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Original Article

Investigation of the Mutator Genes in *Staphylococcus aureus*

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Abstract In contrast to *Escherichia coli*, the mechanisms of hypermutability have been poorly studied in *Staphylococcus aureus*, only the role of *mut*SL has been recently demonstrated. The *in silico* analysis of the genome of *S. aureus* MW2 allowed to identify thirty-four homologues of genes reported as mutators in *E. coli* or *Bacillus subtilis.* Six candidate genes in *S. aureus*, homologues of *mia*A*, nth, ung, rec*A*, rec*G and *mut*S2, were chosen on the basis of their co-existence in *E. coli, B. subtilis* and *S. aureus*, and of a high percentage of similarity of the deduced amino acid sequences (36%, 21%, 54%, 59%, 35% and 26% identity between *E. coli* and *S. aureus* and 48%, 63%, 48%, 68%, 50%, and 50% identity between *B. subtilis* and *S. aureus*, respectively). Individual genes of *S. aureus* RN4220 were inactivated by insertion of the thermosensitive pBT1 plasmid. Only mutants of *nth* gene showed an increase in frequencies of mutation to rifampin (mean 1.6×10^{-7}) and streptomycin resistance (mean 3.1×10^{-6}), *versus* 1.9×10^{-8} and 5.1×10^{-7} in the parent strain, respectively. Complementation of the mutant by an intact *nth* gene allowed to restore the mutation frequency similar to that of the wild-type. To the best of our knowledge, *nth* gene may play a part at the hypermutability other than *mut*SL in *S. aureus*.

Keywords: mutator gene, *Staphylococcus aureus*

INTRODUCTION

Bacteria with elevated mutation rates have been reported amongst natural population in numerous pathogenic species, such as *Escherichia coli, Salmonella enterica*, *Pseudomonas aeruginosa, Neisseria meningitides, Helicobacter pylori* and *Streptococcus pneumoniae* by a defect in bacterial mismatch repair system (MMR).[1,2] MMR deficience is often detected in these mutants that exhibit high mutation and homologous recombination rates. MMR contributes almost 1000-fold to the fidelity of DNA replication and removal of this barrier also enhances the mechanism of acquired antibiotic resistance in those bacteria.[1,3,4] Mutation and subsequent recombination events create genetic diversity, which is subjected to natural selection and have therefore a crucial role in evolution and adaptation of bacteria to changing environmental conditions. However, the advantage of hypermutability for mutator alleles is counter-balanced by a cost due to a heavy deleterious mutation load. Gram-positive bacteria have an MMR system that is functionally equivalent to that of *E. coli*, although there are some important differences for example the lack a MutH equivalent or the discrimination between the leading strand and lagging strand needed for the reparation.[4] In *S. aureus*, hypermutators have also been reported. Recently, our studies showed that 14.6% of *S. aureus* isolated in cystic fibrosis patients were hypermutators. In noncystic fibrosis patients infected by this species, it was also shown that hypermutability of the isolate was significantly associated to resistance to fluoroquinolones that is due to mutations in the target of the antibiotics, the

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bacterial topoisomerases.[5,6] Alterations of *mut*S and *mut*L genes, which form an operon in *S. aureus*, were found in several of the hypermutators. Since some clinical hypermutator isolates did not show any structural modification of *mut*SL, we postulated that impaired expression of *mut*SL or alteration of other mutator genes might occur.[5-7] Nearly thirty mutator genes have been reported in *E. coli* and, so far, remain unidentified in *S. aureus*. In particular, the genes associated with *mutS* and *mut*L in the methyl-directed mismatch repair of *E. coli* have no homologues in *S. aureus.*[8] Probably, *S. aureus* uses another strategy to identify the mutated strand.

Mutator genes other than those belonging to the Mut family have been identified in the *E. coli* and *Bacillus subtilis* chromosome, and some may have homologues in the *S. aureus* chromosome.[8,9] In this study, we have identified in *S. aureus* several homologues of mutator genes and studied by insertional analysis and complementation the putative hypermutator role of six of these genes.

