Vol. 190, No. 4

A Transposase-Independent Mechanism Gives Rise to Precise Excision of IS256 from Insertion Sites in *Staphylococcus epidermidis*[∇][†]

Susanne Hennig and Wilma Ziebuhr*

Universität Würzburg, Institut für Molekulare Infektionsbiologie, Röntgenring 11, 97070 Würzburg, Germany

The mobile element IS256 causes phase variation of biofilm formation in *Staphylococcus epidermidis* by insertion and precise excision from the *icaADBC* operon. Precise excision, i.e., removal of the target site duplications (TSDs) and restoration of the original DNA sequence, occurs rarely but independently of functional transposase. Instead, the integrity of the TSDs is crucial for precise excision. Excision increased significantly when the TSDs were brought into closer spatial proximity, suggesting that excision is a host-driven process that might involve most likely illegitimate recombination.

IS256 is an insertion sequence (IS) element occurring in the genomes of staphylococci and enterococci, either as an integral part of the composite transposon Tn4001 or in multiple free copies (3, 6, 17). Recent studies indicate that IS256 is particularly common in the genomes of multiresistant, biofilm-forming *Staphylococcus epidermidis* isolates resident in the hospital environment (13). *S. epidermidis* biofilm formation is mediated mainly by a polysaccharide intercellular adhesin (PIA) (16), whose synthesis enzymes are encoded by the *icaADBC* operon (11). We demonstrated earlier that *ica* operon expression and subsequent biofilm formation are subject to phenotypic variation (18). In approximately 30% of spontaneous biofilm-negative variants, the switch from on to off in biofilm formation is caused by insertion of IS256 into *icaA* or *icaC* (19). Typically, IS256 creates 8-bp target site duplications (TSDs) on either end upon active transposition. Reversion from off to on was detected after repeated passages of *S. epidermidis ica*::IS256 insertion mutants (19). Interestingly, restoration of PIA production was accompanied by precise excision of IS256, including the initially duplicated target site, from the *ica* sequence (19). While the insertion frequency of IS256 into the *ica* operon is about 10^{-6} per cell generation, the excision frequency was reported to be considerably lower, at $<10^{-8}$ per cell generation, indicating that restoration of the PIA-mediated biofilm by IS excision must be a rare event (19). However, the molecular mechanism of precise excision—complete elimination of IS256, including the duplicated target site, and restoration of the original *ica* sequence—has not been elucidated so far.

Precise excision of IS256 from the *icaC* gene does not require a functional IS256 transposase. To test whether precise excision of IS256 from the *ica* operon is a transposase-driven process, we constructed two mutants of the biofilm-positive, IS256-negative strain *S. epidermidis* CSF41498 (Table 1). The first mutant, *S. epidermidis* CSF41498-1, carried a chromosomal copy of a previously described *icaC*::IS256 insertion (14), including an 8-bp TSD (GGTGGTTA), while the second, *S. epidermidis* CSF41498-2, carried an *icaC*::IS256 insertion with a 20-bp deletion within the *tnp* gene, rendering the transposase inactive (Table 1) (14). Both mutants exhibited a biofilm-negative phenotype. Since it was known from previous experiments that precise excision is a rare event (19), biofilm-positive revertants were enriched by serial passages in tissue culture flasks as described previously (19), with the following modification: at each passage step (every 24 h), the medium was discarded and flasks were washed with phosphate-buffered saline before new Trypticase soy broth was added. After 7 to 14 days, the adhering bacterial cells were scratched from the bottom and appropriate dilutions were plated onto Congo red agar (CRA). In all of three independent experiments, revertants could be detected as rough and black colonies on CRA, for both CSF41498-1 and CSF41498-2. Further analyses of these revertants by PCR using *icaC*-specific primers and by nucleotide sequencing revealed that the majority of them still carried the IS256 insertion (12), while others had lost the IS element completely and restored the original *icaC* sequence. IS256- and *icaC*-specific Southern hybridizations of eight members of the latter group demonstrated that the removal of the element was not accompanied by new IS256 insertions in the *S. epidermidis* chromosome (Fig. 1A and B). These results suggest that precise IS256 excision is not associated with transposition but is mediated by a transposase-independent mechanism.

Alteration of TSD homology abolishes precise excision. In the next step, we tested whether precise excision from the chromosome might occur as a result of a recombination process, with the 8-bp TSDs serving as homologous regions. To answer this question, we introduced a 3-bp deletion (GGT) into the right TSDs of CSF41498-1 and CSF41498-2, thereby reducing the homology between the TSDs to 3 bp (**GGT**G GTTA/**GGT**TACAT). After serial passages of the corresponding mutant strains CSF41498-3 and CSF41498-4 (Table 1), biofilm-positive revertants were isolated as black, rough colonies on CRA. However, despite extensive PCR screening in seven independent experiments, we could not detect precise excision of IS256 in any of the biofilm-forming revertants.

