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SHORT COMMUNICATION

Rapid Method for the Identification of Essential Genes in *Staphylococcus aureus*

Mingming Xia,* R. Dwayne Lunsford,† Damien McDevitt,† and Serban Iordanescu*,[1]

*Public Health Research Institute, New York, New York 10016; and †SmithKline Beecham Pharmaceuticals, Collegeville, Pennsylvania 19426

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A strategy based on a vector host-dependent for autonomous replication, pSA3182, was utilized both for the rapid screening for *Staphylococcus aureus* genes essential for cell viability and for the introduction of specific polarity-neutral deletions in nonessential genes. The results obtained support the use of pSA3182 for both purposes. © 1999 Academic Press

Recently, the complete sequence of several bacterial genomes has been determined (Blattner *et al.,* 1997; Bult *et al.,* 1996; Fleishmann *et al.,* 1995; Fraser *et al.,* 1996; Kunst *et al.,* 1997). Based on this information a variety of studies, both fundamental and applied, now have become possible. In the case of a pathogenic microorganism, such as *Staphylococcus aureus,* the availability of the genome sequence makes possible studies attempting to identify genes that are essential either for viability of the microorganism *in vitro* or for its ability to cause infections. The products of both classes of genes can be considered as potential targets in the effort to develop new classes of antimicrobial agents.

The aim of the present work was to develop a protocol that allowed for the screening of *S. aureus* genes essential for viability. The system was also suitable for the introduction of defined polarity-neutral deletions in genes that are not essential for viability but might be involved in pathogenicity. The approach used to evaluate the essential character of a gene was based on the insertion by a single crossover of a specific DNA sequence both in the middle of a structural gene, with the inherent inactivation of the gene, and at its 3′ end, where the insertion does not affect the structural gene but might have a polar effect on the downstream genes. Comparing the frequency of insertions at these two locations in a gene should give a good estimate on the essential character of the respective gene.

For each studied gene, defined fragments located either in the middle of the coding sequence or at its 3′ end were cloned into a vector host-dependent for autonomous replication, pSA3182. pSA3182 (Fig. 1) was obtained by ligation of three distinct fragments: (i) a region of plasmid pT181 (coordinates 1–1687) (Khan and Novick, 1983) containing the plasmid origins of replication for both the leading (Gennaro *et al.,* 1989) and the lagging strand (Gruss *et al.,* 1987), but not the *repC* gene encoding the essential plasmid replication initiator, RepC (Novick *et al.,* 1982); (ii) a 2.9-kb fragment containing the *tetA* gene conferring tetracycline resistance (Nesin *et al.,* 1990); and (iii) the 1.1-kb *Eco*RI L fragment of phage ø11 which includes the phage DNA packaging site (Novick *et al.,* 1986). As a result of its structure, pSA3182 can replicate autonomously only in strains where RepC is provided in *trans.* Such a permissive strain, SA3528, has been constructed as follows. A *repC* gene in which the overlapping origin of replication has been inactivated by oligonucleotide replacement (Iorda-

[1] To whom correspondence should be addressed at Public Health Research Institute, 455 First Avenue, New York, NY 10016. Fax: (212) 578-0804. E-mail: serban@phri.nyu.edu.