MATERIALS AND METHODS

Homology Searches, Genome Analysis, and Multiple Sequence Alignments

Based on literature data, mutator genes were identified in *B. subtilis* and *E. coli*. Candidate proteins were identified in published sequences of *S. aureus* MW2 (Accession: NC_003923) from the NIH GenBank database, using the BLASTP software available over the internet at the National Center for Biotechnology Information website (http:www.ncbi.nlm.nih.gov/).

Bacterial Strains and Plasmids

E. coli DH10B and EC101, *S. aureus* RN4220 were used as hosts for cloning of the different plasmids, listed in Table 1.

Transformation Procedures

Electrocompetent cells were prepared for *S. aureus* and *E. coli*.[10] Plasmid transformants were selected on brain heart infusion (BHI) agar medium (Difco Laboratories, Detroit, MI) added with antibiotics at the following concentrations when necessary: amoxicillin, 100 μg/ml; chloramphenicol, 20 μg/ml; spectinomycin, 60 μg/ml for *S. aureus*.

DNA Techniques

DNA extraction was carried out with an Instagene™ Matrix kit (Bio-Rad Laboratories, Hercules, Calif.). Plasmids were extracted from *E. coli* transformants with the Sigma GenElute plasmid miniprep kit (St. Louis, MO). PCRs were performed with the primers listed in Table 2. All PCRs involved a precycle of 5 minutes at 94°C and then 35 cycles of 30 seconds at 94°C, 30 seconds at 50°C, and 30 seconds at 72°C (times could be extended for the extension step up to 2 minutes according to the size of the product), followed by a cycle of 10 minutes at 72°C on a PTC-225 DNA Engine Tetrad Thermal Cycler (MJ Research, Watertown, Mass.).

Insertional Inactivation and Complementation

Strains disrupted for the genes of interest were constructed by insertional inactivation of the candidate gene using the pBT1 thermosensitive shuttle plasmid.[11] An internal fragment of the candidate gene was amplified by using the appropriate primers modified with restriction enzyme sites (Table 2) and subsequently cloned into the corresponding sites of the pBT1 plasmid in *E. coli* DH10B. The resulting plasmid was introduced by electroporation into *S. aureus* RN4220 as previously described.[12] The recombination between the construct and the homologous fragment in the chromosome was forced by submitting the strains to three successive subcultures at 42°C in BHI broth containing chloramphenicol. Efficient insertions were verified by PCR with appropriate primers (Table 2). Gene inactivation was considered as completed when the PCR did not amplify a fragment extending from the start to the end of the target gene (outside of the internal fragment) and amplified a fragment located between the *bla* gene of pBT1 and the chromosomal start of the gene sequence.

When inactivation of a candidate gene led to hypermutability of *S. aureus* RN4220, the mutant was complemented with an intact allele of the gene. For this purpose, the entire

gene and its putative promoter were cloned into pUC18 and introduced into *E. coli* EC101 to be then subcloned in the shuttle vector pAT28.[13] The recombinant plasmid was finally introduced by electroporation into competent cells of the *S. aureus* RN4220 mutant and selected on agar containing chloramphenicol and spectinomycin. The presence of the inserted recombinant plasmid and of the complementation plasmid was verified by PCR.

Determination of Mutation Frequencies

For each mutant or complemented strain, the mutation frequencies were determined on BHI agar plates supplemented with 100 µg/ml of rifampin or 50 µg/ml of streptomycin as previously described.[14] For each strain, the experiment was repeated three times, excepted for the *nth* mutants for which the experiments were repeated 20 times. Results are indicated as a mean and standard deviation.

RESULTS AND DISCUSSION

Identification of Candidate Mutator Genes

The *in silico* analysis of the genome of *S. aureus* MW2 allowed to identify thirty-four homologues of genes reported as mutators in *E. coli* or *B. subtilis*.[8] Nearly identical sequences were found in the other 10 genomes of *S. aureus* present in the GenBank database. Six candidate mutator genes in *S. aureus*, homologues of *mia*A, *nth, ung, rec*A, *rec*G and *mut*S2, were chosen on the basis of their co-existence in *E. coli, B. subtilis* and *S. aureus,* and of a high percentage of similarity of the deduced amino acid sequences (36%, 21%, 54%, 59%, 35% and 26% identity between *E. coli* and *S. aureus* and 48%, 63%, 48%, 68%, 50% and 50% identity between *B. subtilis* and *S. aureus*, respectively).