* Corresponding author. Present address: School of Biomedical Sciences, The Queen's University of Belfast, 97 Lisburn Road, Belfast BT9 7BL, Northern Ireland, United Kingdom. Phone: 44 (1)28-9097-2073. Fax: 44 (1)28-9097-2124. E-mail: w.ziebuhr@qub.ac.uk.

TABLE 1. Strains and plasmids used in this study[a]

Strain or plasmid	Relevant characteristic(s)	Reference
S. epidermidis strains		
RP62A	*ica* positive, IS*256* positive	ATCC 35984
CSF41498	*ica* positive, IS*256*-negative biofilm former	8
CSF41498-1	*icaC*::IS*256* insertion (at nt 600)	This study
CSF41498-2	*icaC*::IS*256* Δ*tnp* insertion	This study
CSF41498-3	*icaC*::IS*256* insertion, 3-bp deletion of right TSD	This study
CSF41498-4	*icaC*::IS*256* Δ*tnp* insertion, 3-bp deletion of right TSD	This study
Plasmids		
pBT2	*E. coli*-*Staphylococcus* shuttle vector with a thermosensitive origin of replication for gram-positive bacteria	4
pTH1	pBT2 derivative carrying a *spc*::IS*256* insertion at nt 63, including an 8-bp TSD (AGATCTAT)	This study
pTH2	pTH1 derivative with a truncated IS*256* copy of 166 bp consisting of IR$_L$, IR$_R$, and intact 8-bp TSDs	This study
pTH3	pTH1 derivative with 2-bp deletion in the right-hand TSD	This study
pTH4	pTH1 derivative with 4-bp deletion in the right-hand TSD	This study
pTH5	pTH1 derivative with 6-bp deletion in the right-hand TSD	This study

[a] Details on construction and a list of the oligonucleotides used can be found in Table S1 in the supplemental material.

Analyses of these strains indicated that they had switched from PIA-mediated biofilm formation to the expression of a protein-mediated biofilm, which is described elsewhere (12). Our data demonstrate that a reduction of TSD homology abolishes precise excision or at least diminishes the process to a point below the detection limit of this experimental setup.

Quantification of IS*256* excision events. To quantify the rate of excision events, we constructed shuttle plasmid pTH1, carrying a spectinomycin resistance cassette (*spc*) inactivated by an artificial IS*256* insertion, including 8-bp TSDs (Table 1). The plasmid was transformed into the spectinomycin-sensitive *S. epidermidis* CSF41498 background, and the resulting strain was screened for the occurrence of spectinomycin-resistant colonies that had lost the IS*256* insertion. This positive selection approach allowed us to analyze a considerably larger number of colonies than in the previous approach and to calculate the precise excision rates per cell and generation. The data underline that precise excision is a rare event, on the order of magnitude of 10^{-11} per cell and generation when using vector pTH1 (Table 2). This low excision rate suggests that a host-mediated recombination process is behind precise IS*256* excision. Also, Southern blot analysis revealed no new IS*256* insertions in the chromosome in these strains (data not shown).

Next, we wanted to analyze the minimal homology requirement of the IS*256* TSDs for precise excision. Therefore, we introduced deletions into the right-hand TSD of *spc*::IS*256*, leading to mismatches of two, three, and four base pairs and resulting in plasmids pTH3, pTH4, and pTH5, respectively (Table 1). No precise excision event was detected in any of the TSD mutants, and the probability of precise IS*256* excision was calculated to be $<10^{-13}$ per cell and generation (Table 2).

These data support the hypothesis that the microhomologies at either end of the element are a necessary prerequisite for
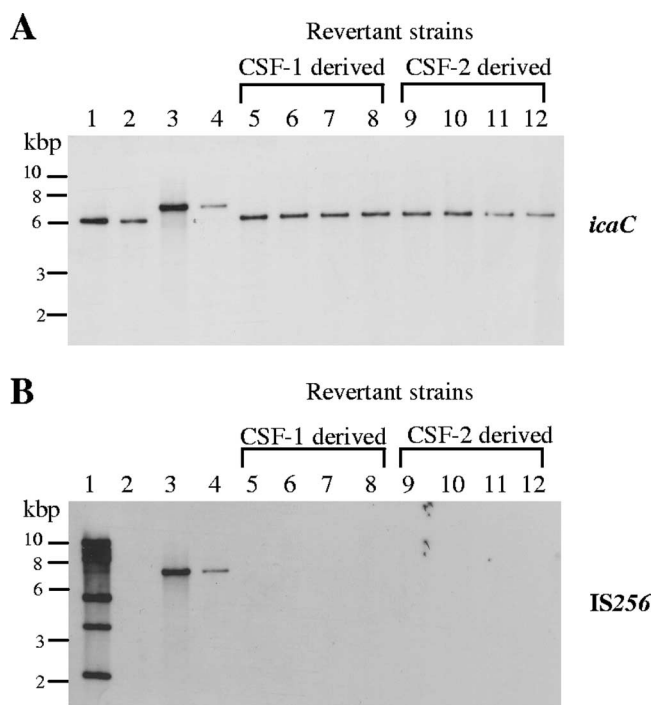
FIG. 1. Genetic analysis of biofilm-forming revertants derived from *S. epidermidis* CSF41498 *icaC*::IS*256* insertion mutants. Southern hybridizations of EcoRI-restricted chromosomal DNA were performed using *icaC*-specific (A) and IS*256*-specific (B) DNA fragments as probes (19). Lanes 1, *S. epidermidis* RP62A; lanes 2, *S. epidermidis* CSF41498 wild type; lanes 3, *S. epidermidis* CSF41498-1; lanes 4, *S. epidermidis* CSF41498-2; lanes 5 to 8, *S. epidermidis* CSF41498-1-derived revertants; lanes 9 to 12, *S. epidermidis* CSF41498-2-derived revertants.

