The *Staphylococcus aureus* chromosomal gene *plaC*, identified by mutations amplifying plasmid pT181, encodes a sigma factor

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ABSTRACT

The *Staphylococcus aureus* chromosomal gene *plaC*, identified by mutations such as *plaC1* that lead to the amplification of plasmid pT181, has been cloned and sequenced. The *plaC* gene encodes a protein with high similarity (79% identity) with the vegetative sigma factor of *Bacillus subtilis*, *sigA*, suggesting that it acts as an RNA polymerase sigma factor in *S.aureus*. The *plaC1* mutation was found to be a C to T transition leading to a proline to serine substitution at amino acid residue 209 of the protein. In other sigma factors this region of the protein is involved in specific recognition of the –10 promoter sequence. The change in sigma factor activity due to this mutation is characterized by its strict specificity for a limited number of promoters and the rather high amplitude of the effect.

INTRODUCTION

Plasmid pT181 is maintained at a much elevated copy number in *Staphylococcus aureus* strains carrying the *plaC1* mutation (1). The effect of this mutation is specific for pT181; other staphylococcal plasmids, including the closely related plasmid pC221 (2), are not significantly amplified in this host mutant.

The *plaC1* mutation was shown to affect the regulation of pT181 replication (3). Two small, untranslated, antisense RNA molecules, called countertranscripts, play an essential role as negative effectors in pT181 replication control (4, 5). In the *plaC1* host, transcription from the pT181 countertranscript RNAs (ctRNAs) promoter is significantly reduced. The reduction in the synthesis of the ctRNAs leads to an increased production of RepC, the specific, plasmid-encoded replication initiator of pT181 (6). This in turn leads to an increased copy number of pT181 in the mutant host (3). The effect of the *plaC1* mutation on the transcription from eight other *S.aureus* promoters was tested. Only one, the P2 promoter of the *agr* chromosomal region (7) was affected in a way similar to the pT181 ctRNA promoter (3).

These data suggested that the *plaC* gene product is involved in transcription, and that the effect of the *plaC1* mutation on this process is rather subtle. To obtain further evidence on the nature

and function of the PlaC product the chromosomal gene has been cloned and characterized. These results are presented in the present report.

MATERIAL AND METHODS

Organisms

S.aureus strains SA20 (NCTC 8325) and its derivative SA1350 carrying the *plaC1* mutation (1) have been used throughout this study.

pSK265 (8) and pRN6321 (9) were used as vectors in *S.aureus* and pBluescriptSK (Stratagene) as a vector in *E.coli* cloning.

Genetic transfers

Protoplast transformation and transduction of *S.aureus* and *Escherichia coli* were carried out as previously described (10, 11, 12).

DNA manipulations

Standard procedures were used for DNA restriction fragment isolation, nick translation and Southern hybridization (13). M13 cloning was done according to the method of Messing (14). DNA sequencing was carried out by the dideoxynucleotide chain termination method (15) using Sequenase (United States Biochemical) and [³⁵S] dATP. The sequence of both strands of the DNA was determined using overlapping fragments cloned in both orientations into the M13 vector.

The amino acid sequences deduced for the proteins potentially encoded by the studied DNA were compared to the GenBank data base using the FASTA program (16).

Cloning of the *plaC* gene

Plasmid pSA0348, a pT181 derivative carrying the *cop-658* mutation, was found to be unable to establish itself in a *plaC1* host. This fact was utilized in the cloning of the wild type *plaC* gene as follows. pT181 DNA has two recognition sites for the restriction endonuclease *NsiI*. The small *NsiI* fragment is dispensable for plasmid maintenance. The purified large *NsiI* fragment of pSA0348 was ligated to *NsiI* cleaved chromosomal

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DNA isolated from a wild type strain. The ligation mixture was transformed into a *placI* host with selection for the tetracycline resistance marker of the pSA0348 plasmid; the plasmid was expected to establish itself only if it carried the wild type *plac* gene.

β -lactamase assay

A specially devised vector, pSA3800, carrying a promoterless β -lactamase (*bla*) gene preceded by stop codons in all three frames and multiple cloning sites (5), has been used for the construction of *bla* transcriptional fusions. Previously described hybrids pSA3804 and pSA3810 (3) have the *bla* gene transcribed under the control of the ctRNA promoter of plasmid pT181 and pC221, respectively. Constructs similar to pSA3804 carrying mutations in the pT181 ctRNA promoter sequence have been obtained in the present work.

The determination of β -lactamase activity was done spectrophotometrically using a chromogenic substrate, nitrocefin (17).

