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Replication-specific conversion of the *Staphylococcus aureus* pT181 initiator protein from an active homodimer to an inactive heterodimer

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The *Staphylococcus aureus* rolling circle plasmid pT181 regulates its replication by controlling the synthesis of its initiator protein RepC. RepC is inactivated during pT181 replication by the addition of an oligodeoxynucleotide, giving rise to a new form, RepC*. We analyzed RepC and RepC* in four classes of mutants: plasmid copy number mutants, two classes of RepC mutants affecting different portions of the protein and *oriC* (origin) mutants. We have found that in the cell with wild-type RepC there are similar relative amounts of RepC and RepC*, regardless of copy number, and that the conversion of RepC to RepC* is replication dependent. Genetic and biochemical evidence is presented that RepC functions as a dimer and that during replication the RepC homodimer is converted to the RepC/RepC* heterodimer.

Key words: initiator inactivation/plasmid replication/protein modification/*Staphylococcus aureus*

Introduction

Bacterial plasmids control their replication autogenously at the initiation step (Pritchard *et al.*, 1969). Many of those that utilize a plasmid-coded initiator protein regulate replication by controlling the rate of initiator synthesis (Scott, 1984). For such plasmids, the concentration of active initiator is maintained at a low, rate-limiting level and it can often be shown that an increase in initiator concentration is accompanied by a corresponding increase in plasmid replication frequency (Sogaard-Andersen *et al.*, 1984; Manch-Citron *et al.*, 1986; Swack *et al.*, 1987). However, control of replication by regulating initiator synthesis would be impractical if the active initiator could be re-used repeatedly. Thus, it seemed likely that plasmids using this type of regulation would devise an inactivation strategy to prevent the accumulation and re-utilization of active initiator and thus ensure stable plasmid replication.

The *Staphylococcus aureus* rolling circle plasmid pT181 is a well-studied example of a protein-initiated replication system. Initiation of replication is regulated primarily by antisense-mediated control of synthesis of the initiator protein RepC (Kumar and Novick 1985). The antisense (countertranscript) RNA (ctRNA), transcribed from the *cop* region (Figure 1), causes premature termination (attenuation) of RepC mRNA (Novick *et al.*, 1989). Replication involves a single molecule of RepC, which performs a specific enzymatic function, namely site-specific nicking with covalent attachment at the 5′ nick terminus (Thomas *et al.*, 1990; Koepsel and Khan, 1987).

Additionally, the rolling circle plasmid offers a contrast to rolling circle phages, such as ϕX174, in which the initiator protein (CisA) is continuously recycled. The protein contains two neighboring tyrosine active sites, both utilized for replication. Termination of one round of replication initiates the next round by a concerted reaction in which the second tyrosine becomes covalently bound to the 5′ terminus of the newly synthesized plus strand, releasing the displaced plus strand as a single-stranded monomer (Brown *et al.*, 1982; van Mansfeld *et al.*, 1986). In ϕX174, of course, this recycling serves the biological requirement of the phage to make as many replicas as possible before the host cell lyses. Such a mechanism would be incompatible with the stably regulated replication that is characteristic of any plasmid. Instead of being recycled, the plasmid initiator must be inactivated (to prevent re-initiation) and a logical point for inactivation would be the point of release from the DNA during termination.

We have shown that RepC is indeed inactivated following (or during) replication and a new form of the protein appears, RepC*, that is inactive as a topoisomerase and as an initiator. We have also shown that RepC* contains a covalently bound single-stranded oligodeoxynucleotide whose sequence corresponds to the 10–12 nucleotides immediately 3′ to the nick site in the origin and have proposed that this may be the cause of RepC inactivation (Rasooly and Novick, 1993). This modification is universal among plasmids of the pT181 family and is host independent (Rasooly *et al.*, 1994).

In earlier studies we have noted an apparent equality between the amounts of the 38 kDa RepC and the 42 kDa RepC* species, suggesting that the two forms may exist as a heterodimer. In this study we have investigated this apparent equality further by analyzing RepC and RepC* in four classes of pT181 replication mutants, representing different plasmid regions and functions related to replication, copy control, origin recognition and binding by RepC. A schematic map of the pT181 replication region showing the location of these mutations is shown in Figure 1.

We found that RepC and RepC* are always present in similar relative amounts in cells with wild-type RepC, regardless of the amount of RepC. This finding raised the questions of why only half of the RepC molecules in the cell are converted to RepC* and why the remaining RepC has little or no activity. Previous results had suggested that RepC might function as a dimer (Rasooly and Novick, 1993), as does the RepD initiator protein for the closely related plasmid pC221 (Thomas *et al.*, 1990). Thus we

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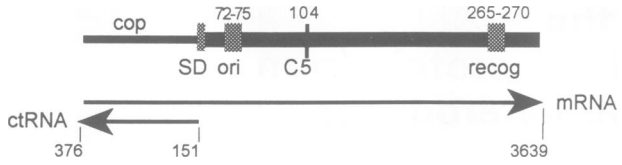

Fig. 1. Schematic diagram of the pT181 replication region. Relevant portions of pT181 are shown: *cop*, the copy control region described in the text; SD, RepC translation start; *ori*, pT181 origin of replication containing the nick site; recog, the region of RepC that determines the specificity of origin recognition. The numbers on top indicate amino acids affected by the mutations used. The numbers below are nucleotides.

considered the possibility that RepC and RepC* are physically linked and that the presence of RepC* prevents RepC function. The results of intragenic complementation and gel filtration experiments presented here suggest that native RepC protein is a homodimer which is converted during replication to a RepC/RepC* heterodimer.