excision. At the present stage of experimental work, we cannot decide unambiguously how TSD-dependent IS*256* excision proceeds on the molecular level. However, the data collected so far strongly imply a host-mediated recombination process as the underlying mechanism. Since RecA-dependent recombination requires sequence homologies of 300 bp or more and site-specific recombination relies on site-specific integrases along with defined DNA recognition sites (2, 10), it is rather unlikely that one of these mechanisms mediates precise IS*256* excision. In contrast, illegitimate recombination by so-called

TABLE 2. Excision rates of IS*256* from the *spc* gene, using a plasmid-based system

Plasmid	Right/left TSD sequence[a]	IS*256* sequence	Excision frequency (per cell generation)[b]
pTH1[c]	AGATCTAT/AGATCTAT	Full length	1.5×10^{-11}
pTH2[d]	AGATCTAT/AGATCTAT	166-bp fragment	9.8×10^{-11}
pTH3[c]	AGATCTAT/AGATA**TT**	Full length	$<2.0 \times 10^{-13}$
pTH4[c]	AGATCTAT/AGAT**TTGG**	Full length	$<4.7 \times 10^{-13}$
pTH5[c]	AGATCTAT/AG**TTGGAA**	Full length	$<1.2 \times 10^{-13}$

[a] Mismatches between the TSDs are shown in bold.
[b] Data are medians for all experiments.
[c] Experiments were carried out in triplicate.
[d] Experiments were repeated six times.

replicational slippage would provide a model for this phenomenon. Precise excision was described for other transposons as well (5, 9), and for Tn*10*, replicational slippage was suggested to be the underlying mechanism (7). Replicational slippage requires short homologous DNA stretches located in close vicinity to each other on the same DNA molecule. In *Escherichia coli*, eight nucleotides appears to be the minimal length required for slippage (1, 8), and slight sequence differences were already shown to reduce the deletion frequency dramatically (15). The same seems to hold true for IS*256* in *S. epidermidis* in this report, as eight homologous base pairs was sufficient for precise excision, while reduction to six base pairs abolished the process to a point below the detection limit.

Narrowing the distance between the TSDs results in an increase of precise excision rates. Last, we tested whether the spatial distance between the target site homologies has an impact on excision frequencies. To this end, we constructed pTH2 (Table 1). In this case, we truncated the IS*256* copy on pTH1 considerably, leaving only the noncoding regions (NCRs) of the element on either end (containing the left and right inverted repeats [IR_L and IR_R, respectively]) as well as the 8-bp TSDs intact, separated by 166 bp of DNA consisting of the 101-bp left NCR, a 15-bp transposase gene remnant, and the 50-bp right NCR. The excision frequency of this truncated IS*256* version in pTH2 (Table 2) showed a significant increase compared to that for pTH1 carrying a full-length IS*256* copy (one-tailed Mann-Whitney U test; $P < 0.025$). From other studies, it is known that a decrease of the distance between repeats increases replicational slippage rates significantly (2, 4). Apparently, this also applies to IS*256*. Thus, the model of replicational slippage provides an interesting hypothesis for future experimental work to investigate precise IS*256* excision in molecular detail.

Concluding remarks. Our data substantiate that IS*256*-mediated phase variation involves two distinct processes, namely, transposition of IS*256* and transposase-independent precise excision. The frequencies of these processes differ dramatically, by at least 5 orders of magnitude, with precise excision being an extremely rare event. Interestingly, we found that with respect to biofilm formation, *S. epidermidis* CSF41498 is obviously able to compensate for the low reversion rate due to precise excision by frequent switching to an alternative, protein-mediated biofilm, highlighting again the functional importance of the biofilm lifestyle for *S. epidermidis* (12). Thus, the latter mechanism appears to be of more biological significance than precise excision of IS*256*. Taken together, the data obtained in this study add to our understanding of IS activity and function in bacterial genomes and their impact on the generation of genetic and phenotypic variety.

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