RESULTS

Cloning of the *plac* gene

The strategy used for the cloning of the *plac* gene took advantage of the observation that a pT181 copy mutant, pSA0348, was unable to establish itself in a *placI* host. The rationale was that if the *plac*⁺ allele is dominant, the cloning of a chromosomal fragment carrying a complete, functional *plac*⁺ gene into pSA0348 would allow the establishment and maintenance of this plasmid in a mutant host.

The use of this approach led to the isolation of three pSA0348 clones, each carrying several *NsiI* chromosomal fragments. The

analysis of these clones has shown that all had in common a 3.1 kb *NsiI* chromosomal fragment, which when recloned by itself into pSA0348 allowed the plasmid to be stably maintained in a *placI* host. The pSA0348 moieties of these clones were still unable to establish in the *placI* mutant, ruling out the presence of secondary mutations in pSA0348.

The 3.1 kb *NsiI* fragment was subsequently cloned into the pSK265 vector (8) generating plasmid pSA2727. This construct was found to efficiently complement a chromosomal *placI* mutation. The same complementation could be obtained with an internal 2.3 kb *ClaI* fragment derived from the original *NsiI* fragment, which therefore carries a complete, functional *plac* gene. Using part of this fragment as a probe, the same 2.3 kb *ClaI* fragment isolated from a *placI* mutant was cloned first in *Escherichia coli* by colony hybridization, and then recloned into *S. aureus*.

Complementation studies

The constructs pSA2728 and pSA2732 carry the 2.3 kb *ClaI* fragment derived from the wild type or the *placI* mutant, respectively, cloned in the same orientation at the *ClaI* site of the vector pRN6321 (9). The effect of the presence of the wild type or mutant *plac* gene on a multicopy plasmid has been evaluated both in *plac*⁺ and *placI* hosts using these constructs. For each situation two parameters have been determined: the copy number of plasmid pT181 and the level of transcription from the pT181 ctRNA promoter, as estimated by the use of a *bla* transcriptional fusion (Table 1). In all cases there was a good inverse correlation between the two parameters.

Figure 1. A. Sequence of the *S. aureus* *ClaI* fragment carrying the *plac* gene. The potential -35 and -10 promoter sequences and ribosome binding site for the *plac* gene are underlined. The change in nucleotide and amino acid sequence due to the *placI* mutation are presented. B. Simplified restriction map of the *ClaI* fragment also showing the main open reading frames identified. C. The ability of different deletions to complement a chromosomal *placI* mutation. The heavy lines show the residual sequences of each construct.

The *plaC*⁺ gene present in multiple copies efficiently complemented a chromosomal *plaC1* mutation but did not have a significant effect in a wild type host. The cloned *plaC1* allele had no effect in either wild type or mutant hosts (Table 1). These results show that the *plaC*⁺ allele is dominant over *plaC1* and that its presence in multiple copies has no effect on the Plac phenotype.

Identification of the *plaC* gene

The complete sequence of the 2.3 kb *ClaI* fragment derived from a wild type strain has been determined and is presented in Fig. 1, along with a simplified restriction map of this region. A single, complete coding sequence of significant length has been identified in the *ClaI* fragment. Two other coding sequences which extend outside of the *ClaI* fragment have also been detected (Fig. 1). To ascertain that the internal coding sequence corresponds to the *plaC* gene, as defined genetically, several deletions in the *ClaI* fragment have been constructed and their ability to complement a chromosomal *plaC1* mutation tested. The results obtained (Fig. 1) supported the identification of the *plaC* gene as the internal coding sequence. This coding sequence starts with an AUG codon preceded by a sequence closely resembling a ribosome binding site.

Promoter activity has been detected 5' to the start of the *plaC* gene by construction of a transcriptional fusion with a promoterless *bla* gene. By comparison of the sequence of this region with the canonical -35 and -10 promoter sequences, two potential promoters have been identified at 50 and 110 bp respectively upstream from the translational start of the *plaC* gene (Fig. 1A). Using this *bla* fusion it was also shown that the level of transcription from the *plaC* promoter(s) was similar in wild type and *plaC1* hosts and therefore the activity of this promoter(s) is not affected by the *plaC1* mutation.

The *plaC* gene can encode a 368 amino acid protein with a molecular weight of 42,177 dal. The amino acid sequence of this protein, as deduced from the DNA sequence, was compared to proteins in GenBank data base. The *plaC* gene product was found to have a 78.8% amino acid sequence identity with the product of the *sigA* gene of *Bacillus subtilis* (Fig. 2), encoding the vegetative sigma factor (18).

