occur mainly every third nucleotide, suggesting that this region is a protein coding region.

INTRODUCTION

Replication of the *Escherichia coli* and *Salmonella typhimurium* chromosomes is initiated at a unique site (*oriC*, origin of the bacterial chromosome) located between the genes *asnA* and *uncB* at 83.5 min on the *E. coli* genetic map (1, 2). The cloned *EcoRI* fragment containing the *E. coli oriC* is 9.0 kilobase pairs (kb) long (3), whereas the *S. typhimurium EcoRI* fragment is 19.4 kb long (2). Both *EcoRI* fragments contain functional *asnA* and *uncB* genes (2, 3; von Meyenburg, personal communication), and the nucleotide sequences of these two *oriC* regions have been determined (4; 5; 6). Within the minimal (7) 245 bp *oriC* region, the sequences of *E. coli* and *S. typhimurium* are 85.3% identical; eleven GATC sites are all conserved; no insertions or deletions are necessary to align the two sequences; and the nucleotide differences are grouped in clusters, with long stretches of complete homology found between these clusters (6).

If nucleotide differences between bacterial origins which function in *E. coli* are not essential for the initiation process, then regions of conservation in *oriC* would define essential regions for initiation, such as binding

sites for required proteins. For this reason, we have extended our study of replication origins to another member of the family Enterobacteriaceae, namely Erwinia carotovora. Among the five subfamilies, or tribes, of the Enterobacteriaceae family (8), E. coli and S. typhimurium are classified in the tribe Escherichieae, while E. carotovora is classified in the tribe, Erwineae. This bacterial pathogen causes soft rotting of plants and has an optimum growth temperature of 30°C. By DNA reassociation studies, it is 28% related to E. coli and 30% to S. typhimurium (9). We show here that plasmids containing oriC from E. carotovora are capable of autonomous replication in E. coli. Comparison of the nucleotide sequences of these three bacterial oriC regions shows a clustering of nucleotide differences, interspersed with regions of homology of up to 19 bp long. The same clusters of nonhomology earlier observed between E. coli and S. typhimurium origin sequences are also observed here. This comparison further suggests that the observed changes in the oriC region have occurred through random substitution of nucleotides.

MATERIALS AND METHODS

Bacterial strains were grown in either YT medium (10) or M9 minimal medium (11) with 0.2% glucose. Antibiotic resistant cells were selected by addition of 50 µg/ml kanamycin (Km, Calbiochem), 15 µg/ml tetracycline (Tc, Calbiochem), or 10 µg/ml ampicillin (Ap, Bristol) to the growth medium. Complementation tests were as described for asnA (12) using E. coli ER (F⁺ asnA asnB thi-1) and for uncB (13) using E. coli AN382 (F⁻ uncB401 argE3 thi-1 mtl-1 xyl-5 galK2 strA704 supE44).

Chromosomal DNA from E. carotovora strain EC153 (14) was isolated from cells grown to late log phase in M9 medium supplemented with 0.05% casamino acids (Difco). Cells were lysed using a lysozyme-sarkosyl procedure (15) with treatment with 0.1% diethylpyrocarbonate (Sigma) at 65°C for 15 min, with 20 µg/ml heat-treated (100°C, 5 min) pancreatic RNase (Worthington) at 37°C for 30 min, and with 500 µg/ml Pronase (Calbiochem-Behring) at 50°C for 1 hr, prior to sarkosyl addition. DNA was isolated via isopycnic ethidium bromide-cesium chloride (dye-cesium) gradient centrifugation. No plasmid DNA band was observed in the dye-cesium gradient of chromosomal DNA from E. carotovora EC153. Plasmid DNA was isolated by a Triton X100-cleared lysate procedure (16) followed by dye-cesium equilibrium centrifugation. Enzymes, reaction conditions, DNA size determinations and purification of DNA fragments were as described (6). Transformation of E. coli (17) and DNA-DNA hybridization (18)

were as described.

