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Characterization of a Phage-Plasmid Hybrid (Phasyl) with Two Independent Origins of Replication Isolated from *Escherichia coli*

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The phage-plasmid hybrid phasyl can replicate as a phage in the presence of a filamentous phage of *Escherichia coli* (M13, f1, fd). The extragenic region of phasyl shows homology with the plus and the minus origins of filamentous phages. Insertion of a Cm^r fragment into the plus origin or of a Km^r fragment into the minus origin resulted in a reduced transduction frequency, while insertion into other parts of the extragenic region did not. This suggests that phagelike replication of phasyl is mediated by an origin that coincides with the two homologous elements in the extragenic region. Autonomous replication of phasyl occurs from a second origin (*oriA*) that is located between positions 297 and 636. This fragment mediates replication if the Arp protein is supplied in *trans*. Arp is the only phage-encoded protein and is essential for plasmidlike replication. No sequence homology to other known origins was found. Phasyl derivatives with either one of the two origins inactivated can be rescued via the alternative replication mode, suggesting that the two replication pathways are independent.

Phasyl is the smallest known naturally occurring replicon in *Escherichia coli* (1,282 bp) (Fig. 1) (25). It is a phage-plasmid hybrid (phasmid). For autonomous replication, the Arp protein (autonomous replication of phasyl) is essential. The Arp protein is the only gene product expressed by phasyl known so far. Any manipulation in this reading frame leads to the loss of autonomous replication capability (25). In the presence of a filamentous phage (M13, fd, f1), phasyl can replicate via the phage mode. Single-stranded DNA (ss-DNA) is packaged, and phage particles are secreted.

The 424-bp extragenic region of phasyl shows two elements which are homologous by sequence and potential secondary structure with the origins for viral (plus)- and complementary (minus)-strand DNA synthesis of filamentous *E. coli* phages (Fig. 2). However, the order of plus- and minus-strand origin is reversed in phasyl when compared with the filamentous phages, and the degree of homology is lower. Also, the morphogenetic signal (domain A) and the A+T-rich enhancer region (domain F), which supposedly are essential features in the intergenic region of filamentous phages, are missing (4, 5, 10, 16, 25, 29).

It was reported previously that gene II of phage fd is sufficient for replication of plasmids carrying the intergenic region of phage fd (20). Derivatives of phasyl which were defective in the *arp* gene could replicate in a strain carrying the gene II product, but the *arp* protein did not complement gene II mutants of phage fd. Also, the *rep* helicase, which is required for the rolling circle replication of single-stranded phages (6), is not essential for autonomous replication of phasyl (25).

From all these arguments, it appeared that two distinct mechanisms of replication are involved during the growth of phasyl as a plasmid or as defective phage. However, it was not known whether the identified origin in the extragenic region, which is similar to that of filamentous phages, is involved in both replication types or whether autonomous replication is initiated from a second, independent origin. In this report, we provide evidence for the existence of independent origins for phage and plasmid replication. We investigated the two different origins of replication by constructing insertions or deletions to characterize their function, size, and location.

MATERIALS AND METHODS

Bacterial strains, phages, and plasmids. Strains used in this study were C600 (18), JM103 (19), and H411 (C600 F′ phage fd gene II) (8). Phage fd109 was described previously (14). The sequence of phasyl was published by Seufert et al. (25). Phasyl derivatives used in this report are shown in Table 1. Plasmid pNON11 is a derivative of pT7-7 (26) with the following modifications. A fragment from positions 76 to 448 is replaced by a 24-bp synthetic linker with an *Xmn*I site. The *Aat*II and *Nsp*I sites at positions 1468 and 635 are mutagenized to *Not*I sites, and the *Hin*dIII site at position 31 is replaced by an *Eco*RV site. pRME1 contains the *Hae*II fragment coding for kanamycin resistance (Km^r) from Tn*903* flanked at both sides by the M13mp19 multicloning site inserted into pBR322 digested with *Hin*dIII.

Cloning procedures and standard techniques. Standard protocols (18) and manufacturer's instructions were followed for preparations of plasmid and phage DNA, restriction enzyme digestion, treatment with calf intestinal phosphatase and Klenow polymerase, ligation, transformation, and agarose gel electrophoresis.

Copy numbers of recombinant clones were estimated by comparing the amounts of DNA obtained for plasmid preparations (2) on agarose gels.

