Pseudomonas chromosomal replication origins: A bacterial class distinct from Escherichia coli-type origins

(initiation/DnaA-binding sites/GATC sites/13-base-pair repeats/autonomously replicating sequence/dnaA-dnaN-rpml-rnpA genes)

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Communicated by Donald R. Henski, October 13, 1989 (received for review June 12, 1989)

ABSTRACT The bacterial origins of DNA replication have been isolated from Pseudomonas aeruginosa and Pseudomonas putida. These origins comprise a second class of bacterial origins distinct from enteric-type origins: both origins function in both Pseudomonas species, and neither functions in Escherichia coli; enteric origins do not function in either pseudomonad. Both cloned sequences hybridize to chromosomal fragments that show properties expected of replication origins. These origin plasmids are highly unstable, are present at low copy number, and show mutual incompatibility properties. DNA sequence analysis shows that both origins contain several 9-base-pair (bp) E. coli DnaA protein binding sites; four of these are conserved in position and orientation, two of which resemble the R1 and R4 sites of the E. coli origin. Conserved 13-bp direct repeats adjacent to the analogous R1 site are also found. No GATC sites are in the P. aeruginosa origin and only four are in the P. putida origin; no other 4-bp sequence is present in high abundance. Both origins are found between sequences similar to the E. coli and Bacillus subtilis dnaA, dnaN, rpmH, and rnpA genes, a gene organization identical to that for B. subtilis and unlike that of E. coli. A second autonomously replicating sequence was obtained from P. aeruginosa that has some properties of bacterial origins.

Central to the process regulating prokaryotic DNA replication are the events that occur at the chromosomal replication origin, oriC. The oriC regions of Escherichia coli and other enteric species have been isolated as autonomous replicons (1, 2), functional as replication origins in E. coli. The oriC region of the gram-positive bacterium Bacillus subtilis has been identified and analyzed (3-7). Enteric bacterial origins differ from each other primarily in base substitutions that preserve orientation and spacing of the conserved elements (for a review, see ref. 8). Within the minimal origin [~245 base pairs (bp)], five 9-bp direct and inverted repeats, the “R sites,” show sequence conservation and identical spacing and orientation (2, 8) and are required for the binding of DnaA protein to DNA (9, 10). DnaA protein also mediates strand opening at three conserved 13-bp direct repeats, prior to assembly of a “prepriming complex” (11). Each of the analyzed enteric minimal origins contains 9-14 GATC sites, and eight are positionally conserved (2).

This conservation of GATC sequence, the recognition site for the dam gene product, a DNA adenine methylase (12), implies an important functional role for Dam methylation in initiation. Evidence indicates a role for Dam methylation in timing between initiation events (13-17) and in segregation of daughter chromosomes (18); however, GATC function in mismatch repair (for a review, see ref. 19) is different from its function in initiation (8, 16, 17).

Nevertheless, viable dam- insertion and deletion mutants of E. coli have been isolated (20, 21); hence, GATC methylation is a dispensable cellular function. Dam methylation appeared quite recently in the E. coli lineage (22). How similar or different are initiation and the origin in bacteria that have no Dam methylation system? This question is examined here for two such bacteria (23), Pseudomonas aeruginosa and Pseudomonas putida. We find that isolated pseudomonad origins function in both P. aeruginosa and in P. putida, but not in E. coli, and the enteric-type origin does not function as an origin in P. aeruginosa. This pseudomonad origin class is then the second class of bacterial origins analyzed in detail. The P. aeruginosa origin contains no GATC sites and the P. putida origin contains only four. Thus, the abundance of GATC sites in the origins of enteric bacteria is indeed related to a fundamental (although dispensable) function of Dam methylation in the normal initiation process.