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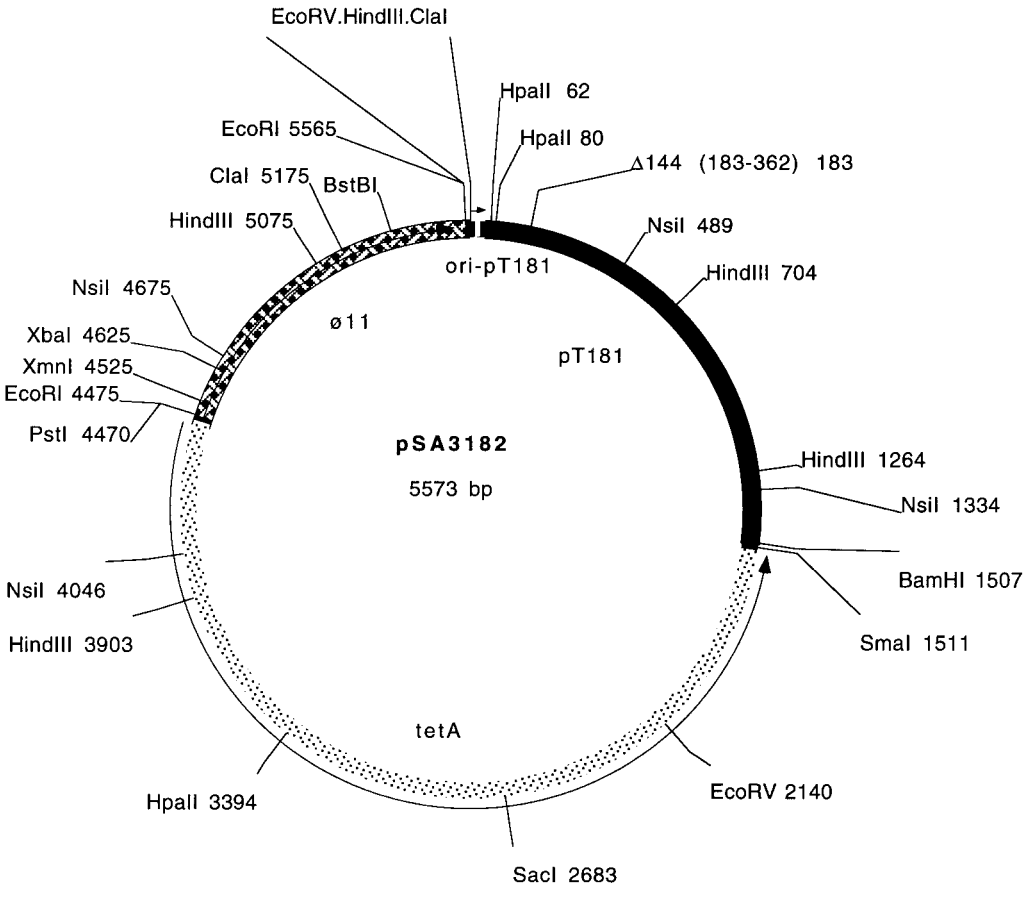

FIG. 1. Restriction map of plasmid pSA3182.

nescu, 1989) has been inserted into transposon Tn917 carried by the delivery vector pTV1(Ts) (Youngman, 1987). Several transpositions of this engineered Tn917 into the chromosome of strain RN4220 (Kreiswirth *et al.*, 1983) have been selected and found to provide similar levels of RepC activity. One of these isolates, SA3528, was used in the present study. A similar strategy was recently reported in the construction of a vector used in another gram-positive organism, *Lactococcus lactis* for a different purpose (Leenhouts *et al.*, 1998).

Due to the presence of the phage ø11 fragment, pSA3182 could be transduced by ø11 into SA3528 at a very high frequency (over 5%) but could not be established in strains that do not provide RepC. However, transduction into RepC$^-$ recipients might occur in the case of pSA3182 derivatives carrying specific gene fragments, due to their ability to integrate into the chromosome based on the homology provided by the cloned fragment, as long as this insertion does not lead to the inactivation of an essential gene.

The approach outlined above was used to test the essential character of four *S. aureus* genes: *nusG*, *div1B*, *dbpA*, and *dbpB*, selected as potential targets for the development of new antimicrobial agents. For each gene, internal (noted K, for knockout) and 3′ terminal (noted P, for polar) fragments were generated by PCR using AmpliTaq DNA polymerase (Perkin Elmer Cetus) under the conditions specified by the manufacturer. The size of all fragments was in the 350 to 550 bp range. The primers used were designed in such a way as to provide *Bam*HI

sites at both ends of each fragment. After amplification, the fragments were digested by *Bam*HI and cloned into the unique *Bam*HI site of pSA3182, using SA3528 as a host. Whenever possible, both orientations of the insert in pSA3182 were isolated. In pSA3182, transcription of *tetA* is toward the unique *Bam*HI site (Fig. 1) and was shown to proceed beyond the end of the *tetA* fragment (Iordanescu, unpublished results). As a result, for one orientation of the insert, noted +, the integration of the hybrid into the chromosome by a crossover at the level of the cloned fragment will be such that transcription of *tetA* will be in the same direction as that of the disrupted gene. Under these conditions, *tetA* transcription should extend downstream and might alleviate possible polar effects of the insertion. This would not occur for the opposite orientation, noted −.