Superinfection with phage fd109. Strain JM103 was transformed with Km^r derivatives of phasyl. Overnight cultures were diluted 1:1,000. After growth in selective medium to 10^7 cells per ml, cultures were infected with 10^8 fd109 phage (Ap$^+$) and grown for 3 h. Phage were separated from cells by centrifugation and treatment with $CHCl_3$. An exponential culture of strain JM103 was incubated for 30 min with the

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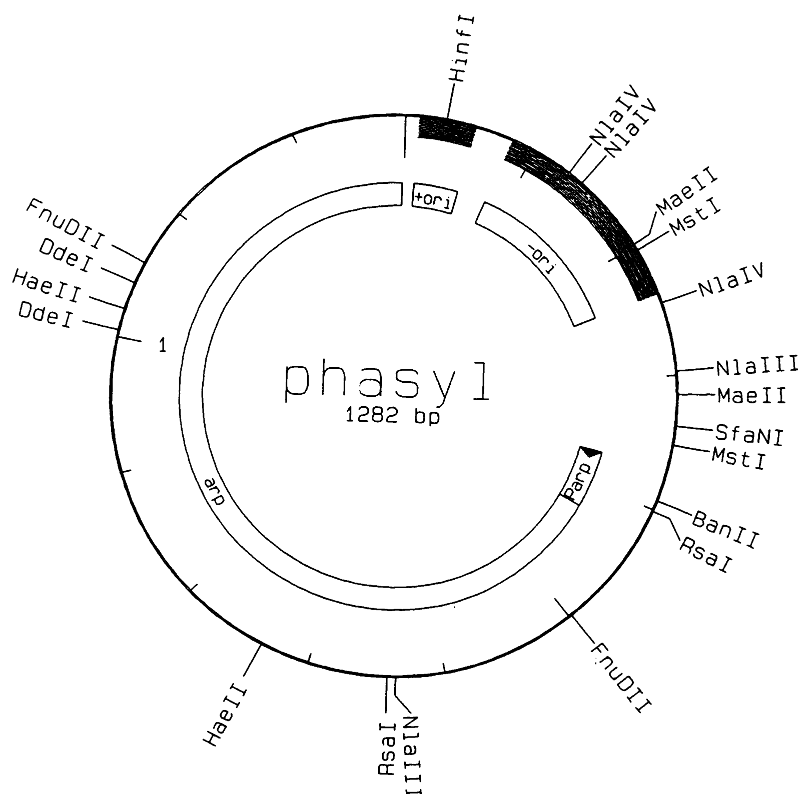

FIG. 1. Plasmid map of phasyl. Extragenic region with plus (+)- and minus (−)-strand origin, promoter, and reading frame for the Arp protein; restriction sites relevant for this report are indicated.

isolated phage. Dilutions were plated on kanamycin-containing plates and on ampicillin-containing plates. Numbers of resistant colonies were counted, and the number of transducing phage particles was calculated.

Nucleotide sequence accession number. The nucleotide sequence accession number for the sequence of phasyl is X56069 of the EMBL DNA library.

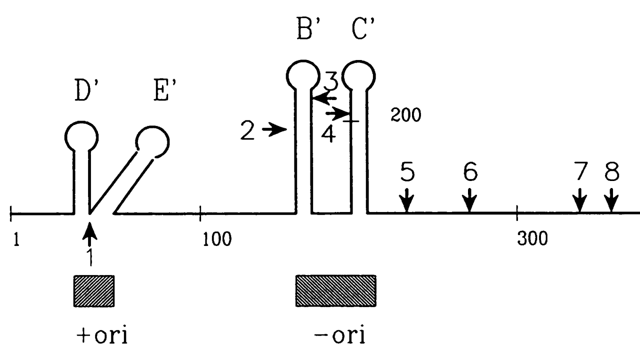

FIG. 2. Potential secondary structure of the extragenic region of phasyl. Loops D' and E' specify the region homologous to the core region of the viral strand origin of filamentous *E. coli* phages, and loops B' and C' specify the element homologous to the complementary strand origin. The order of both elements is reversed with respect to M13, and the enhancer region (domain F) of the viral strand origin and the morphogenetic signal (domain A) are missing (25). Numbers in boldface type indicate the positions of insertion of the Km^r and the Cm^r fragment: 1, pKH1, pCH1; 2, pKN41; 3, pKN43; 4, pKM4; 5, pKN46; 6, pKN31; 7, pKM2; 8, pKS1 (Tables 1 and 2).