Results

RepC and RepC are present in similar quantities in all copy number mutants*

To study the effect of plasmid copy number on RepC and RepC* we have analyzed a series of strains containing pT181 and several copy number mutants (Carleton *et al.*, 1984) providing a range of copy numbers from 20 to 1000. These mutations are in the pT181 *cop* region that is adjacent to *repC* (Figure 1) and affect the regulatory countertranscript, thereby determining the amount of the RepC protein.

Crude lysates from these strains were analyzed for RepC by Western immunoblotting and the results are shown in Figure 2A. Lane 1 shows RepC purified from the *Escherichia coli* strain MB2 (pSK184) containing a cloned *repC* gene (Koepsel *et al.*, 1985). pT181 does not replicate in *E.coli* and no RepC* is present (Rasooly and Novick, 1993). Visual inspection of lanes 2–5, containing RepC preparations from pT181 copy number mutants, show that in strains with high copy number plasmids, such as pT181-*cop608* (800–1000 copies) (lane 2), the amount of RepC and RepC* is high and clearly visible. On the other hand, strains containing low copy number plasmids, such as pT181 (20 copies) (lane 5), the amount of RepC and RepC* is very small and the proteins are seen as very faint bands that reproduce poorly in the figure. In all strains the two forms of RepC are present in a ratio of 1:1 over the entire 40-fold range in copy number, although the spread of RepC* is wider than RepC (lane 2), probably because of the heterogeneity in the length of the oligonucleotide bound to RepC*.

The ratio of RepC to RepC* was measured by densitometric analysis of this immunoblot (Figure 2B). The ratio of intensities of the RepC and RepC* bands varied from 0.89 to 1.13, averaging 1.02. The coefficient of correlation (r) between the amount of RepC and the amount of RepC* measured densitometrically is 0.987 ($P < 0.01$), indicating a very high correlation between these two forms

Replication-defective mutations decrease RepC production*

We have previously demonstrated (Rasooly and Novick, 1993) that RepC* originates from RepC. If RepC* is a

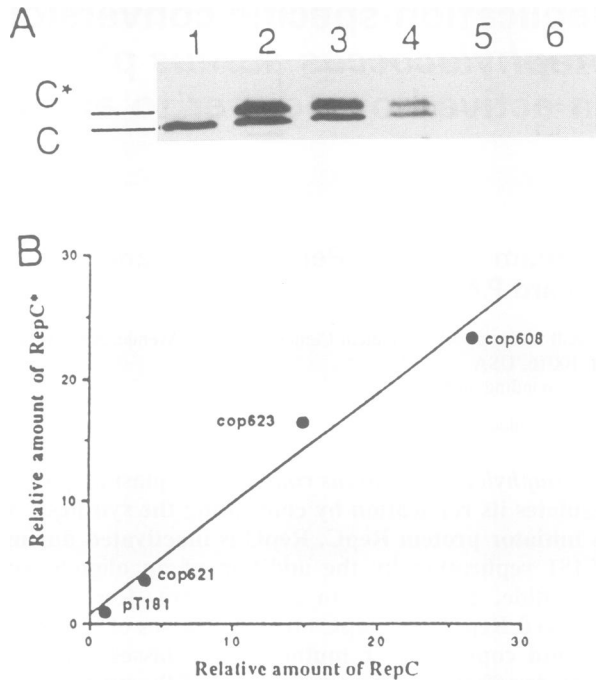

Fig. 2. RepC and RepC* are present in similar quantities. (**A**) Whole cell minilysates were separated by SDS–PAGE and Western blotted with anti-RepC as described in Materials and methods. Preparations from the following strains were analyzed: lane 1, RepC purified from strain MB2(pSK184) *E.coli* containing a cloned *repC* gene; lanes 2–5, RepC purified from strains containing pT181 copy mutants: 2, pT181-*cop608* (800–1000 copies); 3, pT181-*cop623* (400–500 copies); 4, pT181-*cop621* (150 copies); 5, pT181 wild-type (22 copies); lane 6, RN27 (no plasmid). (**B**) Plot of RepC versus RepC*. RepC and RepC* were quantified by measuring the intensity of the RepC and RepC* bands from the Western blot (A) using a Shimadzu densitometer.

product of pT181 replication, then one would predict that mutations interfering with replication would result in decreased production of RepC*. Accordingly we tested several mutants with reduced RepC and *oriC* activities for RepC* production. The *repC* mutations are located in the region of *repC* that determines the specificity of origin recognition (shown in Figure 1; amino acids 265–270) and these *repC* mutations are also *ori*$^-$ (Wang *et al.*, 1992). The *oriC* mutant used was *oriC105*, an origin where part of IR-III of *oriC* is replaced with the corresponding region in *oriD* (Wang *et al.*, 1993).

