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Research paper

Increase in IS*256* transposition in invasive vancomycin heteroresistant *Staphylococcus aureus* isolate belonging to ST100 and its derived VISA mutants

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ABSTRACT

In *Staphylococcus aureus*, transposition of IS*256* has been described to play an important role in biofilm formation and antibiotic resistance. This study describes the molecular characterization of two clinical heterogeneous vancomycin-intermediate *S. aureus* (hVISA) isolates recovered from the same patient (before and after antibiotic treatment) and two VISA derivatives obtained by serial passages in the presence of vancomycin. Our results showed that antibiotic treatment (in vivo and in vitro) could enhance IS*256* transposition, being responsible for the eventual loss of *agr* function. As far as we know this is the first study that reports the increase of IS*256* transposition in isogenic strains after antibiotic treatment in a clinical setting.

1. Introduction

Staphylococcus aureus is an evolving pathogen with the ability to adapt to different environments and acquire virulence determinants and resistance to multiple antibiotics. Resistance to different antibiotic families in *S. aureus* is mediated by the acquisition of mobile genetic elements containing resistance determinants such as plasmid harboring *bla*Z or the chromosomal cassette SCC*mec*, or mutations in different loci.

In recent years it has been suggested that exposure to sub-inhibitory concentrations of antibiotics might have an important role in the development of bacterial resistance to antibiotics. Antibiotics may not only select subpopulations of resistant strains but also affect bacterial physiology, including the generation of genotypic and phenotypic variability, as well as their ability to function as signaling molecules (Andersson & Hughes, 2014).

The effect of sub-inhibitory concentrations of antibiotics on the transposition of insertion sequences and its relatedness to antimicrobial resistance has been also studied in *S. aureus*. For example, sub-inhibitory concentrations of ciprofloxacin and vancomycin lead to the activation of IS*256* transposition in a laboratory strain (Nagel et al., 2011). Additionally, Schreiber et al described that IS*256* transposition is autoactivated in vitro in a strain belonging to ST247 after treatment with sub-inhibitory concentrations of chloramphenicol, linezolid, and spectinomycin (Schreiber et al., 2013).

IS*256* has been detected in the genome of several clinical isolates of *S. aureus* in multiple copies, alone or flanking both ends of the aminoglycoside resistance transposon Tn*4001* (Lyon et al., 1987; Byrne et al., 1989) and transposes by a copy and paste mechanism (Loessner et al., 2002).

The emergence of vancomycin intermediate *Staphylococcus aureus* (VISA) and heterogeneous VISA (hVISA) is of major concern worldwide and to date the underlying mechanism is not yet fully understood (Howden et al., 2014). It has been proposed that VISA emerges by mutations from vancomycin susceptible *S. aureus* (VSSA) by a step-wise process with hVISA as intermediary. A large number of phenotypic features have been described in VISA isolates but the molecular bases have to be clarified (Howden et al., 2014). Some of the most common genetic changes described so far in hVISA/VISA strains are point mutations in different regulatory genes (*vra*SR, *gra*SR, *wal*KR, *rpo*B, *cmk*) (Howden et al., 2008; Watanabe et al., 2011; Matsuo et al., 2013; Shoji et al., 2011; Galbusera et al., 2011). Other researchers, reported IS*256* insertions disrupting different genes implied in cell wall synthesis (*tca*, *walKR*), leading to the VISA phenotype (Maki et al., 2004; McEvoy et al., 2013; Jansen et al., 2007). As far as we know, there are no reports analyzing the effects of antimicrobial treatment on transposition of IS*256* in paired clinical isolates.

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The aim of this study is to characterize two MRSA isolates: recovered from a single patient suffering bone and joint infection, before and after 40 days of vancomycin treatment (D1 and D2 respectively), and two D2 laboratory derived mutants (D23C9 and D2P11) selected by independent serial passages in the presence of increasing concentrations of vancomycin.