RESULTS

Insertional mutation in extragenic region of phasyl. To investigate the extragenic region of phasyl, we inserted a 1,500-bp Km^r fragment from Tn*903* (24) (Km^r fragment isolated from pRME1) into different sites within the extragenic region after partial restriction of phasyl with suitable enzymes. Three insertions were located in the minus-strand origin (insertion 2, pKN41, positions 129 and 130; insertion 3, pKN43, position 141; insertion 4, pKM4, position 199) (Fig. 2), and a fourth was located in the *Hin*fI site in the plus origin at position 31 (insertion 1, pKH1). Four further insertions involved the extragenic region outside of the origins (insertions 5 to 8 in Fig. 2 and Table 1). No apparent change in copy number from wild type was found for these insertional mutants replicating as a plasmid. Thus, it appeared that the autonomous origin was unaffected.

For an analysis of the phage replication and packaging functions, strain JM103 was transformed with the insertion derivatives of phasyl and superinfected with phage fd109 (Ap^+) (14). Phage were isolated, and the number of Km^r (phasyl) and ampicillin-resistant (Ap^r) (fd) transducing particles was determined (Table 2).

Since phasyl is a defective miniphage that carries no marker gene, the replication efficiency of the wild type could not be tested. The constructs with the insertions outside of the origin (insertions 5 to 8: pKN46, pKN31, pKM2, pKS1) were packaged by the fd packaging apparatus with about the

TABLE 1. Plasmids derived from phasyl

Plasmid	DNA fragments or vector
pKH1	1,500-bp *Hinc*II Km^r fragment from pRME1 inserted into *Hin*fI site at position 31 of phasyl
pKM2	Km^r inserted into *Mae*II site at position 314
pKM4	Km^r inserted into *Mae*II site at position 199
pKN41	Km^r inserted into *Nla*IV site at position 129 or 130
pKN43	Km^r inserted into *Nla*IV site at position 141
pKN46	Km^r inserted into *Nla*IV site at 246
pKS1	Km^r inserted into *Sfa*NI site at position 337 or 340
pKN31	Km^r inserted into *Nla*III site at position 276 or 296
pCH1	952-bp *Asu*II Cm^r fragment from pBR325 inserted into *Hin*fI site at position 31 of phasyl
pTOP13-2	*Aat*II site and *Asn*I site introduced into phasyl by oligonucleotide-directed mutagenesis at positions 256 and 260, respectively; insertion of a 1,140-bp *Aat*II-*Nde*I fragment from pT7-7 carrying the *bla* gene and a multicloning site
pAN12-4	941-bp *Ban*II-*Sma*I fragment containing the *arp* gene from pKH1 inserted into the multicloning site of pJF118EH (7), Ap^r replaced by Tc^r
pAR9	pTOP13-2 with deletion of 241-bp *Rsa*I fragment from positions 1516 to 1757 (= positions 401 to 642 of phasyl)
pKH1D7	pKH1 with deletion of 36-bp *Dde*I fragment from positions 2509 to 2545 (= positions 1005 to 1041 of phasyl)
pAD7	pTOP13-2 with deletion of 36-bp *Dde*I fragment from positions 2120 to 2156 (= positions 1005 to 1041 of phasyl)
pKH1R9	pKH1 with deletion of 241-bp *Rsa*I fragment from positions 1905 to 2146 (= positions 401 to 642 of phasyl)
pM1	Phasyl with deletion of 147-bp *Mst*I fragment from positions 204 to 351
pB1	pTOP13-2 with deletion of 147-bp *Ban*II fragment from positions 1361 to 1508
pD7, pH7, pR9, pF2	See reference 25

same efficiency as phage fd. We assume that these transduction frequencies represent wild-type levels.

The three clones carrying an insertion in the minus-strand origin (constructs 2 to 4: pKN41, pKN43, pKM4) gave 10^2 to 10^4 times fewer Km^r transducing particles than plasmids with insertions outside the origin region (pKN46, pKN31, pKM2, pKS1). The number of Ap^r tranducing particles was only slightly reduced in the presence of phasyl derivatives.

TABLE 2. Transductional activity of insertional phasyl mutants[a]

Insertion no.	Plasmid	No. of Km^r transducing particles/ml	No. of Ap^r transducing particles/ml
1	pKH1	1.5×10^{11}	3.3×10^{11}
2	pKN41	1.0×10^{9}	1.3×10^{11}
3	pKN43	1.0×10^{9}	1.3×10^{11}
4	pKM4	2.0×10^{7}	8.0×10^{10}
5	pKN46	1.8×10^{11}	2.3×10^{11}
6	pKN31	1.6×10^{11}	1.7×10^{11}
7	pKM2	1.8×10^{11}	2.3×10^{11}
8	pKS1	3.3×10^{10}	2.3×10^{11}
1	pCH1	1.5×10^{9}	3.9×10^{11}
	None		6.6×10^{11}

[a] The number of Ap^r or Km^r transducing phages was determined as described in Materials and Methods. The locations of insertions indicated in the first column are given in Table 1 and Fig. 2.