For sequence determination, DNA of pJZ55 or pJZ56 was digested as outlined in Fig. 2. The 5' ends were labeled using phage T4 polynucleotide kinase (P-L Biochemicals) and [γ - 32 P]ATP as previously described (19). The DNA fragments subsequently produced by either secondary restriction endonuclease cleavage or melting were separated on acrylamide gels and the labeled bands were eluted and the nucleotide sequence determined (19).

RESULTS AND DISCUSSION

Cloning of the *oriC* Region of *E. carotovora* into Plasmid pMK2004

The 5.19 kb plasmid, pMK2004 (11), possessing three antibiotic resistance genes, *kan*, *amp*, and *tet*, was used as the cloning vehicle. This ColE1 type plasmid requires DNA polymerase I, the product of the *polA* gene, for its replication (20). Plasmids with inserts from *E. carotovora* were selected directly for their ability to confer drug resistance to *E. coli* *polA* mutant cells. In theory, both the *E. carotovora* *polA* gene, as well as *oriC*, could be isolated using this procedure.

A complete *SalI* digest of *E. carotovora* DNA was ligated to pMK2004 previously cleaved at the unique *SalI* site within the *tet* gene and the ligation products were used to transform *E. coli* C2368 (*polA1 thy his rha r_k⁻ m_k⁻*), selecting for Km^r Ap^r colonies. Plasmid DNA was isolated from the twelve Km^r Ap^r colonies obtained and tested for the ability to hybridize to an *S. typhimurium* *oriC* probe (Table 1). One plasmid type consisting of plasmids pNH42, pNH43, pNH44, pNH45, pNH46, pNH47, pNH48, pNH50, and pNH52, hybridized specifically to the *oriC* probe. Restriction endonuclease analysis of some of these plasmids with *SalI* demonstrated that they contain a 7.9 kb fragment in common. Three other *SalI* fragments of 2.6, 0.85, and 0.3 kb are also contained in some of these plasmids, but these fragments may not be contiguous with the 7.9 kb fragment in the chromosome. Plasmids pNH44 and pNH47 were transformed into the *polA*⁺ strain *E. coli* AI214 (*thi pyrE argE his proA leu mtl xyl strA r_k⁻ srl300::Tn300 recA56 F⁻*; ref. 2), for isolation of plasmid DNA. Genetic complementation experiments with these two plasmids showed that both pNH44 and pNH47 carry the *asnA* but not the *uncB* genes. Physical and genetic maps of pNH44 and pNH47 are shown in Fig. 1.

The 7.9 kb *E. carotovora* *SalI* fragment is capable of directing autonomous replication when no other known origin is present. A nonreplicating *SalI* fragment of 5.6 kb containing the *kan* gene from the plasmid pDF11 (11) was

Table 1. Plasmids containing E. carotovora DNA which replicate in an E. coli polA1 strain

Plasmid	Size of cloned <u>E. carotovora</u> <u>SalI</u> fragments (kb)	MMS	Hybridization	Plasmid Loss
pNH41	ND	R	no	7.0
pNH42	7.9, 2.6	S	yes	5.7
pNH43	7.9, 2.6	S	yes	5.0
pNH44	7.9	S	yes	6.0
pNH45	ND	S	yes	2.6
pNH46	7.9, 0.85	S	yes	4.2
pNH47	7.9, 0.3	S	yes	3.1
pNH48	ND	S	yes	3.1
pNH49	ND	R	no	10.2
pNH50	7.9	S	yes	4.7
pNH51	ND	R	no	7.0
pNH52	ND	S	yes	3.1

Abbreviations: MMS, methyl methane sulfonate; R, resistant; S, sensitive; ND, not determined. Hybridization to the S. typhimurium oriC region was shown by Southern blotting analysis (18), using the three oriC region BamHI fragments of the S. typhimurium (6), prepared from plasmid pJZ25, as radioactive probe. Plasmid loss is given as the number of generations for 50% of the cells to lose the plasmid when grown in the absence of the antibiotic.

ligated to the 7.9 kb E. carotovora SalI fragment. The resulting 13.5 kb plasmid, pJC47, replicated in both polA⁺ and polA⁻ E. coli strains and complemented the asnA mutation in E. coli ER. We conclude that the 7.9 kb E. carotovora SalI fragment contains the E. carotovora oriC region in a functional state.

