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Genetic Diversity among *Staphylococcus aureus* Isolates Showing Oxacillin and/or Cefoxitin Resistance Not Linked to the Presence of *mec* Genes

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ABSTRACT Methicillin-resistant *Staphylococcus aureus* isolates lacking *mec* genes ($n = 32$), collected from Belgian hospitals, were characterized for their β-lactamase production and the presence of mutations in *pbp* genes, the *pbp4* promoter, and genes involved in penicillin-binding protein 4 overproduction (*gdpP* and *yjbH*). Twelve isolates were β-lactamase hyperproducers (BHPs), while 12 non-BHP isolates might produce an incomplete GdpP protein. Most isolates showed nucleotide missense mutations in *pbp* genes. A few isolates also showed mutations in the *pbp4* promoter.

KEYWORDS β-lactamase, BORSA, MODSA, PBP

Methicillin-resistant *Staphylococcus aureus* (MRSA) strains carry penicillin-binding protein 2a (PBP2a), a low-affinity PBP encoded by *mecA* and homologues (1, 2). However, isolates with methicillin and/or oxacillin (OXA) resistance but without *mec* determinants (methicillin-resistant lacking *mec* [MRLM] strains) have been reported from the 1980s to recent years (3–10). Their phenotype can be caused by hyperproduction of β-lactamase, which partially hydrolyzes semisynthetic β-lactamase-resistant penicillins (5, 6). These β-lactamase hyperproducers (BHPs) recover full susceptibility to β-lactams in the presence of β-lactamase inhibitors (5, 6). Methicillin resistance has also been associated with multiple unlinked mutations in native *pbp* genes that reduce the affinity of PBPs for β-lactams, as well as with mutations in the *pbp4* promoter and/or in genes (*gdpP* [phosphodiesterase c-di-AMP regulator] and *yjbH* [disulfide stress effector]) that lead to PBP4 overproduction (7–9, 11, 12). BHP isolates are usually named borderline oxacillin-resistant *S. aureus* (BORSA) (6), while isolates with resistance due to mutations are named modified *S. aureus* (MODSA) (3, 10); however, other authors have used the term BORSA for both BHP and MODSA isolates (4).

Data regarding the characteristics of MRLM strains are scarce (5–10), making their nomenclature difficult. In this study, we have determined the occurrence and characteristics, including β-lactamase hyperproduction and mutations in genes and regions involved in β-lactam resistance, of MRLM strains collected at the Belgian National Reference Centre (NRC) for *Staphylococcus aureus*.

The study was a retrospective analysis of 298 human *S. aureus* isolates that were collected from 73 Belgian laboratories and were sent to the NRC, due to diagnostic problems regarding their β-lactam resistance, in 2013 to 2015. The first selection identified isolates resistant to OXA and/or cefoxitin (FOX), as tested by Etest (bioMérieux), combined with the absence of *mecA* and *mecC* (13). Selected isolates were further tested for the presence of *mecB* (14) and PBP2a, by immunochromatographic assay

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TABLE 1 Phenotypic and genotypic characteristics of methicillin-resistant isolates lacking *mec* genes