2. Materials and methods

2.1. Case report

A 30-year-old man suffering M4 acute myeloid leukaemia since 2007, in complete remission and allogeneic bone marrow transplantation schedule was admitted to our University hospital. He had been diagnosed in the emergency department of another hospital with sepsis from probably articular focus; and treated with piperacillin/tazobactam IV. On admission to our hospital, blood cultures and articular puncture were made, and treatment was switched to vancomycin (VAN) (30 mg/kg/day) and imipenem (IMI). Methicillin-resistant *S. aureus* (MRSA) strain D1 (VAN MIC and MBC: 0.5 μg/ml and 16 μg/ml respectively) was isolated from joint puncture and first blood culture. Imipenem was discontinued and amikacin was added for 5 days. Control blood cultures remained negative and vancomycin serum trough concentration was 17.9 μg/ml. Bone and joint infection was diagnosed from ankle MNR and anteroposterior X-ray. On 15th day rifampin was added. Patient persisted with purulent discharge from right ankle and fever recurred. He required multiple articular punctures and rifampin was discontinued. The vancomycin serum through concentration changed to 25 μg/ml and then to 21.5 μg/ml. On 30th day, clindamycin was added. On 40th day, after 40 days of vancomycin treatment sixth arthrotomy with surgical cleaning was performed and MRSA strain D2 was isolated (VAN MIC: 1.0 μg/ml and MBC: 128 μg/ml respectively). On 45th day, ciprofloxacin was added, seventh arthrotomy was made and culture was negative. On 55th day, antibiotic treatment was switched to oral linezolid and levofloxacin. On 72th day, last arthrotomy was performed and culture was negative. Hyperbaric chamber was indicated. The patient presented good outcome and was discharged on 74th day, with oral linezolid and levofloxacin.

2.2. Strains and culture conditions

All strains were grown on BHI broth (Britania) at 37 °C with aeration. In vitro selection of D2 derived mutants (D23C9, D2P11) was performed in two independent experiments by serial passage in BHI broth (Britania, Argentina) with increasing concentrations of vancomycin (Sigma-Aldrich), starting from a concentration of 3 μg/ml. Mutant strains D23C9 and D2P11 were selected from BHI containing 9 and 11 μg/ml of vancomycin respectively.

2.3. Susceptibility testing

Minimal inhibitory concentrations (MICs) for vancomycin (VAN), oxacillin (OXA), ciprofloxacin (CIP) and rifampin (RIF) were determined by broth microdilution method according to Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2012). VISA and hVISA were defined by population analysis profile method (PAP-AUC method) as described by Wootton et al. (2001). ATCC 29213 (VSSA), Mu3 (hVISA), and Mu50 (VISA) were used as control strains.

2.4. Molecular characterization

2.4.1. PCR amplification of mecA, lukS/F-PV, SCCmec and agr typing

Detection of *mec*A and PVL encoding genes (*luk*S/F-PV) was attempted after extraction of genomic DNA as previously described (Lina et al., 1999; Murakami et al., 1991). SCC*mec* type was determined by PCR with a simplified version of Kondo's typing system, including M-PCR-1 and M-PCR-2 (Kondo et al., 2007). Multiplex PCR for SCC*mec* IV J1/J2 regions was performed as previously described (Milheirico et al., 2007). Tn*4001* was detected upstream *mecA*–Δ*mecR1* by PCR amplification of the region between the *mec*A locus and IS*256*L, and the region between IS*256*L and aminoglycoside resistance genes within the Tn*4001* (Sola et al., 2012).

The *agr* locus was genotyped by multiplex PCR, as previously described (Gilot et al., 2002). Disruption of *agrB* gene by IS*256* insertion in strain D23C9 was explored by PCR mapping and confirmed by sequencing (Genbank Accession number KU926958). Primers used for PCR mapping are described in Supplementary material 1.

2.4.2. Typing methods

Genotyping analysis was completed using *spa* typing (Harmsen et al., 2003), Multilocus Sequence Typing (MLST) (Enright et al., 2000) and Pulsed-Field Gel Electrophoresis (PFGE) with *Sma*I (Chung et al., 2000).

2.4.3. Southern blot hybridization

After PFGE the DNA was transferred to a HYBOND-N + membrane by Southern blot standard procedures. Probe hybridization and signal detection were performed using DIG-High Prime DNA Labeling and Detection Starter Kit II (ROCHE), following the manufacturer's recommendations. Hybridization probes for IS*256*, *agr*AC and *mec*A detection were PCR amplified using primers previously described (Murakami et al., 1991; Valle et al., 2007; Adhikari et al., 2004).

