JOURNAL OF BACTERIOLOGY, Sept. 1989, p. 4831–4835
0021-9193/89/094831-05$02.00/0

Vol. 171, No. 9

Staphylococcus aureus Chromosomal Mutation *plaC1* Amplifies Plasmid pT181 by Depressing Synthesis of Its Negative-Effector Countertranscripts

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Received 21 February 1989/Accepted 26 May 1989

A *Staphylococcus aureus* chromosomal mutation, *plaC1*, which leads specifically to the amplification of plasmid pT181 has previously been described (S. Iordanescu, Plasmid 10:130–137, 1983). The mechanism by which *plaC1* amplifies plasmid pT181 has been approached in two ways: determination of the plasmid region required for the specific response to the *plaC1* mutation and evaluation of different parameters of pT181 replication control by using transcriptional and translational fusions with the β-lactamase gene as an indicator gene. The results obtained indicate that the control region of plasmid pT181 represents the target of the *plaC1* effect, which acts primarily by depressing the synthesis of plasmid pT181 countertranscripts, those small, untranslated RNA molecules playing the roles of negative effectors in the replication control mechanism of the plasmid. In turn, the reduction in countertranscript synthesis leads to an increase in the production of the initiator protein RepC, which is limiting for plasmid replication, and a higher plasmid copy number.

pT181 is a well-characterized replicon which is representative of a large class of staphylococcal plasmids (21). A plasmid-encoded protein, RepC, is essential (13) and limiting (14) for the autonomous replication of pT181. The *repC* transcript has a rather long leader sequence. The region corresponding to this leader, referred to as the control region, encodes two small, untranslated RNA molecules. These are transcribed from a common promoter in the opposite direction from the *repC* mRNA and are consequently called countertranscripts, or ctRNA. The countertranscripts act as negative effectors in the control of RepC synthesis (8) and, in turn, of plasmid pT181 replication. The present understanding of the system is that the countertranscripts interact with the *repC* leader and change its conformation in such a way that transcription termination is promoted at a Rho-independent site just upstream from the translation initiation signal for *repC* (R. P. Novick, S. Iordanescu, S. J. Projan, and I. Edelman, submitted for publication). Since countertranscript synthesis is presumed to be constitutive, any increase in the plasmid copy number should lead to a proportional increase in the countertranscript concentration, which in turn should shut off RepC synthesis and stop further plasmid replication. On the other hand, a decrease in copy number should be accompanied by a reduction in countertranscript concentration and derepressed RepC synthesis, enabling the plasmid to restore its normal copy number.

In a wild-type host, pT181 is maintained in about 25 copies per cell. *Staphylococcus aureus* chromosomal mutants in which the copy number of pT181 is about 10 times higher than that in a wild-type host have been isolated (5). One such mutant, SA1350, carrying the mutation *plaC1*, was used in the present work. A striking property of the *plaC1* mutation is its strict specificity for pT181. Even closely related plasmids such as pC221 are not amplified in SA1350.

Though plasmids depend on host functions for their replication, there are few known situations in which a chromosomal function can interfere specifically with the replication control of a plasmid. In *Escherichia coli*, the σ^{32} factor coded by the *rpoH* gene (4) was reported to be required for the transcription of the *repE* gene, which plays a central role in plasmid F replication and its control (23); consequently, mutations in the *rpoH* gene specifically affect plasmid F replication. Mutations in another *E. coli* locus, *pcnB*, were reported to reduce the copy number of plasmid pBR322 (9). The mechanism involved in this case is not yet known. The present paper attempts to elucidate the mechanism by which the *S. aureus* chromosomal mutation *plaC1* can specifically affect the replication control of plasmid pT181. Two questions were addressed: (i) what region of the pT181 genome is responsible for the specific response of this plasmid to the *plaC1* mutation, and (ii) whether (and if so, how) the *plaC1* mutation acts by interfering with the plasmid replication control mechanism. The results showed that amplification of pT181 in a *plaC1* host depends on the presence of a 220-base-pair (bp) pT181 segment (coordinates 158 to 377), including the countertranscript promoter, and that the *plaC1* mutation acts primarily by reducing the activity of this promoter.