The transduction frequency of each hybrid to $RepC^-$ recipient RN4220 was determined and the values were normalized against the transduction frequency of the same hybrid to the $RepC^+$ recipient SA3528. For all hybrids tested the absolute value of transduction frequency to SA3528 was in the range of 5–10%. pSA3182 without any insert was used as a control. The values obtained are presented in Table 1. Note that for *dbpA* and *dbpB* two distinct internal fragments (K1 and K2) were used to avoid misinterpretations due to the possible residual activity of truncated gene products.

For all constructs the efficiency of integration into the chromosome seems rather low, as reflected by a transduction frequency at least 10^4 times lower to RN4220 than to SA3528. This might reflect a less efficient homologous recombination in *S. aureus.* No significant effect of the orientation of the cloned fragments was observed for the hybrids tested. The results obtained (Table 1) suggest that the genes tested fall into two classes. For *nusG* and *dbpB* the transduction frequency was about the same for hybrids with internal or 3′ end fragments, while for the other two genes, the hybrids with the 3′ end had a transduction frequency at least 5- to 10-fold higher than that of those with internal fragments, suggesting that insertions in these structural genes have, at least, some deleterious effects.

TABLE 1

Relative Transduction Frequencies of pSA3182 Derivatives

Gene	Fragment	Transduction frequency[a]
–	–	$<3.0 \times 10^{-7}$
nusG	K+	6.8×10^{-5}
	K−	2.8×10^{-5}
	P+	1.0×10^{-4}
	P−	3.1×10^{-5}
div1B	K+	1.0×10^{-6}
	K−	7.8×10^{-7}
	P+	5.8×10^{-6}
	P−	4.2×10^{-6}
dbpA	K1+	1.4×10^{-6}
	K1−	1.2×10^{-6}
	K2+	4.8×10^{-7}
	K2−	5.8×10^{-7}
	P−	7.2×10^{-6}
dbpB	K1+	4.4×10^{-5}
	K1−	1.7×10^{-5}
	K2+	7.8×10^{-5}
	K2−	1.6×10^{-5}
	P+	7.8×10^{-6}
	P−	1.1×10^{-5}

[a] Transduction frequencies were expressed as a ratio of transductants/ml obtained in RN4220 relative to SA3528. All values are the average of at least three experiments.

For each hybrid, 1–2 transductants isolated in RN4220 were selected and analyzed to establish that they carry the hybrid specifically inserted into the chromosome due to the homology provided by the cloned fragment. In a first step, the specificity of the insertion was evaluated by PCR amplification using as template chromosomal DNA isolated from a transductant and two primers: one complementary to a sequence in *tetA* and the other corresponding to a sequence in the respective gene outside the cloned fragment. For all transductants studied, such PCR amplifications generated fragments of the size expected (data not shown), indicating that indeed integration into the chromosome occurred in a specific way. A second step involved a Southern analysis of the chromosomal region expected to carry the insert. Probes specific both for pSA3182 and for the studied gene were

used. Two types of results were obtained. For all hybrids carrying 3′ end fragments as well as for those with internal fragments of *nusG* and *dbpB,* the Southern analysis indicated that the structure of the chromosomal region carrying the insert was as expected, and no signal corresponding to the wild-type gene could be detected. These results confirm that the inactivation of these genes does not affect viability. Multiple, tandem insertions of a hybrid were observed in some of these transductants. This can be explained by the presence of the hybrids as long concatemers in transducing particles (Novick *et al.,* 1986).