2.4.4. Sequencing of loci related to IS256 transposition

The *sig*B and *rsb*U genes were amplified by PCR (Schreiber et al., 2013; Chen et al., 2011). Purified amplicons were sequenced and compared with the corresponding genes from *S. aureus* N315 genome sequence (NCBI Reference Sequence: NC_002745.2).

2.5. agr locus functionality

The *agr* locus functionality was assessed by the delta haemolisyn assay as described by Traber et al. (2008).

2.6. RTqPCR

The transcription of IS*256* transposase (*tnp*) gene was evaluated by RTqPCR using primers previously described (Valle et al., 2007). The effect of sub-inhibitory concentrations of vancomycin (¼ MIC) on the transcription was further analyzed. RNA was isolated from bacteria grown in BHI broth, with/without ¼ MIC exposure until the optical density ($OD_{620\ nm}$) reached ~0.5–0.7. RNA isolation and all real-time PCRs were performed in triplicate. Bacterial pellets were treated with lysozyme 15 mg/ml (Sigma-Aldrich), for 1 h at 37 °C and RNA was extracted using TRIZOL® Reagent (Invitrogen) with the Pure Link® RNA Mini Kit (AMBION, USA) according to the manufacturer's recommendations. RNA was quantified using NanoDrop™ 1000 SpectroPhotometer (Thermo Scientific) and treated with DNase (3 U/μl, 1 h at 37 °C, RQ1 RNase free DNase, Promega). Reverse transcription was performed using 500 ng of RNA, 200U of M-MLV™ Reverse Transcriptase (Invitrogen, USA) and 50 μM Random hexamers (Invitrogen, USA) according to the manufacturer's recommendations. The qPCR reaction was carried out using a 1/100 dilution of cDNA, SYBR® Select Master Mix (Applied Biosystems, USA) and the primers previously described in a 7500 Real-Time PCR System (Applied Biosystems, USA).

The combination of *gyr*B and *pta* was used as reference genes. Cq values were converted into Normalized Relative Quantities (NRQ) values using normalization to the geometrical average of the reference genes and the specific PCR efficiency for each gene (Valle et al., 2007; Chen et al., 2011; Bustin et al., 2009; Hellemans et al., 2007; Valihrach & Demnerova, 2012; Vandesompele et al., 2002). The complete RTqPCR protocol is described in Supplementary material 2.

2.7. Statistical analysis

The NRQ values of *tnp* transcripts were converted into logarithmic values to obtain symmetrical data, and compared by two-way analysis of variance (ANOVA). Multiple comparisons were performed with the Duncan posttest using Infostat Software (Di Rienzo et al., 2013). *p* values of <0.05 were considered statistically significant.

2.8. Mutation frequency

The mutation frequency was explored for the four strains in comparison with *S. aureus* ATCC 29213. As the clinical strains were rifampin resistant, the mutation frequency to ciprofloxacin (CIP) was evaluated. Briefly, bacteria were grown in BHI broth in the presence of VAN (¼ MIC) or without antibiotic until they reached an $OD_{620\ nm} = 0.7$ and plated in BHIA with/without CIP 4 × MIC (1 μg/ml) for viable counting. The experiment was performed by triplicate and mutation frequency was determined and expressed as the media of M/V (M = number of mutants in a culture; V = number of viable cells in a culture (O'Neill et al., 2001).

3. Results

3.1. Antimicrobial susceptibility and molecular typing

Both clinical isolates were defined as hVISA, while both laboratory mutants were categorized as VISA; D2 strain and their derived mutants displayed changes in the susceptibility to other antimicrobial drugs when compared to D1 (Table 1).

SCC*mec* was not typeable by conventional approaches, and neither *ccr* complexes nor J1/J2 regions could be amplified. Tn*4001* was detected upstream *mecA*–Δ*mecR1*. Clonality was confirmed by PFGE (Fig. 1A). Isolates showed identical *mec*A hybridization patterns suggesting that the changes in oxacillin MIC were not due to SCC*mec* excision (Fig. 1D). All strains homogeneously belonged to ST100, *t002*, and *agr* group II, but *agr* group of D23C9 could not be determined by conventional PCR typing.