Surprisingly, the transduction rate of plasmid pKH1, with the insert in between the two loops of the plus-strand origin, was not reduced. Dotto et al. (4) have reported an insertion of 10 bp into the same *Hin*fI site of phage f1 which results in a complete loss of biological function. Neither nicking reaction nor plus-strand initiation was observed in this construct.

The ends of the inserted Km^r fragment contain part of the inverted repeats of transposon Tn*903*. Plasmid pKH1 may form an alternative secondary structure in which the ends of the insert hybridize, thereby restoring a secondary structure in the double-stranded (ds) (plus) origin similar to the original one. To test this hypothesis, we replaced the Km^r fragment with a chloramphenicol resistance (Cm^r) fragment from pBR325 that has no inverted repeats (plasmid pCH1). The amount of Cm^r transducing particles obtained with pCH1 was decreased by a factor of 10^2 compared with the Km^r transduction rate of pKH1 (Table 2). However, complete inactivation, as found with f1 after the insertion of 10 bp at this site, was not observed.

It was apparent that the insertion of the Km^r or Cm^r fragment into the extragenic region affected the replication as a phage when the plus or minus origin was involved, while autonomous replication was unaffected. We conclude that the origin for autonomous replication is located at a different site, suggesting that the two replication modes are different.

Cloning of the autonomous replicon. (i) Cloning of *arp* gene. To define the region responsible for autonomous replication of phasyl, we first constructed plasmid pAN12-4, which supplies the Arp protein in *trans*. For this purpose, we cloned a 941-bp *Ban*II-*Sma*I fragment from pKH1 into the *Sma*I site of the expression vector pJF118EH (7) under the control of the *tac* promoter. Plasmid pD7, which has a 36-bp deletion in the *arp* reading frame and therefore cannot replicate autonomously (25), was found to be stably maintained with plasmid pAN12-4 in *trans*. Therefore, we conclude that the Arp protein is a *trans*-acting replication protein.

Even without induction, the amount of Arp protein produced by plasmid pAN12-4 is apparently enough to support replication of an *arp*-deficient phasyl. This is consistent with previous observations that the *tac* promoter is not tightly repressed (13). Induction of this promoter with increasing amounts of isopropyl-β-D-thiogalactopyranoside (IPTG) in the absence of pD7 resulted in a decreased number of colonies (only 25% of the cells survived an IPTG concentration as low as 0.05 mM [data not shown]), suggesting that high concentrations of Arp are lethal. We were unable to show overexpression of the Arp protein by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Upon induction with IPTG, no additional band of the corresponding size was found (data not shown).

(ii) Origin of autonomous replication, *oriA*. To delimit the size of the autonomous origin, we constructed deletion derivatives of phasyl. The capability to replicate was tested by cotransformation with the Arp-complementing plasmid pAN12-4. One of the constructs (pB1, Fig 3) was derived from plasmid pTOP13-2, which contains the *bla* gene for selection. The *bla* gene was inserted at position 254 in the extragenic region. Insertion in this region does not alter the replication activity as a phage or as a plasmid, as discussed above.

Establishment of deletion derivatives was only possible if the deletion did not enclose a region of phasyl which contains the *arp* promoter, the N-terminal part of the *arp* gene, and the right half of the extragenic region. The left boundary of the deletion moved progressively from position