The second plasmid type, consisting of plasmids pNH41, pNH49, and pNH51, did not hybridize to the S. typhimurium oriC probe, and E. coli polA1 cells containing these plasmids are no longer sensitive to methylmethane sulfonate (MMS). The MMS resistance of polA1 cells containing these plasmids suggests that these plasmids possess a polA-like gene from E. carotovora which allows replication of these chimeric plasmids from the ColE1 origin of the pMK2004 cloning vehicle. Cells carrying a polA1 mutation are incapable of normal DNA repair and as a result are more sensitive to MMS than are polA⁺ cells (21).

Fig. 1. Physical and Genetic Map of pNH44 and pNH47 and Derivative Plasmids Containing the Origin of *E. carotovora*. (A) pNH44 and Derivative Plasmids. (B) pNH47 and Derivative Plasmids. Open regions designate the *E. carotovora* chromosomal DNA and hatched regions indicate the cloning vehicle pMK2004. The ColE1 origin of pMK2004 is indicated by rep. The scale is in kilobase pairs.

Both plasmid types isolated in this experiment were unstable, in that they were rapidly lost from cells in the absence of antibiotic selection (Table 1).

Subclones of the *oriC* Region of *E. carotovora*

Plasmids pNH44 and pNH47 each have the *E. carotovora* 7.9 kb SalI fragment containing *oriC* inserted into pMK2004, but the inserts are in opposing orientations. These plasmids were digested with either XhoI or PstI, self-ligated, and the preparations used to transform *E. coli* AI214, selecting for either Ap^r or Km^r colonies. Restriction endonuclease analysis of these plasmids along with the results of assays for a functional bacterial origin (*oriC*) or the *asnA* gene were used to determine a more precise location of *oriC* and *asnA* in the 7.9 kb SalI fragment (Fig. 1). The deletion plasmid, pJZ55, which consists of the smaller XhoI fragment of pNH47, had *oriC* function but not a functional *asnA* gene. There is approximately 1.0 kb of the *E. carotovora* chromosome contained in this plasmid. However, the 0.3 kb SalI fragment included in this 1.0 kb piece is not required for *oriC* function since it is missing in

pNH44 (Table 1). Therefore, pNH55 limits the *E. carotovora* *oriC* region to about 700 bp from the *Sal*I to the *Xho*I site. The largest *Bam*HI fragment of pNH44 was cloned into the *Bam*HI site of pMK2004 and the resulting plasmid, pJZ68 (map not shown), contained a functional *oriC* origin and *asnA* gene. This plasmid further limits the *E. carotovora* origin to 374 bp between the *Bam*HI and *Xho*I sites.

Determination of the Nucleotide Sequence of the *E. carotovora* *oriC* Region

Detailed restriction maps of the *E. coli* and *E. carotovora* *oriC* regions are shown in Fig. 2. Very few restriction sites are conserved between the two species both inside and outside of the minimal origin of *E. carotovora*. However, some of those sites conserved between *E. coli* and *S. typhimurium*, notably *Bam*HI and *Bgl*II sites in the *oriC* region, are also conserved in *E. carotovora*.

The strategy for determining the nucleotide sequence of the *E. carotovora* origin is also shown in Fig. 2, including the restriction fragments used and the extent of sequence determined for each fragment. The restriction fragments were isolated from pJZ55 and pJZ56 (Fig. 1). The 374 bp origin-containing *E. carotovora* *Bam*HI-*Xho*I fragment also includes the minimal origin sequence as defined in *E. coli*.