MATERIALS AND METHODS

Organisms. *S. aureus* wild-type strain SA20 (NCTC 8325) and its derivative SA1350, which carries the *plaC1* mutation (5), were used throughout this study as hosts for the naturally occurring plasmids pT181 and pC221 and their derivatives. The vectors used for the construction of translational and transcriptional fusions with the β-lactamase (*bla*) gene have been described elsewhere (25; P.-Z. Wang et al., manuscript in preparation). pSA3800 (Novick et al., submitted) has been used for transcriptional fusions. In the construction of this vector, the *bla* promoter was deleted, multiple cloning sites (pUC19 polylinker) were introduced in front of the *bla* structural gene and its Shine-Dalgarno signal, and stop codons in all three frames were placed 30 bp upstream from the Shine-Dalgarno signal by the insertion of a 16-bp oligonucleotide. A *repC-bla* translational fusion, pWN1804, containing the *Hin*dIII-*Pvu*I fragment of pT181 (coordinates 885 to 0) has been constructed (Wang et al., in preparation). This construct has unique *Hpa*I and *Pvu*I sites located in the pT181 material. Derivatives of this hybrid

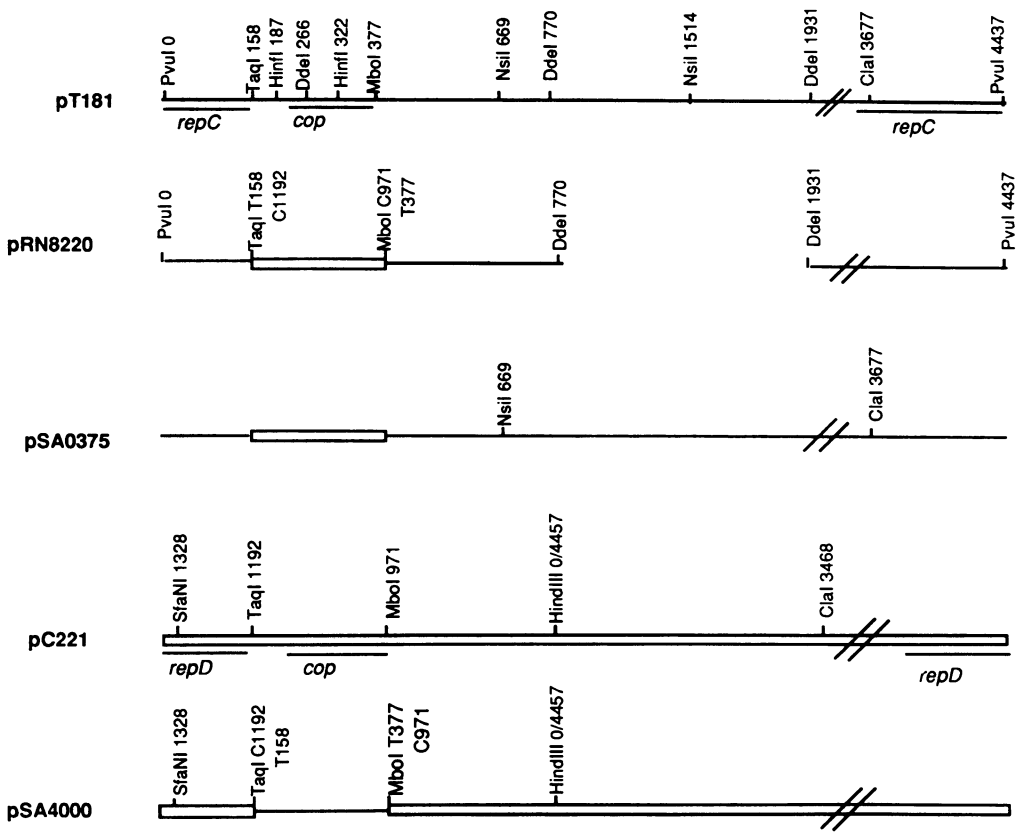

FIG. 1. Schematic structures of pT181-pC221 hybrids. pT181 sequences are shown as a single line, and pC221 sequences are shown as a double line. Only the restriction sites used in the construction of each hybrid are included. Coordinates are from the standard maps of pT181 (7) and pC221 (20). For the hybrids, the pT181 coordinates are preceded by a T, and the pC221 coordinates are preceded by a C. *rep* and *cop* mark the positions of the *rep* coding sequences and copy number control regions, respectively, and do not refer to transcription or translation of these elements. Maps are not to scale, and the major compression is shown.

carrying other control regions in front of the *repC* gene could be constructed by replacement of this *Hpa*I-*Pvu*I fragment.

Genetic transfers. Protoplast transformation and transduction of *S. aureus* were carried out as previously described (3, 6).

Plasmid copy number evaluation. Whole-cell lysates of plasmid-harboring strains prepared from cultures grown under conditions selective for the plasmids were electrophoresed on agarose gels, and evaluation of plasmid copy numbers was done by fluorescence densitometry of the ethidium bromide-stained gels (19).