The site of the *plaC1* mutation

By restriction fragment exchange between pSA2728 (*plaC*⁺) and pSA2732 (*plaC1*), the *plaC1* mutation has been mapped in the *SnaBI-FspI* fragment. The sequence of this fragment derived from the *plaC1* clone has been determined. The *plaC1* mutation has been identified as a C to T change at position 1,444 of the

sequence. The location of the *plaC1* mutation within the internal coding sequence further supports the assignment of this sequence as the *plaC* locus.

The *plaC1* mutation leads to a proline to serine substitution at amino acid residue 209 of the protein. This change occurs in a region of the protein highly conserved in all sigma factors studied so far (19). In the case of other sigma factors, this region has been shown to be involved in a specific interaction with the -10 promoter sequence (20, 21).

The effect of the *plaC1* mutation on the transcription from pT181 cRNA promoters carrying down mutations in or immediately adjacent to the -10 sequence has been tested. Two previously isolated mutations, *cop-619* (21) and *cop-714* (Iordanescu, unpublished data), have been used in this approach. For each mutant a transcriptional fusion having the same structure as pSA3804, which carries the wild type pT181 cRNA promoter (3), has been constructed. The results obtained (Table 2) showed the expected reduction in transcription due to these mutations in a wild type host and a further reduction in the *plaC1* host. The amplitude of the effect of the *plaC1* mutation on the mutated cRNA promoters was about two times less than in the case of the wild type (Table 2). For comparison, a similar fusion with the cRNA promoter derived from the closely related pC221 plasmid, was even less affected by the *plaC1* mutation, despite the presence of a -10 sequence identical to that of pT181 (Table 2).

	10	20	30	40	50	60
plaC protein	MSDNTVKIKKQ	IDPTLTLEDVKKQ	LIEGKKGHLSH	EIAEKLFND	ISDQMDDFD	
sigA protein	MADKQTHETEL	TFDQVKEQLT	SGKKRGVLT	YEEIAERMS	SFEIESDQMD	EYEE
	70	80	90	100	110	
plaC protein	QLNDNDISLV	NEKSSD-----	TDEKLNPS	DLSPPGVK	INDPVRMYL	KEIGRWNL
sigA protein	FLGEGVELISE	NEETEDPN	IQQLAKAE	EEFDNL	SVPPGKIND	PVRMYLKEIGRWNL
	120	130	140	150	160	170
plaC protein	LSAQEEIELAK	RIEQGDEVA	KSRLAENLR	VVSIKRV	VGRGMLF	LDLIQENGMGLKA
sigA protein	LSAKKEIAYA	QKIEEGDE	SKRRLAEN	LRVVSIA	KRVVGRG	MLFLDLIHEGNMGLMKA
	180	190	200	210	220	230
plaC protein	VEKFDYKGF	KFSTYATW	NIQAITRA	IQADQART	IRIPVHM	VETINKLIRVQRQLQDLG
sigA protein	VEKFDYKGF	KFSTYATW	NIQAITRA	IQADQART	IRIPVHM	VETINKLIRVQRQLQDLG
	240	250	260	270	280	290
plaC protein	RDPAPEEIGE	MDLPAEKV	REILKTAQ	EPVSL	ETPIGEED	DSHLGDFIEDQEAQSPSDHA
sigA protein	REPTPEEIA	EDMDLTP	KEVREILK	TAQEPV	SL	ETPIGEEDDSHLGDFIEDQEAQSPSDHA
	300	310	320	330	340	350
plaC protein	AYELLKEQL	EDVLDTL	DR	ENVLR	FLDGR	TRTLEE
sigA protein	AYELLKEQL	EDVLDTL	DR	ENVLR	FLDGR	TRTLEE
	360	370				
plaC protein	RKLHPSRS	KRLKDFMD				
sigA protein	RKLHPSRS	KRLKDFLE				

Figure 2. Comparison of the amino acid sequence of *plaC* with *sigA* from *B. subtilis*. A colon shows identity and a point a conservative substitution.

Table 1. Effect of the presence in multiple copies of wild type and mutant *plaC* gene on the Plac phenotype.

Plasmid	Host	pT181 copy number	Value	Ratio [§]	β -lactamase activity*	Ratio [§]
—	<i>plaC</i> ⁺	22	1.00	6.32	1.00	
	<i>plaC1</i>	250	11.40	0.58	0.09	
pSA2728	<i>plaC</i> ⁺	22	1.00	6.06	0.96	
(<i>plaC</i> ⁺)	<i>plaC1</i>	22	1.00	6.28	1.00	
pSA2732	<i>plaC</i> ⁺	22	1.00	5.24	0.86	
(<i>plaC1</i>)	<i>plaC1</i>	230	10.45	0.73	0.11	

*The β -lactamase activity of pSA3804 carrying the *bla* gene under the control of the pT181 cRNA promoter is expressed in units/mg (dry weight) bacterial cells

[§]The values in the *plaC*⁺ host without any plasmid were arbitrarily taken as 1.00.