3.2. IS256 transposition in hVISA/VISA strains

Southern blot of PFGE showed an increase in the number of bands detected with IS*256* probe in D2 and both independent mutants (Fig. 1B). Since IS*256* is known to transpose by a "copy and paste" mechanism, and considering the identity of PFGE profiles, the change in the Southern blot hybridization patterns should be due to an increase in the number of IS*256* copies.

A shift in the *agrAC* band was detected only in D23C9 (Fig. 1C). This strain did not show delta-haemolytic activity when the *agr* locus functionality was evaluated (Fig. 2). Additionally, the disruption of *agrB* gene by IS*256* insertion was demonstrated in this strain by PCR mapping and sequencing (Accession number KU926958).

The transcription of IS*256* transposase (*tnp*) gene was evaluated by RTqPCR (Fig. 3). The effect of sub inhibitory concentrations of vancomycin (¼ MIC) on the transcription of this gene was further analyzed.

Table 1
Antimicrobial susceptibility determined by MIC (microdilution method) and vancomycin PAP-AUC method.

Strain	PAP-AUC/ PAP-AUC Mu3	Description	MIC(μg/ml)			
			VAN	OXA	RIF	CIP
ATCC 29213	0.42	VSSA	0.5	0.25	0.004	0.25
D1	0.98	hVISA	0.5	128	8	0.25
D2	1.48	hVISA	1	2	4	0.25
D23C9	2.81	VISA	4	8	1	0.25
D2P11	5.70	VISA	8	32	2	0.25

VAN: vancomycin; OXA: oxacillin; RIF: rifampin; CIP: ciprofloxacin.

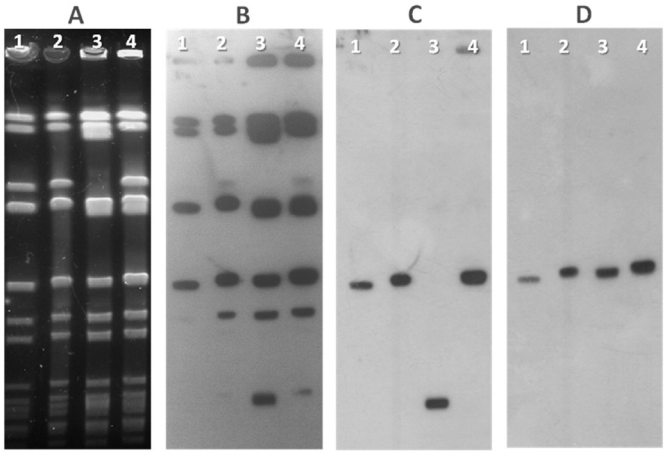

Fig. 1. A) PFGE; B) PFGE Southern Blotting using IS*256* specific probe; C) PFGE Southern Blotting using *agr*AC specific probe; D) PFGE Southern Blotting using *mec*A specific probe. Lane 1, D1; lane 2, D2; lane 3, D23C9; lane 4, D2P11.

Expression levels of the *tnp* gene were significantly different ($p < 0.0001$, two-way ANOVA). The *tnp* transcript level was significantly major for D23C9 ($p < 0.05$, Duncan posttest), and although statistical analysis did not show a significant difference, D2 and D2P11 revealed with a clear trend higher expression levels of *tnp* when compared to D1. This result is in concordance with the Southern blot hybridization patterns (Fig.1B).

Moreover, treatment with sub-inhibitory concentrations of vancomycin (¼ MIC) produced a significant effect on the expression of the *tnp* gene ($p = 0.0335$, two way ANOVA). Mutant strains D2P11 and D23C9 incubated under this condition showed significantly higher expression levels of *tnp* than those obtained without antibiotic, and these levels are significantly higher than those of the parental strain D2 ($p < 0.05$, Duncan posttest).

3.3. Analysis of additional genetic events

No changes in the mutation frequency were found under the conditions tested: ciprofloxacin resistant mutants occurred at frequencies of around 10^{-7}–10^{-8} in all strains even in the presence of subinhibitory concentrations of VAN (Table 2).

None of the four strains carried mutations in *sig*B and *rsb*U genes.