MATERIALS AND METHODS

Bacterial Strains and General Procedures. P. aeruginosa PA02005 argH- recA- (24) and P. putida KT2440 r- m- (25) were used as sources of chromosomal DNA and for cloning. E. coli K-12 strains were: LE392 hasR514 supE44 supF58 lacY1 galK2 galT22 metB1 trpR55 F’ (26), JM101 Δ(lac-pro) supE thi/F’ traD36 proAB lacY1 Δ(lacZ)ΔM15 (27), JM83 ara Δ(lac-pro) proS L thi [δ80 Δ(lacZ)M15] (28), and JZ294 polA1 argH hasR rpsL thyA36 (29). Pseudomonas growth, plasmid isolation, and transformation were as described (30). Antibiotics used were tetracycline (100 μg/ml) for P. aeruginosa and P. putida and carbenicillin (500 μg/ml) for P. putida. E. coli growth and transformation, stability measurements, and recombinant DNA methodologies were essentially as described (31). Nested deletion series fragments for dieoxy DNA sequence determination were generated as described (32), and the two-dimensional method of Brewer and Fangman (33) was used to probe Pseudomonas chromosomal DNA with cloned putative origins.

Copy Number Determination. The Yee and Inouye two-dimensional DNA fingerprint protocol (34) was modified to a horizontal gel format with thin gels and by use of simultaneous in situ digestion with the second enzyme of the unseparated adjacent first-dimension lanes sealed in a Ziploc bag (Dow). Copy number per chromosome was estimated by comparing plasmid mass (easily identifiable) with differing molecular weight chromosomal fragments by using an LKB Ultrascan densitometer.

RESULTS

Origin Cloning and Subclone Isolation. Complete Pst I, EcoRI, and HindIII digests of P. aeruginosa or P. putida chromosomal digests were ligated with pBR322 linearized

Abbreviations: ORF, open reading frame; ARS, autonomously replicating sequence.

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with the corresponding restriction enzyme and transformed into \textit{E. coli} LE392. Plasmid DNA was isolated from pooled colonies, and competent \textit{P. aeruginosa} and \textit{P. putida} cells were transformed. Inability of the narrow-host-range pBR322 origin to function in pseudomonads (25) was used to select \textit{Pseudomonas} autonomously replicating sequences (ARS). Of 10 \textit{P. aeruginosa} transformants, all isolated ARS fragments overlapped with one of two \textit{Pst I} inserts called pae301 and pae310 (Fig. 1; in plasmids pTWY301 and pTWY310 \textit{tet}). Although a \textit{P. putida recA} strain was unavailable, an EcoRI ARS fragment called ppu332 (Fig. 1; in plasmid pTWY332 \textit{tet}) was isolated. This ppu332 insert showed sequence similarity (demonstrated by Southern hybridization) with the pae310 insert (data not shown).

No tetracycline-resistant colonies were obtained by transformation of \textit{E. coli} JZ294 (29), a \textit{polA} strain in which the pBR322 origin is nonfunctional (35), with pTWY301, pTWY310, pTWY332, or pBR322, whereas all of these plasmids transformed the isogenic \textit{polA} parent of JZ294, LE392, at similar rates. The broad-host-range vector pMW79 (36) transformed both \textit{E. coli} hosts equally well, as did the enteric \textit{oriC} plasmids pJZ19 (37) and pJZ70 (29). Thus, the cloned \textit{Pseudomonas} sequences, although not detrimental to \textit{E. coli} viability, do not function as origins in \textit{E. coli}. Conversely, the two enteric \textit{oriC} plasmids failed to transform either \textit{P. aeruginosa} or \textit{P. putida}, whereas the three \textit{Pseudomonas}-origin plasmids transformed both species at rates comparable to that observed for pMW79 (36). Thus, \textit{Pseudomonas} candidate \textit{oriC} plasmids are nonfunctional in \textit{E. coli}, and \textit{E. coli}-type \textit{oriC} plasmids are nonfunctional in these two pseudomonads, as observed (38).