The entire sequence for both strands was determined, and the sequence of all internal restriction sites were resolved by overlapping fragments in order

Fig. 2. Comparison of Restriction Sites of the *E. coli* and *E. carotovora* Origin Regions and Strategies for Sequence Determination of the *E. carotovora* Origin Region. Solid regions designate bacterial DNA, and open regions designate the cloning vehicle pMK2004. The scale, in basepairs, refers to the *E. coli* sequence. Tail of arrows: 32 P-labeled end of each fragment. Head of arrow: extent to which sequence was determined from that fragment. The minimal *oriC* regions yet determined for each organism are indicated.

to eliminate the possibility of missing small fragments. The nucleotide sequence of the E. carotovora oriC region is compared to the nucleotide sequences of the oriC regions from E. coli (4; 5; W. J. Buhk, personal communication) and S. typhimurium (6; D. Smith, unpublished data) in Fig. 3.

The minimal size for function of the E. coli oriC region is between 237 and 245 bp (7; Fig. 3), and this sequence is 67.7% homologous to a 245 bp sequence found within the E. carotovora oriC 374 bp region. Although this degree of homology is less than that found between E. coli and S. typhimurium (85.3%), the nucleotide differences are grouped in clusters similar to those found between E. coli and S. typhimurium (6), with regions of up to 19 bp completely conserved between all three species. Further, the clusters of differences between any of the three pairwise comparisons of these species overlap each other. Only two "deletions" in the E. carotovora sequence are required to completely align the three sequences, and these "deletions" are found in a region which permits extensive intrastrand secondary structure, in part due to the presence of four conserved, nearly equally spaced GATC sequences (region 23 to 70). The regions forming the stem of a proposed skewed "cloverleaf" structure (positions 80 to 88 and 186 to 194) which was based on a comparison of the E. coli and S. typhimurium sequences (6) are completely conserved, and found bracketed by regions of extensive nonhomology. One arm (region 220 to 228) of the potential hairpin structure previously discussed (6; 22; 23; 24) is nearly completely conserved, but the other arm (region 235 to 243) is not. However, a perfect inverted repeat of the conserved arm is found between positions 255 and 263.

The E. carotovora minimal origin contains nine GATC sites, all of which are completely conserved between these three origins, and an additional ten GATC sites are found to either side of the minimal origin. The increased density of these sequences in the oriC region may account for the high degree of conservation of the sequences in this region between these bacterial organisms, as previously proposed (6).

Over twice as many changes are found between the E. carotovora minimal oriC region and either that of E. coli or of S. typhimurium as is found between the E. coli and S. typhimurium minimal oriC region (see Table 2). Of these, there are twice as many "transversions" as "transitions", unlike the equal number of transition- and transversion-type differences between the E. coli and S. typhimurium origin sequences (Table 2). Whereas there are nearly equal numbers of each of the four types of transversions, there are twice as many A,T to G,C transitions as G,C to A,T changes, again unlike the equal

Table 2. Evolutionary Rate Calculations

Compared Sequences	n	n _p	n _q	n _{p+n_q}	Percent Diverg.	K(P,Q)	(P,Q)
<u>E.coli-S.typh</u>	245	18	18	36	14.7%	0.164	0.028
<u>E.coli-E.caro</u>	245	22	52	74	30.2%	0.387	0.049
<u>S.typh-E.caro</u>	245	26	52	78	31.8%	0.414	0.052

n is the number of nucleotide base pairs in the region compared, n_p is the number of transition-type differences, n_q is the number of transversion-type differences, n_{p+n_q} is the total number of nucleotide differences, K(P,Q) is the evolutionary distance between each pair of organisms per site, and (P,Q) is the standard error in K.

number of such changes between E. coli and S. typhimurium. These inequalities account for the differences in A,T-content in the minimal oriC region between these organisms (63.0% for E. carotovora, 59.5% for S. typhimurium, and 58.8% for E. coli). The A,T-content of the regions on either side of the minimal origin in E. carotovora are close to 48.3%, the average A,T-content of the chromosome of E. carotovora EC153 (25).

The nucleotide differences in the "minus" region (positions -138 to -1) are also clustered, suggesting that this region may serve as a regulatory site(s). This region in S. typhimurium contains a promoter for counterclockwise (leftward) transcription (J. W. Zyskind, unpublished data). A unique feature of this region in E. carotovora is the presence of several "insertions" relative to E. coli and S. typhimurium, including one of 11 bp, required to maximally align these sequences.