The mutant *repC* (*ori*$^-$) genes were cloned into a plasmid with a pE5 replicon. RepC from these donor plasmids was supplied *in trans* to an *oriC* target plasmid, pRN6397. The target plasmid requires RepC in order to replicate at 42°C because its other replicon, pE194Ts, functions at 32°C, but not at 42°C. Thus at 42°C replication of pRN6397 depends on the supply of RepC from the donor plasmid and serves as an *in vivo* indicator of RepC activity. With these pE5::*repC* constructs, the amount of mutant protein synthesized is probably constant (all have the same copy number and use the same promoter for RepC) and RepC is not involved in replication of the RepC-producing plasmids, since they lack a functional pT181 origin. The only difference between the constructs is the degree to which the protein functions.

Several different *repC* alleles were used as a source of

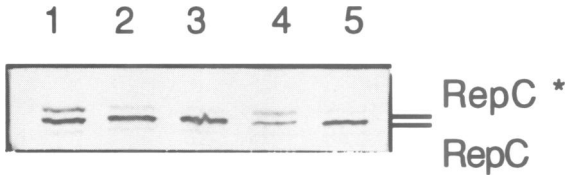

Fig. 3. Effect of RepC mutation on the conversion of RepC to RepC*. Whole cell mini-lysates of various *repC* and *oriC* mutants were analyzed as in Figure 2. Lane 1, pWN8926 (*repC103*, 50 copies); lane 2, pWN9008 (an *oriC105* mutant, copy number 15); lane 3, pWN8944 (*repC126*, 15 copies); lane 4 pWN8816 (wild-type *repC*, 90 copies); lane 5, pWN8925 (*repC102*, copy number <1).

RepC. These mutations varied in their ability to maintain a target plasmid, pRN6397, at 42°C, as measured by the copy number. As seen in Figure 3, the ratio of RepC to RepC* is proportional to the copy number of the target plasmid. Thus with *repC* alleles where little or no replication was detected, because of the severity of the RepC mutation, RepC* is only a small fraction of RepC antigen (*repC102*; lane 5). With *repC103*, which supports pRN6397 at a copy number of 50 at 42°C, the amount of RepC* is nearly equal to that of RepC. A similar result is seen with wild-type *repC* which supports 90 copies of pRN6397 (lane 4). The more functional the RepC protein (able to maintain pRN6397 at a high copy number), the more RepC is converted to RepC*. Note that although occasional cross-reacting bands are seen with this polyclonal antibody, they are unrelated to RepC and its function (Rasooly *et al.*, 1994).

Similarly, we compared RepC and RepC* levels in a *repC* mutation, *repC126* (lane 3), and in an *oriC* mutation, *oriC105* (lane 2). *oriC105* is a point mutation that reduces pT181 replication (Wang *et al.*, 1993). In both mutant strains the pT181 copy number is ~15 and both strains have a low proportion of RepC*. This suggests that it is not the specific mutations *per se* that affect the production of RepC*, but rather the effect of the mutation on replication frequency. These results demonstrate that the amount of RepC converted to RepC* is determined by the functional efficiency of the RepC–ori complex and that is why there are different RepC:RepC* ratios. In the previous experiment, when the amount of wild-type RepC and RepC* produced was varied, the proportion of RepC to RepC* was a constant 1:1.

Allele-specific intragenic complementation between repC mutants

We have shown that strains with RepC that has been used for pT181 replication have very little RepC activity *in vitro* compared with strains with RepC that was not used in replication (Rasooly and Novick, 1993). The question that remained from the experiments described is why there is very little RepC activity detected if, as shown, 50% of RepC in the cell is unmodified (38 kDa form). One possibility is that the 38 kDa molecules are inactivated by another mechanism, without altering their electrophoretic mobility. Another possibility is that the presence of inactive RepC* in the cell inhibits RepC activity stoichiometrically. Consistent with this second hypothesis are some indications suggesting that RepC may function as a dimer (Rasooly and Novick, 1993), because then inactivation of one subunit of the dimer by conversion to RepC* may inactivate the whole molecule. To test this possibility we analyzed RepC function genetically.

One way to test for a functional dimer (or multimer) is by intragenic complementation between mutant alleles. We used one allele with a mutation in the N-terminal region of RepC (*repC5*), shown in Figure 1, and several of the recognition-defective *repC* alleles that were used earlier, with mutations in the C-terminal portion of the protein. Combinations of two mutant alleles were tested for their ability to amplify the target plasmid, pRN6397 carrying *oriC*. The effect of these mutants alone on replication has been analyzed previously (Wang *et al.*, 1992) and it was shown that they cannot maintain the target plasmid at the non-permissive temperature.