The chromosomal structure of clones carrying insertions into the structural genes *div1B* and *dbpA* did not correspond to that expected and a signal corresponding to the wild-type gene was always present regardless of the restriction enzyme used. These data suggest that *div1B* and *dbpA* are essential genes and insertions disrupting their structure can occur only in cells carrying also a wild-type copy of the gene, as part of a duplication of the respective chromosomal region. Such duplications should not necessarily change the size of the diagnostic PCR fragments generated by a single pair of primers, as presented above for these insertions. The presence of duplications in the genome of many bacteria is well documented (Romero and Palacios, 1997). More studies are required to understand the nature of the duplications postulated based on the experiments reported here.

The conclusions, based on the results presented, on the essential character of the four studied genes are in good agreement with data available from other organisms. *nusG,* known to be involved in rho-dependent transcription termination, was recently shown to be nonessential in *B. subtilis* (Ingham *et al.,* 1999). The product of *div1B,* an homolog of *E. coli* FtsQ, is involved in the initiation of cell division. Although *div1B* is not essential in *B. subtilis* at temperatures under 30°C (Beall and Lutkenhorn, 1989), it is essential for the normal rate of growth and cell division at all temperatures (Rowland *et al.,* 1997). *dbpA* and *dbpB* are postulated to encode RNA helicases of the DEAD protein family. Some of these enzymes have been reported to be essential in yeast (Schmidt and Linder, 1992).

pSA3182 could also be used as part of a system for the generation of unmarked deletions in the *S. aureus* chromosome. Such systems allow for the delivery of precise polarity-neutral mutations into bacterial chromosomes in a site-specific fashion (Reyrat *et al.,* 1998). The test locus in this approach was the *resDE* response regulator pair from *S. aureus.* A 679-bp region of upstream homology together with a 716-bp region of downstream homology was generated by crossover PCR as described (Link *et al.,* 1997), except that in addition an in-frame termination codon was included directly before the overlap region. The final deletion was 201 bp and removed a C-terminal portion of the response regulator along with an N-terminal region of the histidine kinase component. The deletion disrupted the translational coupling of the pair and, together with the in-frame termination codon, assured inactivation of the locus. The final PCR product contained terminal *Bam*HI sites for insertion into pSA3182. The resulting construct, pHR321, was introduced into RN4220 by electroporation, and single insertion clones generated by homologous recombination with one of the two chromosomal fragments were isolated and analyzed by diagnostic PCR with appropriate primers as described above (Fig. 2A). The second step involved the isolation of clones in which a second crossover has occurred resulting in the loss of the integrated pSA3182. The procedure used took advantage of the fact that a pT181 origin cannot be maintained in the presence of a source of RepC in excess (Iordanescu, 1995). Plasmid pSA7592 overproducing RepC and conferring erythromycin resistance was introduced into clones carrying the original pHR321 insertion and the transductants screened for the loss of the pSA3182 tetracycline-resistance marker (Fig. 2B). The resulting tetracycline-sensitive clones were examined for either abortive allelic exchange (regeneration of the wild-type locus) or successful allelic exchange (introduction of the 201-bp deletion) (Bloomfield *et al.,* 1991) (Fig. 2C) by diagnostic PCR and Southern hybridization. A clone containing the specific deletion was iso-