BORSA type and strain	MIC (mg/liter)[a]						*blaZ*	PDDT	BHP	ST/lineage[b]
	OXA	FOX	PEN	AMP/SAM	AMX/AMC	CPT				
BHP										
001	**3**	4	**>32**	4/2	12/1	0.5	+	+	+	ST25/CC25
002	**3**	4	**>32**	8/2	12/1	0.38	+	+	+	ST25/CC25
003	**4**	4	**>32**	6/1.5	8/1	0.38	+	+	+	ST25/CC25
005	**8**	4	**>32**	4/1.5	8/1	0.38	+	+	+	ST25/CC25
006	**4**	3	**4**	3/1.5	8/0.75	0.25	+	+	+	ST3407/CC25
007	**3**	3	**>32**	2/0.5	4/0.38	0.25	+	+	+	ST30/CC30
008	**6**	4	**>32**	16/3	24/1	0.38	+	+	+	ST25/CC25
009	**8**	4	**>32**	8/2	12/1	0.38	+	+	+	ST8/CC8
010	**6**	**6**	**0.75**	4/2	4/2	0.38	+	+	+	ST9/CC9
023	**4**	**6**	**>32**	12/6	16/1	0.38	+	+	+	ST25/CC25
030	**4**	**6**	**3**	3/1.5	6/1.5	0.38	+	+	+	ST34/CC30
032	**6**	**6**	**2**	4/2	12/2	0.5	+	+	+	ST7/CC7
Non-BHP										
004	**12**	**6**	**4**	2/2	3/1	0.38	+	+	−	ST3405/CC8
011	**4**	**8**	**1.5**	1.5/2	2/1.5	0.5	+	+	−	ST5/CC5
012	**8**	**6**	0.125	0.19/0.25	0.75/0.5	0.5	+	+	−	ST582/CC15
013	**4**	**6**	**2**	2/2	3/1.5	1	+	+	−	ST5/CC5
014	**4**	**6**	**3**	3/3	4/2	1	+	+	−	ST7/CC7
015	**6**	**6**	**6**	2/2	3/1.5	1	+	+	−	ST101/CC101
017	1.5	**6**	**0.75**	1/1.5	2/1	0.5	+	+	−	ST1327/CC22
018	2	**6**	**1.5**	2/2	3/1.5	1	+	+	−	ST45/CC45
019	**4**	**8**	**0.75**	1.5/1.5	2/1.5	1	+	+	−	ST669/CC97
025	**6**	**6**	**12**	3/4	3/2	0.75	+	+	−	ST3412/CC101
027	1	**6**	**2**	2/2	3/1	0.38	+	+	−	ST3385/CC30
028	0.75	**6**	**1.5**	1/2	2/1	0.25	+	+	−	ST109/CC9
029	0.5	**6**	**0.75**	1.5/1	2/1	0.5	+	+	−	ST22/CC22
031	**12**	**8**	**4**	4/3	4/2	1	+	+	−	ST3384/CC1
016	0.5	**6**	**0.94**	0.19/0.25	0.38/0.38	0.25	−	−	−	ST3411/CC8
020	**3**	**6**	**0.75**	0.38/0.25	0.38/0.38	1	−	−	−	ST101/CC101
021	**8**	**6**	**0.38**	0.38/0.19	1/1.5	0.50	−	−	−	ST101/CC101
022	**6**	**6**	**0.94**	0.125/0.5	0.25/0.75	0.38	−	−	−	ST1/CC1
024	**4**	**6**	**0.19**	0.50/0.75	0.75/0.75	1	−	−	−	ST5/CC5
026	**4**	**6**	**0.25**	0.50/1	1/0.75	1	−	−	−	ST398/CC398

[a]Bold type indicates resistance values according to EUCAST (15).
[b]ST, sequence type.

after induction with OXA/FOX disks (13), using the Clearview Exact PBP2a assay (Alere). OXA- and/or FOX-resistant, *mecA*-, *mecB*-, *mecC*-, and PBP2a-negative isolates were studied further.

MICs for penicillin (PEN), ampicillin (AMP), amoxicillin (AMX), amoxicillin-clavulanic acid (AMC), ampicillin-sulbactam (SAM), and ceftaroline (CPT) were determined by the Etest method. MICs for PEN and CPT were interpreted according to EUCAST guidelines (15). β-Lactamase production was determined by the penicillin disk diffusion test (PDDT) (15). The presence of the β-lactamase gene *blaZ* was determined by PCR (16). Isolates were classified as BHPs if they were PDDT and *blaZ* positive with ≥2-fold MIC reductions for AMC and/or SAM, compared to AMX and AMP (5). The genes encoding native PBPs (*pbp1*, *pbp2*, *pbp3*, and *pbp4*), the *pbp4* promoter, *gdpP*, and *yjbH* were amplified and sequenced by using primers described previously (see the supplemental material). Molecular typing was performed using multilocus sequence typing (MLST) (17).