To determine the minimal DNA sequences required for origin function, deletion derivative plasmids (Fig. 1) were constructed by partial restriction with \textit{Hae III}, addition of linkers, and insertion into pBR322. The minimum-size derivative so obtained for pTWY332 spanned \textit{Sal I}, \textit{HindIII}, and \textit{BamHI} sites. Functional testing of subclones of the \textit{Sal I}, \textit{BamHI} and \textit{HindIII–BamHI} fragments showed that the \textit{Sal I}–\textit{BamHI} fragment, but not the \textit{HindIII–BamHI} fragment, contained a functional origin. Transformation experiments showed that all tested plasmids carrying functional subclones of pae310 or ppu332 were able to replicate within both \textit{P. aeruginosa} and \textit{P. putida} but not within \textit{E. coli} JZ294. Sizes


![Fig. 2. Two-dimensional gel analysis of Pseudomonas-origin candidates. (A–I) Predicted streak patterns for different types of branched DNA molecules (33, 39). (J) \textit{Pst I}-digested \textit{P. aeruginosa} DNA hybridized with the pae310 \textit{Pst I} fragment. (K) \textit{EcoRI}-digested \textit{P. putida} DNA hybridized with the ppu332 \textit{EcoRI} fragment. (L) \textit{EcoRI}-digested \textit{P. aeruginosa} DNA hybridized with the 1.2-kb pae310 \textit{EcoRI} fragment. (M) \textit{Pst I}-digested \textit{P. aeruginosa} DNA hybridized with the pae301 \textit{Pst I} fragment.](image2)
of the minimal origins so obtained were 800 bp for pae301, 502 bp for pae310, and 657 bp for ppu332.

Stability, Incompatibility, and Copy Number of Pseudomonas-Origin Plasmids. In the absence of selection, all tested Pseudomonas-origin plasmids were highly unstable. Even when plated directly from selective medium, about 40% of P. aeruginosa cells initially containing pTWY310 and 12% of P. putida cells initially containing pTWY332 were tetracycline sensitive. Decrease of plasmid-bearing cells in nonselective medium occurred at a rate of 10–12% per generation for both cases and at a rate of 15–20% for either pTWY470 in P. aeruginosa or pTWY451 in P. putida. These rates are comparable to those observed for enteric origin plasmids in E. coli (31).

Incompatibility (Table 1) was examined via transformation interference (lowered transformation by an incoming plasmid due to a resident plasmid). Plasmids showed moderately strong interference with each other and with themselves. Transformation efficiencies were depressed from 4-fold to more than 25-fold in all cases except for pTWY461 as resident plasmid. Thus, all three Pseudomonas-origin plasmids exhibit mutual incompatibility, independent of the source species.

Two-dimensional DNA fingerprinting was used to determine plasmid copy numbers; these were all low (Table 1). Some correlation is seen between size-of-origin-region present, copy number, level of incompatibility, and plasmid stability. In general, the lowest copy-number plasmids are also the least stable.

Identity of the Chromosome Origin with the Cloned Origins. In the two-dimensional gel method of Brewer and Fangman (33), branched molecules show retarded mobility in the second, vertical dimension (shape separation) compared with the first, horizontal dimension (mass separation). When probed, these generate streak patterns characteristic of the type of branched molecule (Fig. 2 A–I). Pseudomonas chromosomal DNA from balanced-growth cultures was probed with the cloned ARS DNA. Because of unit copy of chromosomal sequences, attainable signal-to-noise ratios were relatively poor. The 1.2-kb EcoRI fragment to the right of the minimal pae310 origin (Fig. 1) provided a nonorign sequence control. A simple arc is observed (Fig. 2L), arguing that this