Table 2. Effect of changes in the -10 promoter sequence on the response to the *plaC1* mutation

-10 promoter sequence*	Derivation	β -lactamase activity (u/mg) [§]		
		<i>plaC</i> ⁺	<i>plaC1</i>	Ratio <i>plaC1/plaC</i> ⁺
CGTTTAAT	pT181 wild type	6.32	0.58	0.09
CTTTTAAT	pT181 <i>cop-714</i>	1.13	0.20	0.18
CGTCTAAT	pT181 <i>cop-619</i>	0.51	0.10	0.20
CGTTTAAT	pC221 wild type	5.37	2.16	0.40

*The sequence presented includes two nucleotides 5' to the actual -10 sequence in order to show the site of one of the mutations studied.

[§]*bla* transcriptional fusions with the same structure as pSA3804 (3) were used in this experiment.

An independently isolated mutation, *plaC3*, which has a phenotype similar to *plaC1*, was found to have the same sequence alteration as *plaC1*.

DISCUSSION

The *S. aureus* *plaC* gene was identified by mutations leading to the amplification of plasmid pT181. The results presented here show that the *plaC* gene encodes a protein with high similarity with the vegetative sigma factor of the RNA polymerase from *B. subtilis*. Based on this fact, and the previously described effect of *plaC* mutations on transcription from specific promoters (3), it seems safe to assume that the product of the *plaC* gene acts as a sigma factor in *S. aureus*.

Many bacteria have, in addition to a major vegetative sigma factor responsible for transcription from most promoters, several other minor sigma factors acting on certain classes of promoters which control genes that are normally expressed only under specific conditions. At the present time it is difficult to conclude if the product of the *plaC* gene represents the major sigma factor of *S. aureus* or a minor species. No other region with homology with the *plaC* sequence was detected by Southern analysis in the *S. aureus* chromosome (data not shown). This fact, together with the strong similarity with the vegetative sigma factor from *B. subtilis*, supports the idea that *plaC* encodes the major sigma factor of *S. aureus*. On the other hand, the organization of the region encoding the major sigma factor is quite similar in *Escherichia coli* and *B. subtilis* (23). In these species the vegetative sigma is encoded by the third gene of an operon in which the second gene codes for DNA primase. In contrast, the *plaC* gene of *S. aureus* is directly preceded by an active promoter and the proteins potentially encoded by the adjacent open reading frames do not have any significant similarity with DNA primase or any other protein in the GenBank data base. Attempts to determine if *plaC* gene function is or is not essential for cell viability, which are in progress, will help clarify this aspect of the problem.

Knowledge of the role of the *plaC* gene product in *S. aureus* will also help in evaluating the properties of the prototype *plaC1* mutation. The effect on transcription of *plaC1* is specific for a small number of promoters; this might either represent the consequence of a subtle change in the specificity of the sigma factor due to the mutation, or it may be that the *plaC* sigma factor acts only at a limited number of promoters.

The *plaC1* mutation leads to a proline to serine substitution in a region of the protein which in other sigma factors is involved in the specific recognition of the -10 promoter sequence. However, there is presently no evidence that the *plaC1* mutation affects the specificity of the sigma factor for the -10 sequence. The pC221 ctRNA promoter, having an identical -10 sequence to the pT181 ctRNA promoter, is much less affected by the *plaC1* mutation. On the other hand, the P2 *agr* promoter responds to the *plaC1* mutation with a decrease in activity similar to that observed for the pT181 ctRNA promoter (3) although the sequence of the P2 promoter (24) has no homology with the pT181 ctRNA promoter. Two different mutations in or immediately near the -10 sequence of the pT181 ctRNA promoter still responded to the *plaC1* mutation, although the effect for both of them was somewhat less than that observed with the wild type promoter. All these results argue against a change in recognition specificity of the -10 promoter sequence due to the *plaC1* mutation.

The amplitude of the effect of the *plaC1* mutation on the transcription from the pT181 ctRNA promoter is higher than that reported for most other sigma factor mutations (25, 26, 27), suggesting an important change in the way in which the mutated *plaC* sigma factor interacts with the pT181 ctRNA promoter. No mutations in the pT181 ctRNA promoter which suppress the effect of the *plaC1* mutation have yet been isolated (Iordanescu, unpublished results).

The fact that two independently isolated mutations, *plaC1* and *plaC3*, have the same change in sequence suggests that only a limited number of changes in the sigma factor could lead in a specific way to an impaired transcription from the pT181 ctRNA promoter.

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