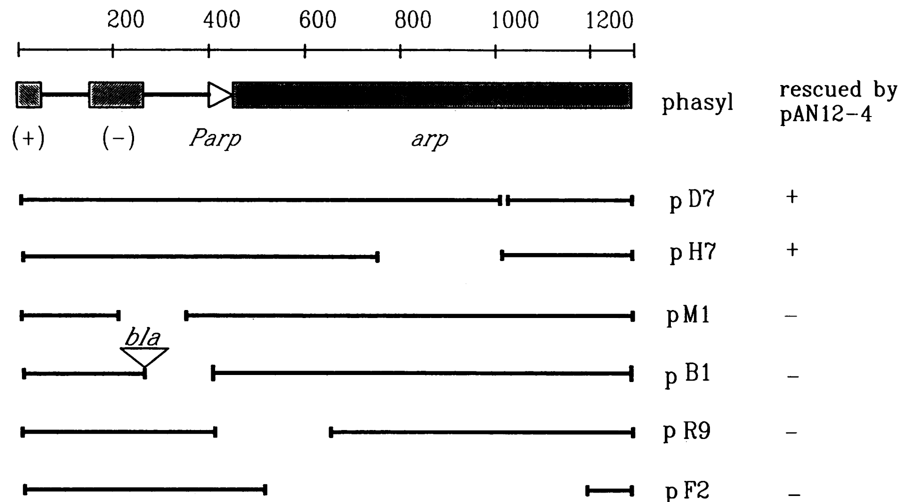

FIG. 3. Deletion derivatives of phasyl and pTOP13-2. A linear map of phasyl is shown with plus and minus origins in the extragenic region and the *arp* gene with its promoter. The restriction sites used for the construction of the deletion derivatives are reflected in their names: *Dde*I (D), *Hae*II (H), *Mst*I (M), *Ban*II (B), *Rsa*I (R), and *Fnu*DII (F). pB1 was derived from pTOP13-2, which carries a *bla* gene and a multicloning site. As shown in the right column, only the constructs pD7 and pH7 were found to be stable after cotransformation with the Arp-expressing plasmid pAN12-4. All other constructs, in which the *arp* promoter and part of the extragenic region are deleted, could not be detected after cotransformation.

204 (pM1) to positions 260 (pB1), 401 (pR9), 498 (pF2), 736 (pH7), and 1005 (pD7) (Fig. 3). Only pH7 and pD7 were found to replicate with the Arp-complementing plasmid in *trans*, suggesting that the right border of the origin for autonomous replication must lie between positions 498 and 736.

The right boundary of the deletion moves to the left in constructs pB1 and pM1 from positions 393 to position 351 (Fig. 3). Since these plasmids were not rescued with pAN12-4 in *trans*, the left border of the origin for autonomous replication must be left of position 351.

All these deletion derivatives could replicate in strain H411, providing fd gene II in *trans*, with the exception of clone pM1, which was found to be inactive in both strains. The lack of replication activity in the constructs without the region from positions 351 to 498 suggests that the origin of autonomous replication is inactivated by these deletions and that plasmid replication is therefore blocked.

Two independent origins. Apparently, there are two functionally different origins in phasyl. One, similar but not identical to the replication origin of the filamentous phages of *E. coli*, is responsible for phagelike replication. The second origin is located in a region where the *arp* promoter is also found. It is functional in autonomous plasmid replication. To test this hypothesis, we constructed a set of clones carrying either one of the inactivated origins and tested them under different replication conditions. The clones were either transformed into strain H411 (gene II of phage fd inserted into F′), in which constructs with a functional phage origin are able to replicate, or the clones were cotransformed with the Arp-expressing plasmid pAN12-4 into C600 (Fig. 4 and Table 3), in which constructs with a functional plasmid origin should be able to replicate.

Plasmid pAR9 has a deletion of 241 bp that removes the promoter and the N-terminal part of the *arp* gene. The phage origin is not altered. This plasmid could be established in strain H411 but could not be successfully cotransformed with pAN12-4. Therefore, only the origin for autonomous replication must have been affected by the deletion.

Plasmid pKH1D7 carries the Km^r insertion in the plus origin of phasyl and a 36-bp deletion outside the region of the putative origin of autonomous replication. This plasmid replicated with pAN12-4 in *trans* but could not be established in strain H411, suggesting that the phage origin is inactivated but not the plasmid origin. Plasmid pAD7 was constructed as a control. It carries an active phage origin and the 36-bp deletion in the *arp* gene outside the *arp* promoter region. It could be transformed into both strains, i.e., the two origins for phage and plasmid replication were unaffected.

It was impossible to obtain plasmid pKH1R9, compatible with both origins being inactivated. For transformation efficiency data, see Table 3.

These experiments suggest that the two replication modes of phasyl operate via two distinct origins. The origin for phage replication is located in the extragenic region, while the origin for plasmid-type replication, *oriA*, is located in the region of the *arp* promoter. It extends from left of position 351 to a point between positions 498 and 736.

Fine mapping of *oriA*. For further analysis of the borders of the origin of autonomous replication, *oriA*, we cloned different fragments derived from phasyl derivative pTOP13-2 (Fig. 5 and Table 1) into the origin test vector pNON11. pNON11 carries the ColE1 origin on a *Not*I cassette and a multicloning site. Tested fragments were inserted into this multicloning site. The original ColE1 origin was subsequently excised with *Not*I. The religated products could be successfully transformed in a suitable strain if the inserted fragments possessed origin activity.