A. P. aeruginosa Autonomously Replicating Sequence:

B. P. aeruginosa Origin of DNA Replication:

C. P. putida Origin of DNA Replication:

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**Fig. 3.** Pseudomonas-origin DNA sequences. (A) The pae301 origin. Numbering is relative to the EcoRI site. (B) The pae310 origin. The minimal Hae III origin is enclosed. (C) The ppu332 origin. The enclosed origin is based on the Sal I–BamHI minimal ppu332 origin and the Hae III minimal pae310 origin. N-terminal ends of the dnaA and rpmH genes are shown. Numbering is relative to the Sal I site. Putative DnaA binding sites are enclosed, probable R1 and R4 sites are so labeled, and 13-bp direct repeats are underlined. GenBank (40) accession numbers: M30124 (A), M30125 (B), and M30126 (C).
fragment is an “ordinary” fragment (Fig. 2A). In contrast, the pae310 Pst I fragment as probe yielded a pattern of retarded streaks characteristic of growing replication bubbles converting to forks (Fig. 2J). The presumptive pae310 origin region is closely centered within the Pst I fragment; thus, the streak of forks should be very short (see Fig. 2C). This appears to be the case (Fig. 2J). However, there is an abundance of molecules characteristic of intermediates containing short bubble regions, and the number of molecules with bubbles nearly the size of the complete Pst I-fragment is very small. Similarly, the P. putida ppu332 EcoRI fragment as probe yielded a discontinuous streak pattern characteristic of a functioning origin (Fig. 2K). Probing with the P. aeruginosa pae301 Pst I fragment yielded puzzling results (Fig. 2M), somewhat suggestive of a replication terminus (Fig. 2B). This pattern is neither that of a typical origin-carrying fragment nor that for an ordinary chromosomal fragment.

DNA Sequence Determination. Using deletion-derivative origin plasmids (see Fig. 1) as DNA sources, nucleotide sequences were determined for both strands and are shown in Fig. 3.

**DISCUSSION**

Two distinct DNA sequences were isolated from *P. aeruginosa* PAO2003 and one from *P. putida* KT2440 that are functional origins in each *Pseudomonas* species but not in *E. coli*. The following evidence argues that the *P. aeruginosa* pae310 sequence and the *P. putida* ppu332 sequence are the true bacterial replication origins. (i) These sequences both hybridize to chromosomal DNA sequences that show properties expected of origins of DNA replication (Fig. 2J and K); this is not true of the pae301 sequence (Fig. 2M). The profile observed for an “ordinary” DNA fragment (Fig. 2L) is decidedly different from those obtained using the three putative origin fragments. None of the profiles is like that expected for unidirectional initiation events (see Fig. 2 E–G).

(ii) Sequences of both minimal origin regions show definitive homology to the *E. coli* and *B. subtilis* rpmH and rnpA genes to one side and to the *E. coli* and *B. subtilis* dnaA and dnaN genes on the other side (T.W.Y., unpublished data). The *P. putida* dnaA-like and upstream sequences agree completely with those found by Fujita et al. (41). These upstream sequences contain the *P. putida* origin region, but origin function was not demonstrated (41). Thus, chromosomal bracket these origins, and these genes are those expected for a genetic organization similar to that of *B. subtilis* (5). (iii) Each origin contains three 13-mer direct repeats and several 9-bp DnaA binding sites. Two of these DnaA sites have the same spacing and orientation of the R1 and R4 sites in the enteric origin, and the position of the 13-mer direct repeats relative to R1 is the same as for the enteric origins (Figs. 3 and 4). Transformation using isogenic strains and positive and negative control plasmids demonstrated that these origins function as origins in both *Pseudomonas* species and not in *E. coli*, and that the enteric bacterial origin is nonfunctional in *P. aeruginosa*. The *Pseudomonas* origins thus comprise a class of bacterial origins functionally different from the enteric-origin class.

The bacterial origin has not yet been isolated from *B. subtilis* as an autonomously replicating sequence. However, three regions are likely candidates. The first two, rich in the 9-bp DnaA binding sequence, are found between the *B. subtilis* dnaA-like and rpmH-like open reading frames (ORFs) and between the dnaA-like and dnaN-like ORFs (5). Hybridization experiments with initially replicating fragments from germinating spores favor the latter as the origin (3), although recent mutant studies indicate strong incompatibility between the first region and chromosomal initiation (7). This incompatibility is overcome by mutations in the 9-bp DnaA binding sites (7). In contrast, similar hybridization experiments with *B. subtilis* dnaB37 cells suggest that initiation begins 10 kb downstream toward the *rrnO* operon (4), and it may be that *B. subtilis* uses two closely linked origins (3, 6). The *E. coli*-type and pseudomonad-type origins are to date the only two classes of bacterial origins precisely located functionally and physically.