To carry out the experiment we constructed strains bearing three plasmids as shown in Figure 4A.

(i) A RepC donor plasmid pWN9161 containing the temperature-sensitive *repC5* allele (P114S) (Iordanescu, 1976) cloned into the pUB110 replicon (*oril*). This plasmid contains a functional pT181 *ori* which is not considered to interfere with the experiment.

(ii) A second RepC donor-containing plasmid with one of a series of recognition-defective *repC* alleles (*repC101*, *repC102*, *repC110* and *repC112*) cloned into a pE5 replicon (*oriL*) (Wang *et al.*, 1992). These plasmids lack a functional pT181 *ori*.

(iii) pRN6397, a target plasmid carrying *oriC* which requires active RepC supplied in *trans* in order to replicate at 42°C.

The results of this experiment are shown in Figure 4. Figure 4B shows the temperature effect on RepC5. There is very little replication of the target plasmid at 42°C (lane 2) compared with 32°C (lane 1). Similarly, Figure 4C shows the temperature effect on the target plasmid in the presence of RepC102. There is no replication of the target plasmid at 42°C (lane 3) or on a similar target plasmid with *oriD* (lane 4) compared with 32°C (lanes 1 and 2). However, as shown in Figure 4D, one of the four mutants, *repC102* (lanes 5 and 6) complemented *repC5* for replication of the target plasmid, which was amplified approximately 12-fold as determined by densitometry. The other three (*repC110*, *repC112*, and *repC101*) did not complement *repC5* (Figure 4D, lanes 2, 3 and 4). The allele-specific intragenic complementation observed with *repC102* and *repC5* demonstrates that the RepC protein functions as a dimer or a multimer. Similar observations have been made by S.Iordanescu (in preparation).

In these experiments only partial restoration of RepC activity may be seen, even if RepC functions as a dimer or multimer. For example, if RepC functions as a dimer, three types of dimers would form in these cells: RepC5 homodimers, RepC102 homodimers and RepC5/RepC102 heterodimers (Figure 4A). Since only 50% of RepC in the cell would be found in heterodimers, only partial RepC activity would be restored, even if there is complete complementation. If functional RepC has three or more subunits, an even lower proportion of wild-type RepC activity would be restored.

*The dimeric structure of RepC and RepC**

To investigate the nature of the RepC multimer we studied the structure of native RepC. We purified RepC that had not been used for replication from a strain containing