After excision of the *Not*I fragment, the religated pNON derivatives were cotransformed with the Arp-expressing plasmid pAN12-4. Three of them were found to replicate. The smallest fragment which showed origin activity was a 339-bp *Nla*III fragment that extended from position 1412 to position 1751 (positions 297 to 636 in phasyl). This fragment was sufficient to enable the ColE1 origin-deprived plasmid to replicate with the Arp protein in *trans*. No transformants were found if plasmid pAN12-4 was omitted.

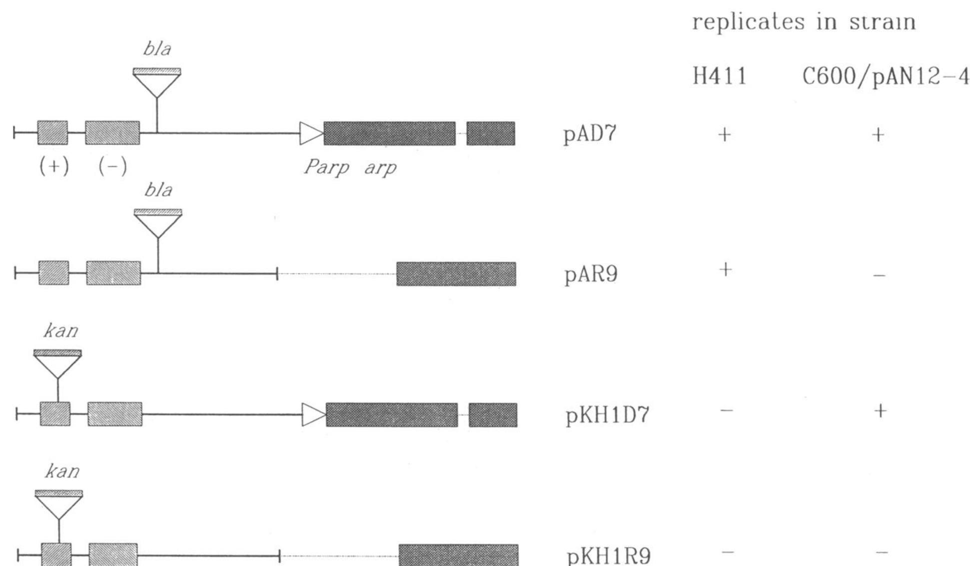

FIG. 4. Linear maps. Plus and minus origins and the *arp* gene with its promoter are indicated. In pKH1D7 and pKH1R9, the plus origin is inactivated by insertion of a Km^r fragment. In pAR9 and pKH1R9, the postulated origin for autonomous replication is removed by deletion of 241 bp. The *arp* gene is inactivated by a 36-bp deletion in plasmids pAD7 and pKH1D7 which does not affect the putative origin for autonomous replication. The plasmids were tested for replication in strain H411 (gene II positive; replication in the phage modus) or in strain C600 with plasmid pAN12-4 (Arp^+, autonomous replication). Replication capabilities under the different conditions are given in Table 3.

The right border of the autonomous origin *oriA* therefore must be located between positions 1629 and 1751 of pTOP13-2 (corresponding to positions 514 and 636 of phasyl), while the left boundary must be located rightward from position 1412 (position 297 of phasyl). This is in agreement with the results discussed above (Fig. 3). Combining these results, we show that the left border of the plasmid origin *oriA* of phasyl is located between positions 297 and 351, while the right border can be mapped between positions 514 and 636.

DISCUSSION

The phage-plasmid hybrid phasyl has two distinct modes of replication. For autonomous replication, the Arp protein is essential. Phasyl also grows as a phage via ssDNA in the presence of a filamentous phage (M13, fd, f1).

The extragenic region of phasyl contains two regions homologous to the origins of filamentous phages for viral (plus)- and complementary (minus)-strand DNA synthesis, respectively. For conversion of ss viral DNA to the ds form, RNA polymerase binds to the minus-strand origin (10) and synthesizes a primer which is elongated for synthesis of the complementary strand (9). The synthesis of ssDNA and of ds replicative intermediates is initiated and controlled by gene II protein, which binds to the plus origin and produces a nick (21). This nick serves as a primer for the rolling circle type of replication of the viral strand. The extragenic region of phasyl contains neither a morphogenetic signal (domain A [5]) nor the A+T-rich enhancer region (domain F [16]) of the viral origin, which are essential in filamentous phages (25, 29).