DNA manipulations. Plasmid DNA was isolated by CsCl-ethidium bromide density gradient centrifugation of cleared lysates (15). Restriction enzymes were purchased from Boehringer GmbH (Mannheim, Federal Republic of Germany) and New England BioLabs, Inc. (Beverly, Mass.); T4 DNA ligase was from Collaborative Research, Inc. (Waltham, Mass.); and T4 DNA polymerase was from U.S. Biochemicals Corp. All enzymes were used as specified by the manufacturers. Restriction fragment isolation was done by extraction from polyacrylamide gels (10). M13 cloning was done by the method of Messing (11). DNA sequencing was carried out as described by Sanger et al. (22).

β-Lactamase assay. The determination of β-lactamase activity was done spectrophotometrically with a chromogene substrate, nitrocefin (17), and the activity was expressed in units per milligram (dry weight). One unit corresponds to 1 μmol of substrate hydrolyzed per h.

Construction of pT181-pC221 hybrids. The construction of pT181-pC221 hybrids is shown in Fig. 1. pSA0375 was derived from pRN8220, a construct in which a 220-bp fragment from pT181, including most of the control region (158 to 377), was substituted for by the corresponding region of pC221 (20). pRN8220 was missing a *Dde*I fragment (770 to 1931) from pT181 (Fig. 1). The complete genome of pT181 was restored in pSA0375 by ligating the *Cla*I-*Nsi*I fragment (3677 to 669) of pRN8220 to the pT181 fragment (669 to 3677) obtained by *Cla*I digestion and incomplete *Nsi*I digestion.

The reciprocal hybrid, pSA4000, was obtained by ligation of three fragments from pC221 (*Taq*I-*Sfa*NI, *Sfa*NI-*Hin*dIII, and *Hin*dIII-*Mbo*I) to the pT181 *Taq*I-*Mbo*I fragment (158 to 377). pSA4000 carries the pT181 control region in an otherwise unchanged pC221 plasmid (Fig. 1).

The structure of each of the hybrids was confirmed by restriction endonuclease mapping and sequencing of the control region and its boundaries.

RESULTS

Control region of plasmid pT181 is the target for the *plaC1* effect. The approach used to determine the pT181 region responsible for the amplification of this plasmid in SA1350 took advantage of two facts: (i) pT181 but not pC221 is amplified in SA1350 (5); and (ii) the two plasmids have a similar organization and a high degree of homology in their replication regions, so that the exchange of homologous regions can generate functional replicons (20).

TABLE 1. Correlation between copy numbers and β-lactamase activities of *rep-bla* translational fusions in wild-type (SA20) and *plaC1* (SA1350) hosts

Plasmid	Copy no.			β-Lactamase activity[a]		
	SA20	SA1350	Ratio (SA1350/SA20)	SA20	SA1350	Ratio (SA1350/SA20)
pT181	25	250	10.0	2.0	20.0	10.0
pC221	25	40–50	1.5–2.0	0.6	1.8	3.0
pSA0375	25	40–50	1.5–2.0			
pSA4000	25	250	10.0			

[a] The β-lactamase activities of *rep-bla* translational fusions carrying the control region of pT181 (pWN1804) or pC221 (pSA2652) are expressed in units per milligram (dry weight).

pT181 and pC221 encode Rep proteins with different specificities which under normal conditions can initiate replication only from the corresponding origin. The two plasmids also differ in the specificities of their replication control mechanisms. To determine which of these elements is responsible for the different behavior of the two plasmids in a *plaC1* host, two reciprocal hybrids were constructed (see Materials and Methods). pSA0375 is a pT181 replicon in which a 220-bp fragment encompassing most of the control region has been substituted for by the corresponding fragment from pC221, while in pSA4000, the pT181 control region has replaced the control region of an otherwise unchanged pC221 plasmid (Fig. 1). The copy numbers of these two hybrids in SA20 and SA1350 were determined. pSA4000 was amplified in SA1350 to the same degree as was pT181, while the copy number of pSA0375 was characteristic of pC221 (1.5 to 2.0 times higher in SA1350 than in SA20) (Table 1). The results given above show that the 220-bp control region (158 to 377) of pT181 is solely responsible for the plasmid amplification in SA1350. It can therefore be concluded that the *plaC1* mutation does not directly affect the Rep protein-origin interaction but interferes instead with the plasmid-encoded replication control mechanism.

RepC synthesis is derepressed in a *plaC1* host. The control region of pT181 contains several elements that could be targets of the *plaC1* effect: the major *repC* promoter, the promoter for the countertranscripts, and the region believed to be involved in the first step of the countertranscript-*repC* mRNA interaction, where most *cop* mutations are located (1).