Inactivation of Candidate Mutator Genes

For each of the six candidate genes, an internal fragment was cloned in the pBT1 thermosensitive shuttle vector plasmid and introduced into *E. coli* DH10B. and the resulting plasmid was used to obtain a chloramphenicol-resistant *S. aureus* RN4220 mutant with inactivated gene by homologous recombination at a non-permissive temperature (Figure 1).

Effets of Inactivated Candidate Mutator Genes and Complementation on Mutation Frequencies

The spontaneous mutation frequencies to rifampin and streptomycin resistance of mutants were compared to that of the parent strain *S. aureus* RN4220. Seven determinations

Table 1. Plasmids and mutant strains used

Plasmid or mutant strains	Relevant properties[a]	Reference or source
Plasmid		
pBT1	Cm^r Am^r (G-), thermosensitive shuttle cloning vector	11
pUC18	Am^r *lacZ',* cloning vector	15
pAT28	Sp^r *lacZ',* cloning vector	13
Mutant *S. aureus* strain		
RN4220/pBT1Ωfragment *nth*	Mutant of RN4220 after homologous recombination of an internal fragment of *nth* gene by non-permissive temperature, Cm^r	This study
RN4220/pBT1Ωfragment *mia*A	Mutant of *mia*A gene, Cm^r	This study
RN4220/pBT1Ωfragment *rec*A	Mutant of *rec*A gene, Cm^r	This study
RN4220/pBT1Ωfragment *rec*G	Mutant of *rec*G gene, Cm^r	This study
RN4220/pBT1Ωfragment *ung*	Mutant of *ung* gene, Cm^r	This study
RN4220/pBT1Ωfragment *mut*S2	Mutant of *mut*S2 gene, Cm^r	This study
RN4220/pBT1Ωfragment *nth*+ pAT28Ω*nth*	Complementation of *nth* gene by pAT28 plasmid, Cm^r Sp^r	This study

[a]Am^r, ampicillin resistant; Cm^r, chloramphenicol resistant; Er^r, erythromycin resistant; Sp^r, spectinomycin resistant; G-, resistance expressed only in gram-negative bacteria; *lacZ'*, *lacZα* gene.

in a mutant inactivated for the *nth* gene showed frequencies ranging from 1 x 10^{-7} to 2.5 x 10^{-7} (mean 1.6 x 10^{-7} ± 0.85) for rifampin and from 2.81 x 10^{-7} to 1.1 x 10^{-5} (mean 3.1 x 10^{-6} ± 3.5) for streptomycin. These frequencies were higher than those calculated for *S. aureus* RN4220.

Complementation of the *nth* mutant by the intact gene cloned in plasmid pAT28 restored a low mutation frequencies from 3.6 to 4.5 x 10^{-8} (mean 4.1 x 10^{-8} ± 0.41) for rifampin and from 7.3 to 8.4 x 10^{-7} (mean 7.8 x 10^{-7} ± 2.58) for streptomycin, similar to those for RN4220 (1.9 x 10^{-8} ± 2.94 and 5.1 x 10^{-7},