Among the isolates in the *S. aureus* collection ($n = 298$), 32 isolates showed resistance to OXA ($n = 8$), FOX ($n = 6$), or OXA and FOX ($n = 18$) (Table 1) and were *mecA*, *mecB*, *mecC*, and PBP2a negative. This proportion of MRLM strains seemed high (10.7%), compared to other studies of clinical collections (6), but is probably biased by the sampling method (isolates were referred to the NRC because of discordance in OXA and/or FOX resistance results). The isolates were recovered from different patients attending 20 hospitals located in Flanders ($n = 10$), Wallonia ($n = 4$), or Brussels ($n = 6$). The

isolates were recovered mostly from nasal/skin screening samples ($n = 18$) but also from wound/skin infection ($n = 8$), ear, nose, and throat ($n = 3$), blood ($n = 1$), urine ($n = 1$), and unknown ($n = 1$) samples. The carriage rates of BORSA isolates have been the subject of only a few studies, but they have been detected colonizing the nares of asymptomatic healthy carriers, as well as being involved in skin and soft tissue infections, surgical wounds, and urinary tract infections in hospital and community settings (10).

Most isolates ($n = 26$ [81%]) carried an active β-lactamase (*blaZ*), but only 12 were PDDT positive and BHPs (Table 1). Although their β-lactam resistance phenotype may be due β-lactamase hyperproduction, they carried mutations (Table 2) that cannot be disregarded as influencing the resistance phenotype. The remaining 20 isolates (including 14 *blaZ*-positive/PDDT-positive isolates and 6 *blaZ*-negative/PDDT-negative isolates) were non-BHPs and had diverse mutations (Table 2).

The 32 isolates were associated with 13 lineages, with a predominance of clonal complex 25 (CC25) ($n = 7$ [21.8%]). CC25 has been described as the most frequent lineage with the MRLM phenotype in Canada (7), but this clone is rarely found in Belgian hospitals (18). MRLM strains belonging to CC1, CC8, CC15, and CC45 in clinical settings and strains belonging to CC45 and CC398 in livestock were described previously (9, 19, 20).

Amino acid (AA) substitutions in the transglycosylase and transpeptidase domains of native PBPs may have different effects on β-lactam resistance. Certain AA substitutions affecting β-lactam resistance (A405V and Q629P in PBP2) were described previously (7, 9, 21). Some AA substitutions (Y336C, T371I, and H499Y in PBP1 and S364F in PBP3) were detected previously in MRLM strains from CC1, CC8, and CC15 (9). The non-BHP CC22 (CPT MIC of 0.5 mg/liter) showed AA substitutions (S629T and S664T in PBP1, T691A in PBP2, and D98E in PBP4) in common with CPT-intermediate-resistant (CPT MIC of 2 mg/liter) MRSA CC22, although the former carried additional mutations not present in MRLM strains (17). Isolates of CC25 and CC101 showed specific mutations that may have lineage origins.

Overexpression and/or mutations in PBP4 have been associated with low-level methicillin resistance (11). PBP4 overexpression can be mediated via mutations in its promoter (12) or via AA substitutions and/or loss of function of GdpP and YjbH proteins (22–24). In our study, a few isolates showed mutations in the *pbp4* promoter, although no duplications or deletions were detected. One promoter mutation (a nucleotide change from C to T 298 bp upstream of the *pbp4* start codon) was located between the −35 and −10 promoter sequences. Only one isolate showed AA substitutions in YjbH, but most carried AA substitutions in GdpP. In fact, 12 of the 20 non-BHP isolates may produce an incomplete GdpP. Among them, one isolate carried a *gdpP* gene interrupted by the insertion of a putative IS*30* family transposase. GdpP is a phosphodiesterase that controls the intracellular levels of the secondary messenger c-di-AMP, which influences cell wall architecture, biofilm formation, and resistance/tolerance to β-lactams (24–26). The deletion of *gdpP* results in increased levels of c-di-AMP, which increase *pbp4* transcript levels (24, 25). Moreover, mutations in this gene have been related to CPT tolerance (27, 28). Interestingly, some ($n = 7$) of the non-BHP isolates producing an incomplete GdpP have a borderline CPT MIC (1 mg/liter).

The clinical importance of MRLM strains is still unclear. However, these isolates have been involved in cases of clinical failure (29) and outbreaks (19), and they have been observed at high incidence rates in different patient populations (5, 30, 31). In Belgium, MRLM strains represent a heterogeneous group, with different patterns of resistance against β-lactams. Their overall prevalence may be underestimated due to the general use of the FOX test as a unique marker of methicillin resistance. Some isolates were BHPs, but most may be a mixture of BHP and MODSA, underlining the difficulties in their nomenclature.