In this study, we investigated the influence of insertions and deletions on the two replication modes of phasyl. The insertion of a 1,500-bp Km^r fragment into the minus-strand origin of phasyl led to a decreased number of Km^r transducing phasyl particles. This effect varied depending on the location of the insertion (Fig. 2). Insertion into the B loop

TABLE 3. Transformation efficiency of phasyl constructs with manipulated origins[a]

Plasmid	Transformation efficiency (transformants/μg of DNA) in strain: H411	C600 (pAN12-4)
pAR9	1.8×10^4	0[b]
pKH1D7	0[b]	8.8×10^3
pAD7	1.4×10^4	1.4×10^4
pKH1R9	0[b]	0[b]

[a] Transformation efficiency of plasmids derived from phasyl with either the phagelike origin or the autonomous origin inactivated (for details, see text and Table 1).

[b] No transformants found in three independent experiments.

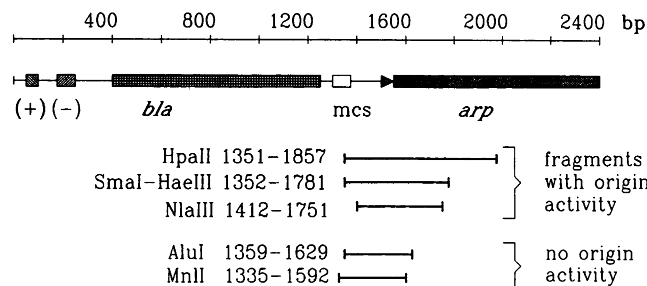

FIG. 5. Size and location of the origin of autonomous replication, *oriA*. A linear map of phasyl derivative pTOP13-2 is shown. Plus and minus origins, *bla* and *arp* genes, and the multicloning site (mcs) are indicated. Fragments of different sizes were cloned into the origin test vector pNON11. The ColE1 origin of the plasmids was excised, and the resulting clones were tested for replication activity with plasmid pAN12-4 in *trans*. Fragments that contain the origin are marked.

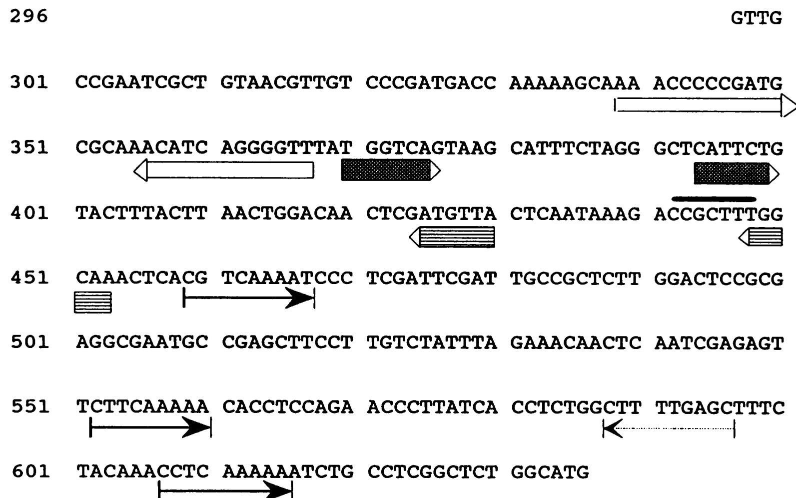

FIG. 6. Sequence of the 339-bp *Nla*III fragment from phasyl, containing the origin for autonomous replication, *oriA*. The −10 and −35 regions for the *arp* promoter and for the promoter of the antisense transcript (to be published elsewhere) are indicated by hatched and striped boxes, respectively. A 9-bp direct repeat, which is found three times, is shown by solid arrows. A fourth repeat, which has the reverse orientation and is less conserved, is indicated by a stippled arrow. A putative n′ site with one mismatch is marked by a solid bar. Also shown is a stem-loop structure (open arrows), which serves as terminator for the antisense transcript.

(pKN41, pKN43) led to a decrease by a factor of 10^2; insertion into the C loop, where binding of RNA polymerase has been found (10), led to a decrease by a factor of 10^4 (pKM4). This is in agreement with results published previously (17, 22). Insertion between loops D and E of the plus-strand origin in pKH1 did not alter the transduction rate, and replacement of the Km^r fragment by the Cm^r fragment without inverted repeats at its ends lowered the transduction frequency only by a factor of 10^2. This contradicts results published previously (5, 15). A complete inactivation (biological activity, $<10^{-5}$) of phage fl was observed after insertion of 10 bp into the same *Hin*fI site we used. It was demonstrated that gene II product can bind to the defective plus-strand origin of this construct but that the nicking reaction is not performed (11, 15). The viral strand origin of filamentous *E. coli* phages and phasyl contains four repeats of 5 bp (TGGAA/C): α and β, located in the D loop, and γ and δ, located in the E loop. Interaction of gene II product with repeats β, γ, and δ is necessary for initiation of ssDNA replication (11). Repeat α is required for termination of DNA synthesis (3). Therefore, replication initiation by interaction with only one loop seems unlikely.