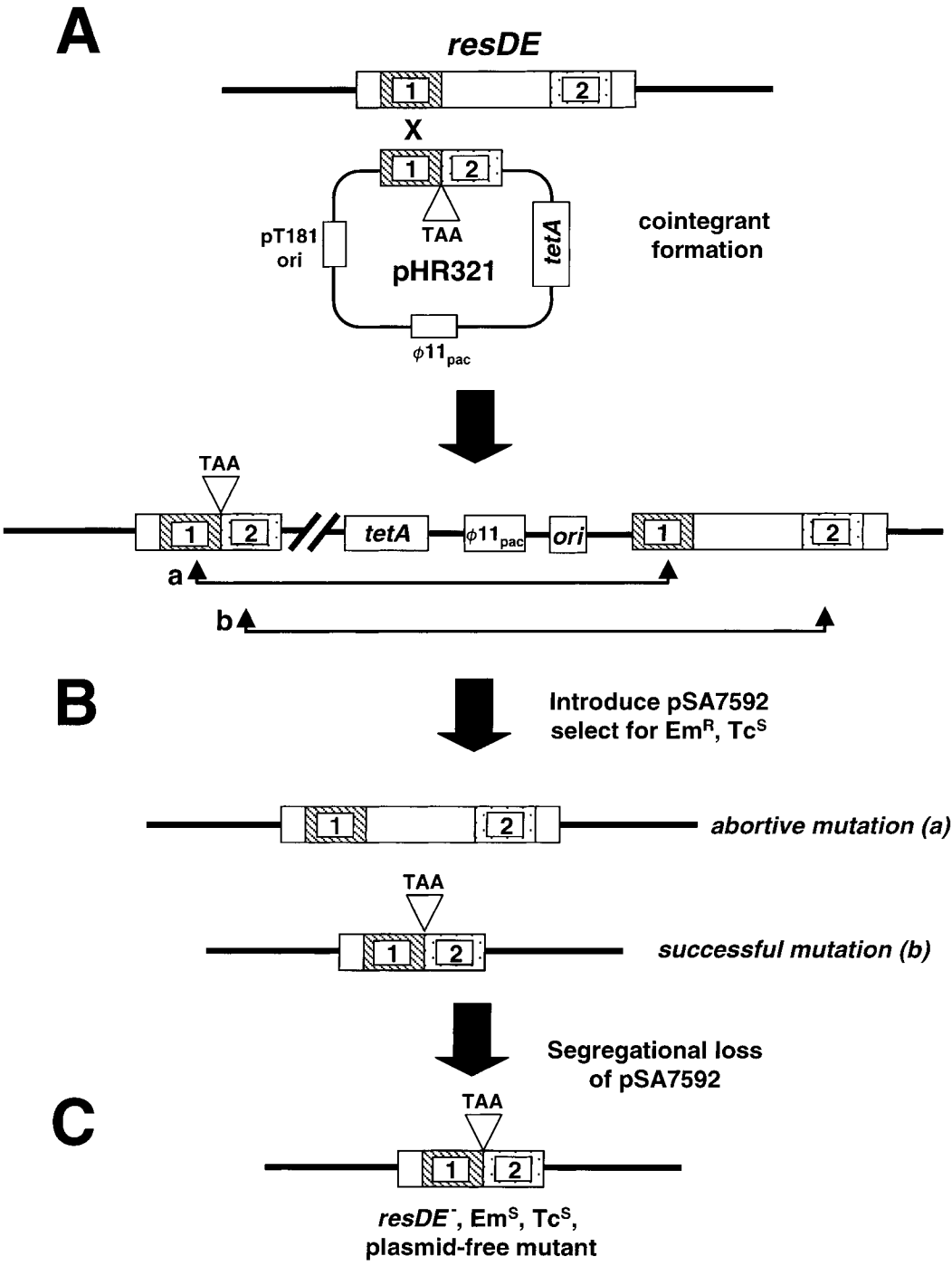

FIG. 2. Generation of an unmarked deletion in the *resDE* response-regulator pair. (A) Initial integration of pHR321 into the locus via homology block 1. Not shown is integration at homology block 2. Both events were possible and generated identical results. The deleted targeting cassette also contained an in-frame termination codon (TAA). (B) Structure of the plasmid cointegrant. Upon activation of the pT181 *ori* by the introduction of pSA7592, excision of pSA3182 could occur via homology blocks 1 (pathway *a*) or homology block 2 (pathway *b*). Pathway *a* would regenerate the wild-type locus and yield an abortive mutation event. Pathway *b* would yield a successful deletion event. (C) Loss of pSA7592 due to segregational instability to produce an unmarked plasmid-free deletion mutant.

lated in this way. Subsequently, plasmid pSA7592 was lost from this strain by growth in nonselective medium. The resulting antibiotic-susceptible, plasmid-free clone could be tested for the effect of the specific deletion on growth and virulence.

The results reported show that the system developed is efficient both as a rapid screening method for the identification of essential genes in *S. aureus* and as a way to generate specific, polarity-neutral deletions in genes that are not essential but might be involved in pathogenicity.

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