In contrast, the region clockwise from the minimal origin is characterized by a nonclustering of nucleotide differences, a preference for a difference every third nucleotide (55% to 75% of all changes), and the absence of any relative deletions required for alignment of these sequences. These properties suggest that this region codes for a protein, although in E. coli it may be required for bidirectional replication from oriC (24) and appears to bind a membrane protein B' (26) which exhibits specificity for the oriC (27). The relevant amino acid reading frame, when translated toward the minimal origin, contains a single conserved stop codon, TAA, at position 291, and a 16 kilodalton protein is synthesized in vivo from plasmids carrying only this region of the E. coli chromosome (F. G. Hansen, S. Koefoed, K. von Meyenburg, and T. Atlung, in "Structure and DNA-Protein Interactions of Replication Ori-

gins", ICN-UCLA Symposia on Molecular and Cellular Biology, vol. XXI, in press). The absence of stop codons in this reading frame, together with the above comparative properties, suggest that E. coli, S. typhimurium, and E. carotovora each code for a similar protein.

Comparison of nucleotide sequences for functional regions permits calculations of evolutionary rates between these species. To date, this has been done only for genes coding for specific proteins such as the trpA genes of E. coli and S. typhimurium (28), for 5S rRNA species (29) and 16S rRNA species (30), and for transcriptional control regions such as the trp operon operator-promoter region (31). The method of Kimura (32), which assumes nucleotide changes to be neutral in evolution, separately accounts for transition-type and transversion-type differences. Assuming equal probability of substitution of any base at a given site, twice as many transversion-type differences would be expected as transition-type differences. Kimura's method can be applied to both coding and noncoding sequences, and is applied here to the minimal oriC region. Two parameters, P, the fraction of nucleotides showing transition-type differences, and Q, the fraction of nucleotides showing transversion-type differences, are used to calculate K, the evolutionary distance (total number of substitutions) per site (Table 2). Comparison of these values shows unambiguously that E. coli and S. typhimurium are more closely related than is either to E. carotovora, consistent with the Bergey classification of these three genera (8). In the phylogenetic tree obtained from the K values (Fig. 4), E. coli and S. typhimurium have diverged from a common

Fig. 4. Phylogenetic Trees Determined from the Minimal Origin. The sum of the branching values given between any two species gives the best-fit value for the $K \times 1000$ value between these two species. The point of divergence of E. coli and S. typhimurium from E is the average of the two branching values given. The branching values for E and E. carotovora from B are in parentheses, since only their sum can be determined from the $K \times 1000$ values; this sum has been arbitrarily divided to give a common point in time for divergence of E and E. carotovora from B.

Escherichieae tribe ancestor, called E, and the ancestor E and E. carotovora have diverged from a common Enterobacteriaceae ancestor, called N. Since K is a measure of rate of nucleotide change for a constant time, eg. the time since E. coli and S. typhimurium diverged from E, or conversely is a measure of the time since divergence assuming a constant rate of evolutionary change, the branching values shown in Fig. 4 as calculated from the K values (Table 2) suggest that the rate of nucleotide change has been slower in E. coli than in S. typhimurium for the minimal origin since these two species diverged from ancestor E. Analysis of the distribution of nucleotide differences for the minimal oriC region using the approach of Nichols and Yanofsky (28) for the trpA genes of E. coli and S. typhimurium shows that the differences between E. coli and E. carotovora, and between S. typhimurium and E. carotovora, are statistically consistent with a random distribution of differences if, and only if, the E. carotovora sequence is considered to be the more ancestral of the pairwise compared sequences. This result is consistent with the notion that nucleotide differences between bacterial origins functional in E. coli are not essential for the initiation process. Extension of such analysis of this interesting region to other related bacteria, with improved analysis of more extensive sequence data, is a promising approach for evolutionary studies of these organisms.

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