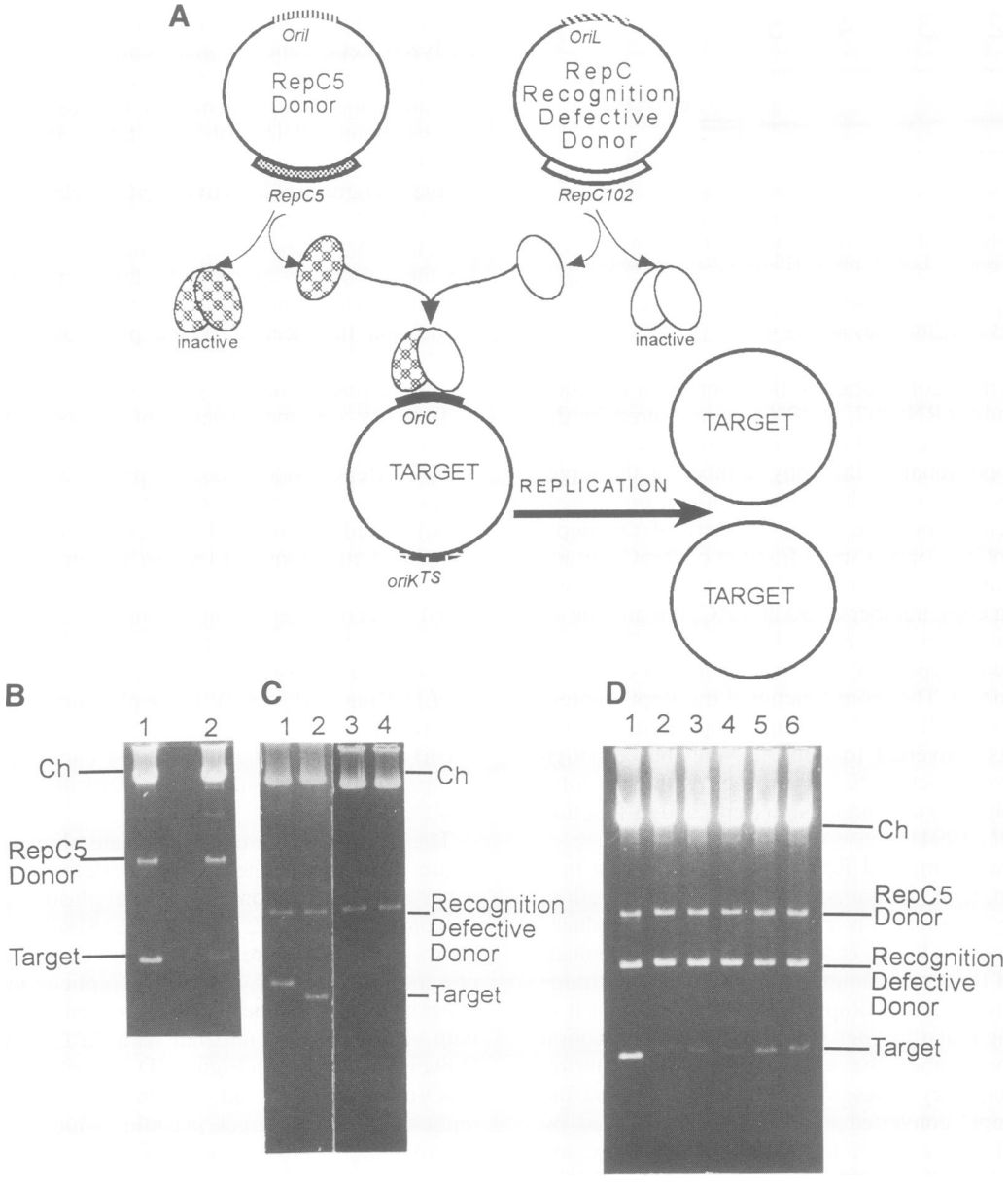

Fig. 4. Intragenic complementation between repC mutants. (**A**) An experiment using three plasmids to study allele-specific intragenic complementation between repC mutants. There are two mutant RepC donor plasmids: *repC5*, a temperature-sensitive mutant maintained on a plasmid with a pUB110 replicon (*oriI*), and a RepC recognition-defective donor maintained on a plasmid using a pE5 replicon (*oriL*). RepC from these plasmids was used to amplify target plasmid RN6397 carrying *oriC*. At 42°C the target plasmid cannot replicate using its temperature-sensitive pE194 replicon (*oriK* ts) and thus depends on a supply of active RepC from the donor plasmids. (**B**) Replication of the target plasmid pRN6397 (with *oriC*) at 32°C (lane 1) and at 42°C (lane 2). (**C**) Replication of the target plasmids in the presence of RepC102: lanes 1 and 2 at 32°C, lanes 3 and 4 at 42°C. The target plasmid was pRN6397 with *oriC* in lanes 1 and 3 and pWN895 with *oriD* in lanes 2 and 4. (**D**) Intragenic complementation between *repC* mutants. Several recognition-defective donors were used to complement *repC5*. Lane 1, the system at 32°C, lanes 2–6 at 42°C. The recognition defective mutants used are: lane 2, *repC110* (pWN8817); lane 3, *repC114* (pWN8912); lane 4, *repC101* (pWN8924); lanes 5 and 6, *repC102* (pWN8925).

pRN6759 (RN7805). In this construct, RepC synthesis is under the control of an inducible *blaZ* promotor (see Materials and methods). However, the plasmid replicates using a different replicon (pC194) and there is no active pT181 origin, because the *repC* gene used was mutated by replacing bases 72–75 (AGAG) with GCTA, a synonymous replacement which did not change the amino acid sequence of the resulting RepC protein (Iordanescu, 1989). Using this *ori*$^-$ construct, no RepC* is made. Partially purified RepC, separated under native conditions by using gel filtration, was detected in fractions corresponding to a molecular weight range of ~100–70 kDa. Western blots of SDS–PAGE of these fractions (denaturing conditions) revealed a single band with a molecular weight of 38 kDa (Figure 5A), the same size as the predicted protein from sequencing data. Therefore, the native molecular weight suggests that RepC purifies as a homodimer, as does RepD (Thomas *et al.*, 1990).

We then prepared RepC/RepC* from a strain with functional pT181 replication (RN4111 with the plasmid