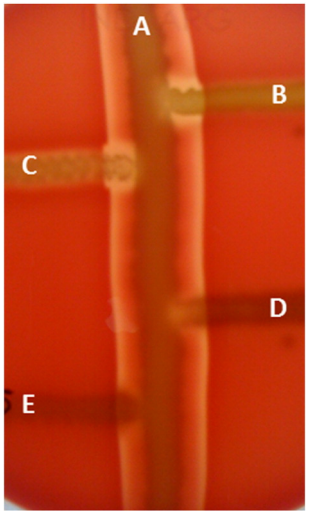

Fig. 2. Evaluation of *agr* locus functionality by delta hemolysin assay.A) *S. aureus* RN4220; B) D2; C) D2P11; D) D23C9; E) *S. aureus* N315.

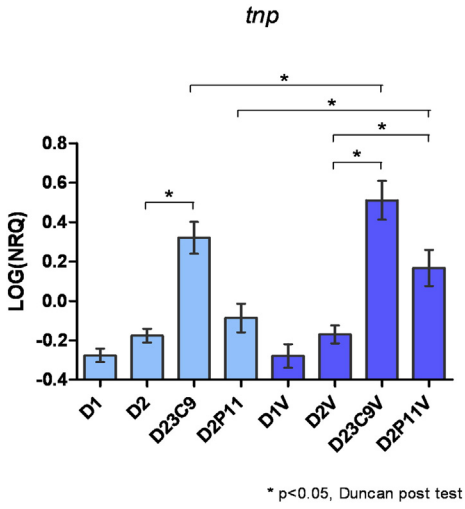

Fig. 3. Expression level of the *tnp* gene by RTqPCR. Strains named with "V" where incubated with VAN (¼ MIC). NRQ: Normalized relative quantity. Bars represent the media and the Standard Error of 3 determinations. A two-way analysis of variance was conducted to analyze the effect of two variables: strain and vancomycin treatment. Expression levels of *tnp* gene were significantly different ($p < 0.0001$) and affected by vancomycin treatment ($p = 0.0335$). There was no significant interaction between strain and vancomycin treatment ($p = 0.1978$). Horizontal bars show the differences detected by Duncan posttest.

4. Discussion

This study describes the increase of IS*256* transposition in hVISA strain during antibiotic therapy, and the accumulation of multiple IS*256* copies in two VISA derivatives obtained by serial passages in the presence of vancomycin.

Transposition of IS*256* has been described to occur in vitro as a result of antibiotic pressure in other VISA strains, mostly belonging to ST239, and ST247 lineages (McEvoy et al., 2013; Jansen et al., 2007). This paper highlights the occurrence of this phenomenon after antibiotic treatment in a hVISA strain recovered from a clinical setting. Furthermore, our results represent the first description of the association of IS*256* with ST100 hVISA/VISA strains. This ST is part of Clonal Complex 5 (CC5), which is widely distributed in Argentina (Sola et al., 2012; Gardella et al., 2005; Gardella et al., 2008). PFGE and MLST indicate that these strains belong to Argentine Pediatric clone (ST100-SCC*mec* IV_{NV}) (Sola et al., 2012), however complete SCC*mec* typing was not achieve by traditional approaches probably due to genetic rearrangements in SCC*mec*. Even more, a complex mosaic structure in SCC*mec* has been previously described in $MRSA_{ZH47}$ strain, which also harbors Δ*mecR1* interrupted by Tn*4001* and belongs to ST100 (Heusser et al., 2007).

Interestingly, the incubation of both VISA mutants (D23C9 and D2P11) with VAN sub inhibitory concentrations (¼ MIC) increased the expression of the IS*256 tnp* gene. Nagel et al. (2011) demonstrated that treatment with subinhibitory concentrations of vancomycin or ciprofloxacin resulted in an activation of transposition frequency of the insertion element IS*256* in the standard laboratory strain *S. aureus* HG001. Both findings have a relevant clinical impact since the increase in transposition could take place in those clinical situations where adequate antibiotic concentrations are not reached, such as suboptimal treatment regimes, or infection sites where the drug has poor distribution and penetration.

Table 2
Mutation frequency expressed as the media of M/V (M = Number of ciprofloxacin resistant mutants in BHI culture; V = number of viable cells in BHI culture).

Strain	Mutation frequency (BHI)	Mutation frequency (BHI + VAN ¼ MIC)
ATCC 29213	5.11×10^{-8}	7.19×10^{-8}
D1	5.32×10^{-8}	3.44×10^{-7}
D2	1.18×10^{-7}	2.1×10^{-7}
D23C9	1.0×10^{-8}	1.0×10^{-8}
D2P11	1.57×10^{-7}	4.71×10^{-8}

BHI: brain heart infusion; VAN: vancomycin.