In contrast to the transduction of plasmid pKH1, in which no decrease in efficiency was observed, plasmid pKH1D7 (which likewise has the Km^r fragment inserted into the plus origin but lacks a 36-bp fragment of the *arp* gene) could not be established in strain H411, which provides gene II protein of phage fd. This indicated that insertion of the Km^r fragment into the plus origin severely impairs phagelike replication if autonomous plasmidlike replication is blocked simultaneously.

There are two possible explanations for this phenomenon. Replication from the origin for autonomous replication could compensate for the reduced activity of the plus-strand origin by increasing the number of available copies of phasyl. Alternatively, replication from the plasmid origin might substitute, albeit less efficiently, for the inactivated plus origin by providing ss replication products which can be packaged into phage particles. However, since plasmid replication of phasyl can occur in the absence of an active minus origin, contrary to phage replication, this interpretation implies a second ss-to-ds conversion signal associated with the autonomous origin. This signal must be insufficient for the conversion of an ss molecule, as it is synthesized by phagelike replication; it should act only in autonomous replication.

Plasmids of gram-positive bacteria replicate via ssDNA intermediates (12, 23). Our second interpretation implies a similar mechanism for phasyl. Additional suggestions for such a mechanism come from preliminary experiments in which ssDNA replication intermediates have been found and from the structure of the Arp protein. It shows homology to the replication proteins of phage ϕX174 and pC194-like plasmids. The amino acid motif around the active site of the ϕX174 Rep protein (**G**F**Y**V**AKY**V**NK** [conserved residues are in boldface type]) is also found in phasyl, although less conserved (**G**R**Y**V**G**K**Y**IS**K**) (17a). No homology to the sequences of plus or minus origins of ssDNA plasmids from gram-positive bacteria were found.

The observed decrease by a factor of 10^2 in transduction activity of plasmid pCH1 compared with pKH1 indicates that the structure of the inserted fragment may also contribute to the restoration of transduction activity. Plasmid pKH1 may form an alternative secondary structure by hybridization of the inverted repeats at the ends of the Km^r fragment, thereby allowing an occasional formation of a structure similar to the active form of the plus-strand origin.

In conclusion, we propose that the different effects observed after insertional inactivation of the plus origin of phasyl and bacteriophage fl reported here and by Dotto et al. (4) are caused by the length and structure of the used inserts and by a backup mechanism for the inactivated plus origin provided by phasyl. This backup could be an increased copy number of phasyl or an alternative synthesis of ssDNA intermediates.

The investigation of the plasmid replication mode revealed *oriA*, the origin of autonomous replication of phasyl. *oriA* is located within the 339-bp *Nla*III fragment (for the sequence,

see Fig. 6). The left border of *oriA* was mapped between positions 297 and 351, the right between positions 514 and 636. This fragment displays origin activity if the Arp protein is provided in *trans*. Two smaller fragments did not display replication activity in this assay. They may represent either inactive or overactive origins, in which control elements are deleted.

oriA includes 100 bp of the right part of the extragenic region, the promoter, and the N-terminal part of the *arp* gene. Additionally, a second promoter is found, which is transcribed in the opposite direction. This transcript is terminated after 83 nucleotides (9a). Computer search and comparison of *oriA* with other sequences did not reveal any particular homologies. Also, we did not find A+T-rich domains, GATC sites, or DnaA boxes, which are characteristically found in the origin regions of other replicons, such as ColE1 or *oriC*. The only known element found was an n′ site at position 442, which shows one mismatch to the consensus sequence (GAAGCGG) (28). Since this sequence is not located in a hairpin loop as are the n′ sites of phage φX174 and plasmids pBR322, pCM959, and pACYC177, primosome assembly at this site seems unlikely. A 9-bp sequence (5′-CnTCAAAAA/T-3′) is found three times in the reading frame for the *arp* gene. A fourth repeat, although less conserved, is found in the opposite orientation (Fig. 6).

The origin of autonomous plasmid replication, *oriA*, encoded in this fragment could not be classified into a group with an origin already identified, since no structural or sequence homology to other origins was found. Nevertheless, the existence of repeated sequences shows analogy to other systems such as P1 (1) and F (27). In these plasmids, the repeats reflect binding sites for the replication protein and are an essential feature for replication. The mechanism of initiation at this new origin, *oriA*, and the properties of the Arp protein are under investigation.

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