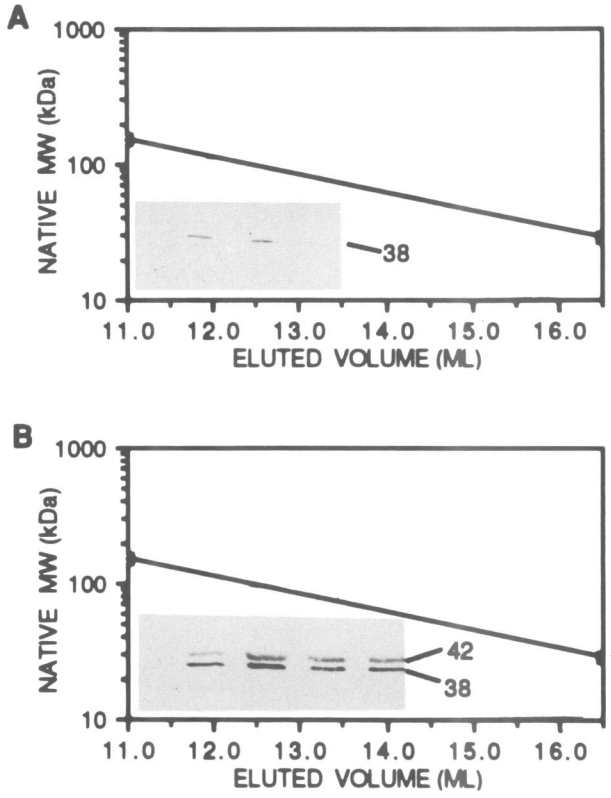

Fig. 5. RepC/RepC* molecular weight determination. Partially purified RepC and RepC/RepC* preparations were analyzed by gel filtration. Column fractions were assayed for RepC by SDS–PAGE and Western blotting as described in Materials and methods. Only the relevant fraction range is shown in the figure. (**A**) Elution profile of a RepC sample from RN7805 (pRN6759 *repC*$^+$ *ori*$^-$ strain). (**B**) Elution profile of a RepC/RepC* sample from RN4111 (pT181-*cop623 repC*$^+$ *ori*$^-$ strain).

pT181*cop623*) with equal proportions of RepC and RepC*. The results from gel filtration show (Figure 5B) that this material was also detected in fractions corresponding to a molecular weight in the range of 100–70 kDa and again, under denaturing conditions, the RepC (38 kDa) and RepC* (42 kDa) bands are seen.

Discussion

In this study we present quantitative data on the equivalence of RepC and RepC* over a wide range of plasmid copy numbers. We also show that RepC functions as a multimer, by demonstrating intragenic complementation between two *repC* mutant alleles. Gel filtration studies show that the apparent molecular weights of native RepC and RepC* molecules are consistent with dimer formation. However, these experiments do not distinguish between a RepC/RepC* heterodimer or two homodimers, RepC/RepC and RepC*/RepC*, in a 1:1 ratio (reflecting the observed RepC to RepC* ratio).

The existence of a RepC/RepC* heterodimer is supported by two lines of evidence. First, the fact that this mixture has little or no RepC activity is consistent with heterodimer formation. Second, we have specifically bound the RepC/RepC* complex, but not pure RepC, by using a column of an oligonucleotide of the complementary sequence adjacent to the nick site which was covalently

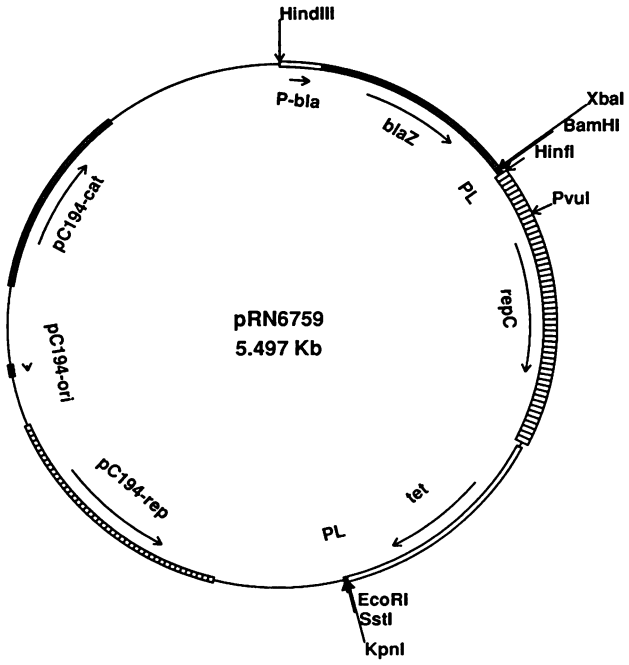

Fig. 6. pRN6759 map. pRN6759 is a derivative of pC194 (left half of map, between *Hin*dIII and *Eco*RI sites) which contains a cloned composite segment consisting of the pI258 *blaZ* promoter (P-bla) and two-thirds of the β-lactamase structural gene (*blaZ*), followed by the region of pT181 containing *repC* and the 5′ half of the tetracycline resistance gene (*tet*). The *repC* (*ori*$^-$) component contains its native Shine–Dalgarno site but lacks its 5′ regulatory sequences and is out-of-frame with *blaZ*. PL, part of the pUC19 polylinker; pC194-*rep*, initiator gene of pC194; pC194-*ori*, leading strand origin of pC194; pC194-*cat*, chloramphenicol transacetylase gene of pC194. Only relevant restriction sites are indicated.

linked to magnetic beads (Rasooly and Novick, 1993). When the bound material was eluted from the beads, a 1:1 mixture of RepC and RepC* was observed. This result suggests that there is no detectable amount of RepC/RepC in the RepC/RepC* mixture and thus confirms that it is a heterodimer. The replication-dependent conversion we demonstrated (Figure 3) is therefore conversion of a RepC homodimer to a RepC/RepC* heterodimer, explaining the observed 1:1 RepC/RepC* ratio (Figure 2).