The effect of the *plaC1* mutation on each of these elements was tested by using recently developed vectors for the construction of translational and transcriptional fusions (25). In these constructs, the β-lactamase (*bla*) gene of plasmid pI258 was used as an indicator. The activity of β-lactamase can be easily and accurately determined (12, 17). The replicon active in *S. aureus* present in these vectors is plasmid pC194, which is maintained in the same copy number in both wild-type and *plaC1* hosts (5).

In-frame *repC-bla* translational fusions carrying the control region of either wild-type pT181 or some of its Cop mutants have been constructed (Wang et al., in preparation). The effect of the *plaC1* mutation on the activity of β-lactamase expressed by each of these constructs was determined. For the construct pWN1804 carrying the wild-type pT181 control region in front of the *repC-bla* translational fusion, the activity of β-lactamase was about 10 times higher in SA1350 than in SA20 (Table 1).

***plaC1* mutation decreases activity of the pT181 countertranscript promoter.** The effect of *plaC1* on derepression of RepC synthesis might be the consequence of either an increased activity of the *repC* promoter(s) or an interference with the control mechanism of RepC synthesis. To differentiate these possibilities, two transcriptional fusions with the *repC* promoter were constructed: pSA2603 carried the *Nsi*I-*Dde*I pT181 fragment (669 to 266) which does not contain the countertranscript promoter, while pSA2601 had the pT181 *Nsi*I-*Taq*I fragment (669 to 158) which includes the countertranscript promoter. The β-lactamase activities expressed by these constructs were similar and were not affected by the *plaC1* host mutation (Table 2). Two conclusions could be drawn from these results: (i) the presence or absence of convergent transcription from the ctRNA promoter seems to have no significant effect on the activity of the *repC* promoter, which is not true in other examples of convergent transcription (26, 27); and (ii) the *plaC1* mutation does not directly affect the activity of the *repC* promoter. This last conclusion was also supported by the study of *repC-bla* translational fusions with Cop mutants in which the part of the control region containing the countertranscript promoter was deleted (unpublished results). The β-lactamase activity of these constructs was also not affected by the *plaC1* mutation, a fact which is in accord with the maintenance of such mutants at the same copy number in SA20 and SA1350 (data not shown).

TABLE 2. β-Lactamase activities of transcriptional fusions

Hybrid	Promoter used	β-Lactamase activity[a]		
		SA20	SA1350	Ratio (SA1350/SA20)
pSA2603	*repC* (ctRNA promoter absent)	20.0	20.0	1.00
pSA2601	*repC* (ctRNA promoter present)	19.6	19.8	1.00
pSA3804	pT181 countertranscript	18.0	2.0	0.11
pSA3810	pC221 countertranscript	16.0	6.4	0.40
pWN101	*bla*	180.0	140.0	0.77
pWN1803	*pre*	3.0	2.8	0.93
pRN6676	*agrA*	2.2	2.0	0.90
pWN1802	*B. subtilis* p43	360.0	320.0	0.89
pRN6675	*agr* p2	10.0	1.2	0.12

[a] Units per milligram (dry weight).

Since the activity of the *repC* promoter was not affected in a *plaC1* host, two other possibilities had to be considered: (i) countertranscript synthesis is decreased in SA1350, or (ii) *plaC1* reduces the interaction between countertranscripts and the *repC* mRNA.

To test these hypotheses, a transcriptional fusion in which the synthesis of β-lactamase was under the control of the pT181 countertranscript promoter (pT181 *Hin*fI fragment [187 to 321] cloned in the correct orientation into the vector pSA3800) was constructed. This construct, pSA3804, was introduced into SA20 and SA1350, and the activity of β-lactamase was determined. The β-lactamase activity expressed by this hybrid was 8- to 10-fold lower in the *plaC1* host than in the wild-type host (Table 2). On this basis, it can be concluded that the *plaC1* mutation acts primarily by reducing transcription from the pT181 countertranscript promoter, that this leads to an increased RepC synthesis and more plasmid replication rounds per cell cycle, and that the end result is plasmid amplification. The results described above also show a very good correlation between the reduction in pT181 countertranscript transcription and the increase in RepC synthesis, as estimated by the *bla* fusions.

This fact makes unlikely a secondary effect of the *plaC1* mutation on the interaction between countertranscripts and *repC* mRNA.