Table 2. Primers used in this study

Oligonucleotide primers	Restriction enzyme used	Size (bp)	Reference
nth			
nth1:5'-AAG-AAA-AAA-GCT-TTA-GAA-ATC-3'	*Hind*III	630	This study
nth2:5'-TTT-ATA-AGC-TTT-TTG-TCC-TTC-3'			
***mia*A**			
miaA1: 5'-AAA-CAA-AGC-TTA-GCA-TAG-AAC-3'	*Hind*III	693	This study
miaA2: 5'-CTT-TAT-ATC-CAA-AAG-CTT-GCA-3'			
***rec*A**			
recA1:5'-GGT-AAA-GGA-TCC-GTA-ATG-AAG-3'	*Bam*HI	565	This study
recA2:5'-GTG-TAG-TCT-CTG-GAT-CCC-CGA-3'			
***rec*G**			
recG1:5'-GAA-TCA-AGC-TTA-AGA-TCA-ATC-3'	*Hind*III	656	This study
recG2:5'-CAC-TGG-ATT-TCT-AAG-CTT-CAG-3'			
ung			
ung1:5'-GCA-AAG-AAT-TCA-TGA-CAT-AAC-3'	*Eco*RI	590	This study
ung2:5'-CAT-TCG-CTT-TGG-AAT-TCG-GTT-3'			
***mut*S2**			
mutS2-FW2 : 5'-CTT-ATC-TAA-AGC-TTC-AGC-ATT-3'	*Hind*III	660	This study
mutS2-RV2:5'-CTA-TAT-CAA-GCT-TTT-GCG-ATT-3'			
Fragment verifications			
blaF: 5'-AAC-GTT-TTC-CAA-TGA-TGA-GC-3'	-	-	16
blaR: 5'-GTC-TAT-TAA-TTG-TTG-CCG-GG-3'			
catF: 5'-AAT-ACA-GCT-TTT-AGA-ACT-GG-3'	-	-	17,18
catR: 5'-ATT-AAT-GAA-TTT-TCC-TGC-TG-3'			
M13FM: 5'-GTT-GTA-AAA-CGA-CGG-CCA-G-3'	-	-	
M13RM: 5'-GGA-TAA-CAA-TTT-CAC-ACA-GG-3'			
nth-bla-cat: 5'- GGT-AAG-TAA-GAA-AAA-AGC-AT -3'	-	658	This study
Nth-end: 5'-TCA-CGC-TTC-TTT-CAA-ACT-AG-3'			
miaA-bla-cat: 5'- GAA-TAA-AAA-TAA-GCC-TTT-TA-3'	-	793	This study
miaA-end: 5'-GAC-AGC-ATC-TTC-ATA-AAT-CA-3'			
recA-bla-cat: 5'-GGA-TAA-CGA-TCG-TCA-AAA-AG-3'	-	783	This study
recA-end: 5'-ATA-ATG-TCA-ACT-TCA-GCT-AC-3'			
recG-bla-cat: 5'-TTG-GCT-AAA-GTA-AAC-TTA-AT-3'	-	987	This study
recG-end: 5'-TTG-GCT-AAA-GTA-AAC-TTA-AT-3'			
ung-bla-cat:5'-GGA-ATG-GTC-GCA-AAT-TTT-TC-3'	-	655	This study
ung-end:5'-CTACGCCTCACTTTCACACC-3'			
mutS2-bla-cat:5'-TGA-GAC-AAA-AAA-CAT-TAG-AC-3'	-	992	This study
mutS2-end: 5'-CGT-TTC-AAT-ATC-TTC-CAT-AA-3'			
Complementation			
nth-cloneFW2: 5'- AAC-GTG-AAT-TCC-TAG-AAT-TAC-3'	*Eco*RI	1239	This study
nth-cloneRV2 :5'- CTA-TGG-AAT-TCA-TCC-AAG-TAG-3'			

Under lines are the restriction enzyme sites.

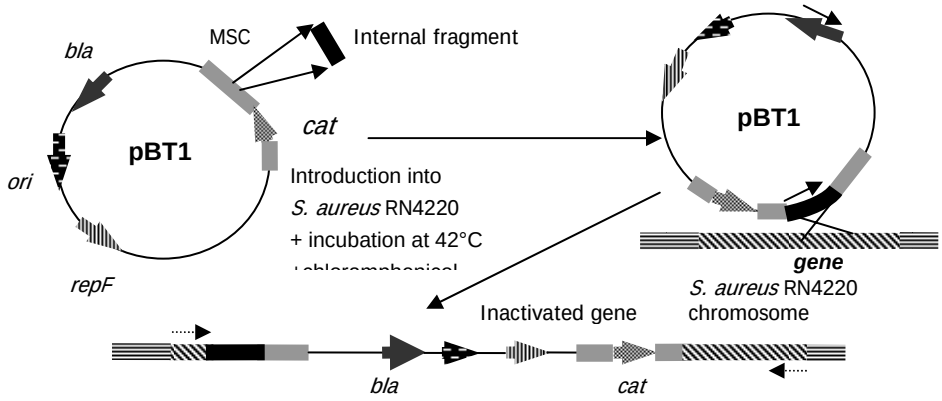

Figure 1. Cloning strategy for the insertional inactivation of a target gene by pBT1 plasmid (PCR verifications used by combined pair of primers of the small arrows).

respectively). Mutants of *mia*A, *rec*A, *rec*G, and *mut*S2 displayed mutation frequencies similar to those of *S. aureus* RN4220.