The pae301 origin contains only two 13-mer repeats, and none of its DnaA sites show nine of nine homology to the DnaA consensus sequence (Fig. 3). Further, pae301 sequences show no significant similarity to any GenBank (40) DNA sequences. Thus, although this origin shows more features of bacterial origins than do bacteriophage or plasmid origins, the pae301 sequence is the likely chromosomal origin.

Common features of the pseudomonad origin are seen in a consensus sequence (Fig. 4), obtained with the PIR align program (42). The pae310 *Hae* III minimal origin includes part of the rpmH-like ORF (Fig. 3B), whereas the ppu332 *Sal* I-*Bam*II minimal origin includes part of the dnaA-like ORF (Fig. 3C). No known bacterial origins contain coding sequences. A 73-bp insertion in the ppu332 origin relative to the pae310 origin is an unusual feature. This insertion shows no significant similarity to GenBank sequences and is unlikely to

![Fig. 4. Pseudomonas-origin sequence comparisons. In the consensus sequence, capital letters indicate identical nucleotides in both sequences; no letter indicates different nucleotides in the two origins, and dashes indicate a relative deletion. Sequences within the boxed minimal pseudomonad origin (ppu332 Sal I site to pae310 *Hae* III site) are shown; the probable true minimal origin, based on the positions of 13-mers (underlined), DnaA sites (boxed), the 73-bp relative insertion, and the enteric bacteria origin is enclosed. Other features are as in Fig. 3. Numbering is relative to the Sal I site in the *P. putida* sequence.](image)
be in the true minimal origin. The probable true minimal origin is boxed in Fig. 4. Four of the eight 9-bp DnaA sites of the ppu332 origin align perfectly with each of the four present in the pae310 origin (Fig. 4; boxed sites), as well as to four found in the pae301 origin.

These origin regions are A+T-rich (48%, 51%, and 46% for pae310, ppu332, and pae301, respectively, versus 35% for the pseudomonad chromosome), and all three origins abound in potential inverted repeat structures. The pae310 minimal origin contains no GATC sites and there are only four in each of the ppu332 and pae301 minimal origin regions; no other 4-bp sequence is present in unusually high abundance. The most abundant are the nonpositionally conserved pairs of sequences GAAA/TTTC and AAAG/CTTT, found 11/7 and 9/9 times in the pae310 and ppu332 origin regions. No methylation process comparable to enteric GATC methylation is known for the pseudomonads (22, 23).

Three 13-bp direct repeats in the E. coli origin provide the site to open oriC duplex DNA for entry of DnaB helicase to form a "prepriming complex" (11). Similar direct repeats are found in other enteric origins (2) as well as in the B. subtilis origin region (5). One of two sets of positionally conserved direct repeats in the pseudomonad-origin regions (Fig. 4) may serve a similar initiation function. The first set (positions -70, -31, and 37) is highly conserved between the two origins but is outside the minimal Sal1-BamHI ppu332 origin and includes the 73-bp relative insertion in the P. putida origin sequence. The second set (positions 156, 180, and 205; underlined in Figs. 3 and 4) is a highly A+T-rich region, and the spacing between these repeats and to the DnaA sites mimics those of the enteric origins (8, 11).

Fundamental features of any bacterial origin likely then include: (i) presence of at least four 9-bp DnaA sites; (ii) spacing of ~180 bp between two of these (R1 and R4), which are inverted repeats; and (iii) presence of three or more 13- to 16-bp A+T-rich direct repeats adjacent to the R1 site. Spacing between the 13-bp repeats and between the DnaA sites, plus sequence differences and DNA modification mechanisms, may then provide the host specificity for each class.

We thank B. J. Brewer for detailed protocols and preprints, and D. O. Wood for plasmid pMW79. This work was supported by National Institutes of Health Grant GM31839 to D.W.S.