Response of the pC221 control mechanism to the *plaC1* mutation. Translational and transcriptional fusions similar to those described above have been constructed in order to analyze the behavior of plasmid pC221 in a *plaC1* host. In hybrid pSA3810, the synthesis of β-lactamase is under the control of the pC221 countertranscript promoter (pC221 fragment [1189 to 1026] cloned in the correct orientation into the pSA3800 vector). The β-lactamase activity expressed by this hybrid was about 2.5 times lower in the *plaC1* host than in the wild-type host (Table 2). pSA2652, a *repC-bla* translational fusion in which the 220-bp pT181 control region was substituted for by the homologous region of pC221, was constructed. For this hybrid, a 3.0-fold increase in β-lactamase activity was observed in the *plaC1* host (Table 1).

The results described above show that the pC221 countertranscript promoter is influenced less by the *plaC1* mutation than is the countertranscript promoter of pT181, and as a consequence, the increases in Rep synthesis and plasmid copy number are also smaller.

Responses of other promoters to the *plaC1* effect. The results presented so far have shown that the *plaC1* mutation acts by reducing the transcription from the pT181 countertranscript promoter and thus increasing the rate of synthesis of the Rep protein. This promoter effect seems to be quite specific: the related pC221 countertranscript promoter was significantly less affected, whereas the activity of the main RepC promoter was similar in SA20 and SA1350.

To ascertain the degree of specificity of the *plaC1* mutation, the responses of several other promoters from gram-positive organisms to this mutation were tested. The approach made use of transcriptional fusions with the *bla* gene as an indicator. The expression of β-lactamase under the control of all but one of the tested promoters was similar in both SA20 and SA1350. The unaffected promoters included the native pI258 *bla* promoter; the promoter for the pT181-encoded recombinase gene, *pre* (2); the promoter of *agrA*, an *S. aureus* chromosomal gene involved in the regulation of the synthesis of accesory proteins (18); and the *Bacillus subtilis* promoter p43 (24) (Table 2). The only exception was promoter p2 of the *agr* chromosomal region (18), which responded to the *plaC1* mutation with a reduction in expression of the same order as that seen in the pT181 countertranscript promoter (Table 2). The functional relationship, if any, between these two promoters affected by the *plaC1* mutation is not yet clear.

DISCUSSION

The *plaC1* mutation in *S. aureus* has been identified by its specific effect on the replication control of plasmid pT181 (5). The results presented in this paper show that the *plaC1* mutation acts by depressing the activity of the pT181 countertranscript promoter. This, in turn, should lead to a reduction in the countertranscript concentration, an increase in RepC synthesis, and a higher plasmid copy number. In the case of plasmid pT181, it is therefore worth underlining the precise correlation between the degree of reduction in countertranscript synthesis and the increase in RepC production in *plaC1*—both estimated by using *bla* transcriptional and translational fusions—and the increase in the plasmid copy number.

The reduction in pT181 countertranscript synthesis per plasmid copy in the *plaC1* host correlates well with the previously reported absence of Inc3A in this host (5) because it has been shown that the countertranscripts represent the effectors for the Inc3A effect (14, 16).

The effect of the *plaC1* mutation presents a high degree of specificity. The pC221 countertranscript promoter, which is quite homologous to that of pT181 (20), is much less affected, while most other promoters are indifferent to the *plaC1* mutation. On the other hand, *plaC1* was also found to interfere with the activity of the *agr* p2 promoter, which plays a central role in the activity of the *agr* system, which in turn is involved in the control of an entire class of genes expressed postexponentially (18). The amplification of pT181 in SA1350 does not depend on the growth phase of the culture (data not shown). It can be suggested that *plaC* codes for an activator of the transcription of a specific class of genes which are not essential for cell survival, inasmuch as the growth of the cells is not significantly affected by the *plaC1* mutation under normal conditions. The fact that the promoter controlling the synthesis of the negative effectors in pT181 replication control falls into this class might not be fortuitous. This might provide a way in which the countertranscript synthesis could be modulated by conditions affecting cell growth. Such a modulation might, in turn, play a role in plasmid stability by permitting increased RepC production under specific conditions. The cloning of the *plaC* gene, which is in progress, will help to explain the biological significance of the dependence of the pT181 countertranscript promoter on the function of this host determinant.

In the case of plasmid pC221, the increase in copy number in the *plaC1* host was somewhat smaller (1.5- to 2.0-fold) than that expected (3-fold) on the basis of the increase in RepD synthesis as evaluated by *bla* fusions. It is possible to speculate that in this case, some other factor(s) can interfere with the ability of the plasmid to respond to higher amounts of Rep protein with a proportional increase in replication.

ACKNOWLEDGMENTS

This study was supported by Public Health Service grant AI-24327 from the National Institutes of Health.

I thank Richard Novick and Ellen Murphy for critically reading the manuscript. The technical assistance of Julie Lin-Breidt is gratefully acknowledged.

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