The *mut*SL operon of genes has a major role in hypermutability in *S. aureus*, similarly to *E. coli*.[7,8] It was shown that several other homologues of *E. coli* and *B. subtilis* mutator genes were present in the chromosome of *S. aureus*. Homologues of the six genes that we selected were shared by the three bacterial species. The product of *mut*S2 belongs to the MutS family; two sub-families of MutS homologues are described, the MutS-I family, which includes *E. coli* MutS, and the MutS-II family.[19] The role of the MutS2 protein has been recently shown in *H. pylori* and *B. subtilis*.[20,21] In *H. pylori*, it does not seem to play a role in mismatch repair but increases efficiency of exogenous DNA incorporation not only between molecules with identical sequences but also between molecules with divergent sequences, therefore inhibiting both homologous and homeologous recombination.[22,23] In this study, *mut*S2 mutants did not display increased mutation frequency, suggesting that in *S. aureus* the gene might have a similar function.

The *rec*A gene has been characterized in *S. aureus*, but its function has been poorly studied in this species although mutant strains have deficiencies in genetic recombination and DNA repair.[24] In other bacteria, it has been shown that the RecA protein is required for genetic recombination and plays a key role in the SOS response.[25] A *rec*G gene has been previously reported in *S. aureus*; the gene appeared to be involved in the repair of DNA damage resulting from quinolone treatment.[26] RecG is an ATP-dependent helicase that allows repair of damage replication in *E. coli*, and can be found as a mutator gene in some species.[8] Recently, authors reported that the inactivation of *rec*G stimulates the RecF pathway during lesion-induced recombination in *E. coli*.[27] Again, *rec*A and *rec*G inactivation in *S. aureus* did not lead to hypermutability in our study.

Homologues of the *mia* and *ung* genes were also found in *S. aureus*. *E. coli mia*A mutants, which lack a specific tRNA modification (methylthioisopentyladenosine), showing a 3-9-fold increase in GC to TA transversions in nutritionally limited cells. This mutational specificity, together with the observation that *mia*A mutations suppress the mutator phenotype of *mutY*, *mutM* and *mutY-mutM* strains in growing cells, suggests a possible involvement in the generation or repair of oxidized guanine residues.[8,14] Ung, a uracil glycosylase is a widely conserved enzyme that removes uracil bases from single- and double-stranded DNA, thereby counteracting the deamination of cytosine. Consequently, *E. coli* cells with mutations in the *ung* gene show a 10- to 15-fold elevated

spontaneous mutation rate for GC to AT transitions.[28]

Finally, only inactivation of the *nth* gene conferred hypermutability in our strain of *S. aureus*. The role of *nth* was proven by inactivation of the gene and complementation by an intact allele that restored the initial mutation frequency. In *E. coli,* endonuclease III which is encoded by *nth* and endonuclease VIII encoded by the *nei* gene are both glycosylases with associated abasic (AP)-lyase activities, that recognize a broad range of oxidatively damaged pyrimidine residues and release the damaged base.[29,30] The gene *nth* does not have a mutator phenotype, although in one study,[29] but not in another one[30] a 20-fold increase in spontaneous mutation rate has been detected in a *nth–nei* double mutant. To the best of our knowledge, this is a first report of a mutator gene other than *mut*SL in *S. aureus*. The role of this gene should be assessed by further investigations among hypermutable clinical isolates of *S. aureus* without *mut*SL alterations. Also, the ability of this gene to prevent recombination between partially divergent sequences should be investigated as previously realized with *mut*SL genes.[7]

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