These data support the model suggesting that RepC is initially found as a homodimer that is specifically converted to a RepC/RepC* heterodimer as a direct consequence of its use for replication. The model is based on the result that RepC which was not used in replication (from a strain lacking *oriC*) is found as a homodimer, while RepC which was used in replication is found only as a RepC/RepC* heterodimer. Furthermore, RepC recognition mutants that vary in their ability to be used for replication also vary in their ability to convert RepC to RepC*, suggesting that the conversion of RepC homodimer to RepC/RepC* heterodimer is replication dependent.

Previously we had shown that RepC antigen isolated from cells with a functioning pT181 origin had less than 10% of the replication or relaxation activity seen with RepC antigen isolated from cells lacking a functional pT181 origin. We also showed that RepC inactivation was correlated with the appearance of a new form of the protein, RepC*, which is modified and inactivated by the covalent attachment of an oligodeoxynucleotide. Here we suggest that because the RepC enzyme is a dimer,

Table I. Bacterial strains and plasmids

	Description	Source
Strain		
RN27	derivative of NCTC8325	
RN7805	RN27(pRN6759/pI524)	this work
RN3259	RN27(pT181)	Carlton *et al.* (1984)
RN3266	RN27(pT181-*cop*608)	Carlton *et al.* (1984)
RN4111	RN27(pT181-*cop*623)	Carlton *et al.* (1984)
RN3291	RN27(pT181-*cop*621)	Carlton *et al.* (1984)
WN2701	RN27(pRN6397/pWN8936	Wang *et al.* (1992)
WN2702	RN27(pRN6397/pWN8944	Wang *et al.* (1992)
WN2703	RN27(pRN6397/pWN8816	Wang *et al.* (1992)
WN2704	RN27(pRN6397/pWN8925	Wang *et al.* (1992)
WN2705	RN27(pRN6397/pWN9008	Wang *et al.* (1993)
WN2706	RN27(pRN6397/pWN8817/pWN9161	this work
WN2707	RN27(pRN6397/pWN8912/pWN9161	this work
WN2708	RN27(pRN6397/pWN8924/pWN9161	this work
WN2709	RN27(pRN6397/pWN8925/pWN9161	this work
Plasmid		
pT181	naturally occurring plasmid (20–25)[a]	Iordanescu (1976)
pT181*cop*608	copy mutant (800–1000)[a]	Carleton *et al.* (1984)
pT181*cop*621	copy mutant (150–200)[a]	Carleton *et al.* (1984)
pT181*cop*623	copy mutant (400–500)[a]	Carleton *et al.* (1984)
pRN6397	pT181 ori cloned to pRN5101	Wang *et al.* (1992)
pWN8926	donor of *RepC103*(S268A)	Wang *et al.* (1992)
pWN8944	donor of *RepC126*(H265S,R266K,S268T,R269K)	Wang *et al.* (1992)
pWN8816	donor of wt RepC	Wang *et al.* (1992)
pWN8925	donor of *RepC102*(N267N)	Wang *et al.* (1992)
pWN9008	donor of *oriC105*	Wang *et al.* (1993)
pWN9161	donor of *RepC5* TS	this work
pI524	β-lactamase repressor	Novick *et al.* (1979)
pRN6759	RepC($^+$), pT181 ori($^-$)	this work
pRN6366	RepC($^-$), pT181 ori ($^+$)	Gennaro and Novick (1986)
pSK184	pUC7 with cloned *repC*	Koepsel and Khan (1985)

[a]Copies per cell

inactivation of one subunit (RepC*) may inactivate the whole molecule, even though the second subunit (RepC) may be unaffected.

Complementation studies support this model. A temperature-sensitive mutant, *repC5*, complemented a recognition-defective mutant allele of *repC*. The *ori* recognition and binding region of RepC has been localized in the six C-terminal amino acids (265–270) and the mutation in *repC5* is located in the N-terminal region (amino acid 114), which is not known to participate in *ori* recognition and binding. Thus it is possible that these two subunits complement each other by supplying a function the other lacks for replication. The allele-specificity of the complementation is characteristic of intragenic complementation and these results suggest that RepC functions as either a dimer or a multimer in replication.

Another question that arises is why the RepC enzyme functions as a RepC homodimer but not as a RepC/RepC* heterodimer. The model for pT181 replication is based on the replication of ϕX174. The ϕX174 initiator protein, CisA, is a monomer that has two tyrosine active sites, allowing cleavage and transfer of the newly synthesized strand, while RepC has only one tyrosine active site. We suggest that the second RepC tyrosine active site (needed for cleavage and transfer of the newly synthesized plus strand) may be supplied by the second subunit of the RepC dimer. However, CisA is repeatedly recycled, as the end of one round of replication leads to the start of another round for the lytic ϕX174 bacteriophage. This recycling is not feasible for a plasmid, since it would lead to runaway replication. Thus one round of pT181 replication ends in termination and disabling of the initiator protein, forming the inactive RepC/RepC* heterodimer.