The frequency of IS*256* transposition has been shown to be controlled by *rsb*U and *sig*B (Schreiber et al., 2013; Valle et al., 2007). Schreiber and coauthors demonstrated that the 3′ end of *rsb*U, encoding the positive regulator of the stress factor sigma B, is a hot spot for spontaneous IS*256* insertion in *S. aureus* SA137/93G. By contrast, in the strains described herein, no mutations in *sig*B or *rsb*U genes were found. Thus, the increase in IS*256* transposition observed after selective pressure by vancomycin in vivo/in vitro might be explained by alternative mechanisms including differential expression of the *sig*B operon or mutations/changes in expression of another locus.

The association between the mutation frequency and the evolution of hVISA/VISA phenotype is controversial. While Schaaff et al. (2002) suggested that an elevated mutation frequency favors the development of vancomycin resistance in *S. aureus*, other authors did not find this association (O'Neill & Chopra, 2003). In this study, there was no change in the mutation frequency to ciprofloxacin. The use of this antibiotic as the selection marker is not the best approach, but rifampin could not be used as the clinical strains are rifampin resistant. However, point mutations in other genes responsible for the molecular changes described herein could not be disregarded.

It is known that transposition of IS*256* may impact in virulence. Recently, it has been described that the insertion of IS*256* into the promoter sequence of the repressor of toxins (Rot), enhances cytotoxin expression (Benson et al., 2014). IS*256* has also been described to disrupt the *agr* locus, presumably causing the attenuation of *S. aureus* virulence (McEvoy et al., 2013; Benson et al., 2014; Shopsin et al., 2010). In this study, the insertion of IS*256* into *agr* locus leads to the loss of *agr* function only in one VISA mutant selected in vitro after vancomycin pressure. As it was described by Howden et al. (2010) the altered expression of *agr* in VISA strains is intriguing. Many VISA strains showed reduced *agr* expression, but this finding is not completely understood as some strains harbored point mutations in *agr* locus, while others present reduced expression without any mutation.

Additionally, the disruption of certain genes by IS*256* or its insertion into regulatory regions (e.g. *tca*A gene, *yyc*FG promoter region) has been previously described to alter vancomycin susceptibility in *S. aureus* (Maki et al., 2004; McEvoy et al., 2013; Jansen et al., 2007). The increase in transposition frequency of IS*256* described in this study could be involved in the development of vancomycin resistance of these strains, or be a separate event triggered by exposure to antibiotics. Furthermore, the possible association between IS*256* transposition and the change in susceptibility to other antibiotics should be explored (Table 1). While the decrease in oxacillin resistance was already reported in VISA strains (Adhikari et al., 2004; Reipert et al., 2003), it is particularly interesting that oxacillin MIC of D23C9 and D2P11 mutants increase in comparison with the MIC of parental strain D2. Previous studies have shown an increase in mutational frequency, and the activation of SOS system associated to the selection of a β-lactam homoresistant population expressing higher levels of methicillin resistance from a heteroresistant strain (Cuirolo et al., 2009; Tattevin et al., 2009).Therefore, changes in susceptibility to other families of antibiotics could be related to a state of hypermutability (point mutation and/or IS*256* transposition).

Finally, increasing transposition is an important factor contributing to the genetic flexibility of these hVISA/VISA strains. More studies are necessary for the complete understanding of the impact of transposition events on virulence and antibiotic resistance in *S. aureus*.

5. Conclusions

This study describes how exposure to antibiotics is associated with an increase in the transposition of IS*256*. The results obtained herein agree with the previously suggested hypothesis: antibiotic stress may lead to an activation of IS*256* transposition (Nagel et al., 2011; Schreiber et al., 2013), but more importantly, it demonstrates that this phenomenon is possible in a clinical environment. In ST100 *S. aureus* strains selective pressure of vancomycin and other antibiotics during long periods of treatment may increase transposition frequency and ultimately generate diverse genetic rearrangements, such as the loss of *agr* locus function, which may contribute to the genetic flexibility and evolution of this pathogen.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.meegid.2016.05.001.

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