Materials and methods

Bacterial strains, plasmids and growth conditions

The strains and plasmids used in this work are listed in Table I. pRN6759 (Figure 6) was used to supply RepC in *trans*. Its construction is described below. All strains were grown in CY broth (Novick and Brodsky, 1972) at 37°C. Growth was monitored turbidimetrically using a Klett–Sumersen colorimeter with a green (540 nm) filter. The *repC* complementation experiment was carried out as described by Wang *et al.* (1992).

DNA isolation and manipulation

Plasmid DNA was isolated by CsCl/ethidium bromide gradient centrifugation of cleared bacterial lysates (Novick *et al.*, 1979). Restriction enzymes were obtained from Boehringer GmbH. T4 DNA ligase and calf alkaline phosphatase were obtained from US Biochemicals. Plasmid DNA was introduced into *Staphylococcus aureus* cells by transduction or by protoplast transformation (Chang and Cohen, 1979).

Construction of pRN6759 and pWN9161

pRN6759, first mentioned in a previous work (Rasooly and Novick, 1993), was constructed starting with the vector pRN6725, a derivative of pC194 containing the pUC19 polylinker at its unique *Hin*dIII site and an 845 nt *Hin*dIII–*Xba*I fragment of pI258 containing the *blaZ* promoter and two-thirds of the structural gene (Bargonetti *et al.*, 1993). pRN6725 was digested with *Kpn*I and *Xba*I and ligated to the *Hin*fI–*Kpn*I fragment of pT181, which contains *repC* and part of *tet*, using a synthetic

*Xba*I–*Hin*fI adapter at the 5′ end of *repC* that restored the native *repC* Shine–Dalgarno site. Since the pT181 leading strand origin is within *repC*, we used an *ori*⁻ mutant with synonymous replacement of four nucleotides surrounding the nick site (Gennaro *et al.*, 1989). The *repC* and *blaZ* reading frames are different, so that this is a transcriptional fusion. pWN9161 was constructed by cloning an *Eco*RI fragment from pSA1900 containing *repC5*, a temperature-sensitive allele of *repC* (affecting amino acid 114) that produces functional RepC at 32°C but not at 42°C (Iordanescu 1976), into the *Eco*RI site of pUB110. (See Figure 6.)

Preparation of whole cell mini-lysates

An aliquot of an exponential CY culture (1.5 ml) at 10^9 bacteria/ml was centrifuged, the pellet washed with TE (10 mM Tris–HCl, pH 8, 1 mM EDTA), resuspended in 30 µl TE with lysostaphin (100 µg/ml) (a generous gift from AMBI Inc., New York, NY) and incubated for 30 min at 37°C. Lysis mixture (30 µl; 0.25 M Tris–HCl, pH 6.8, 4% SDS, 4% glycerol, 1% β-mercaptoethanol, 0.01% bromophenol blue) was added and the cells were vortexed for 10 min and then subjected to three freeze–thaw cycles.

RepC purification

Cells were grown in 1 l CY at 37°C to a density of 10^9/ml. The cells were harvested by centrifugation at room temperature, washed with TE and resuspended in 5 ml cold TE containing 150 mM KCl. The resuspended cells were placed on ice, lysostaphin (200 µg/ml) was added and the cells were incubated for 30 min, followed by three freeze–thaw cycles. The lysate was then centrifuged for 30 min at 19 000 r.p.m. in a Sorvall SS34 rotor. The supernatant was treated with streptomycin sulfate (3% w/v) for 30 min on ice, then centrifuged for 30 min at 19 000 r.p.m. The resulting pellet was resuspended in TE, ammonium sulfate was added to 50% saturation and the resulting preparation re-centrifuged at 19 000 r.p.m. for 30 min. The pellet was resuspended in TE and precipitated again with ammonium sulfate to reduce residual DNA contamination.

SDS–PAGE and immunoblotting

Samples (10 µl) of whole cell minilysates were fractionated by 12.5% SDS–PAGE (Laemmli, 1970). Samples were heated at 90°C for 2 min immediately before being loaded. The gels were run at 150 V for 2 h, then Western immunoblotted with rabbit anti-RepC antisera followed by goat anti-rabbit Ig–alkaline phosphatase conjugate. Antibodies were prepared commercially (BAbCO) as described (Rasooly *et al.*, 1994). RepC was quantified by measuring the intensity of the RepC band on a Western blot using a Shimadzu densitometer.

Gel filtration

The molecular weight of native RepC was measured using gel filtration. Partially purified RepC or RepC/RepC* (100 µl) was loaded on a 50×1 cm Bio-gel P100 (Bio-Rad) column, prepared according to the manufacturer's instructions. The column was calibrated and run with 20 mM Tris, pH 8.6, 1 mM EDTA, 100 mM KCl, 0.02% Triton X-100 at 10 ml/h. The column was calibrated with molecular markers (Sigma).

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