TABLE 2 Location of mutations in the *pbp4* promoter and AA substitutions in the *pbp*, *yjbH*, and *gdpP* genes identified in methicillin-resistant isolates lacking *mec* genes[a]

Lineage and strain	Location of mutations/AA substitutions						
	PBP1 (*pbp1*)	PBP2 (*pbp2*)	PBP3 (*pbp3*)	Upstream of *pbp4* start codon	PBP4 (*pbp4*)	YjbH (*yjbH*)	GdpP (*gdpP*)
CC1							
031	–	–	–	–	–	–	A109T, Q163[b]
022	–	–	–	**–**	**R200L**	–	R504[b]
CC5							
011	–	–	–	–	–	–	D105[b]
013	–	–	–	–	–	–	D105N, P392S, A601E
024	–	T284I	–	–	–	–	D105N, P392S, V609[b]
CC7							
014	–	**Q629P**	–	–	–	–	E396[b]
032	–	**Q629P**	–	–	–	–	I203N
CC8							
004	–	–	–	–	–	–	V490E
009	–	P10L, **A405V**	N685K, K686N, K687[b]	–	–	–	F54L, P312L
016	–	–	P659[c]	–	–	–	–
CC9							
010	T39I, **Y336C**, **T371I**, **H499Y**	*A132V*, **L451I**	**S634F**	T→A at 266 bp	–	–	–
028	–	–	D195N	–	–	–	T307I
CC15							
012	–	H200Y	–	–	–	–	M313I, E314[b]
CC22							
029	S629T, S664T	**T439V**, T691A	**K584N**	C→T at 407 bp, C→T at 298 bp, G→T at 62 bp	D98E	–	–
017	S629T, S664T	**T439V**, T691A	**K584N**	C→T at 407 bp, C→T at 298 bp, G→T at 62 bp	D98E	–	V430[b]
CC25							
001	D149E	**Q629P**	K6N	–	–	–	–
002	D149E	**Q629P**	K6N	–	–	–	–
003	D149E	**Q629P**	K6N	–	–	–	–
005	D149E	**Q629P**	K6N	–	–	–	–
006	D149E	*G142C*, **Q629P**, S679T	K6N, **D644G**	–	–	–	–
008	D149E	**Q629P**	K6N, F24L	–	–	–	–
023	D149E	**Q629P**	K6N	–	–	–	–
CC30							
007	–	*N81S*	–	–	–	–	F54L
027	–	–	–	C→T at 171 bp	–	–	V609D
030	–	–	–	C→T at 171 bp	–	–	G208S, S403I
CC45							
018	**D480E**, S664T	E269Q	S225A, L201F, **M376V**, **D599E**	G→T at 62 bp	**Y208F**, V381F, R430I	–	Q56[b]
CC97							
019	**H499Y**, **S571G**	–	**S634F**	–	–	–	A210S
CC101							
015	D593E	**A591T**	–	**–**	**E218K**	–	Q258[b]
020	D593E	**A591T**	–	**–**	**E218K**	–	Q293[b]
021	D593E	*T117C*, **A591T**	–	**–**	**E218K**	K259I	Q642[b]
025	D593E	**A591T**	–	**–**	**E218K**	–	E486K
CC398							
026	**F405L**, **D480E**, D662N, S664T	D270E, **D489E**, **T439V**, T691A	D684N	A→G at 371 bp, G→A at 265 bp, A→G at 72 bp	–	–	D231*Tn*

[a]The isolates are grouped according to their lineage and/or AA substitutions. AA substitutions in the transglycosylase and transpeptidase domain of the PBPs are in italics and bold, respectively. The methicillin-sensitive *Staphylococcus aureus* strains ATCC 25923, ATCC 9144, NCTC8325, and MSSA476 were used as references for the *pbp1*, *pbp2*, *pbp3*, *pbp4* (including its promoter), *yjbH*, and *gdpP* genes. The AA substitutions A405V and Q629P in PBP2, affecting β-lactam resistance, were described previously (7, 9, 21). The AA substitutions Y336C, T371I, and H499Y in PBP1 and S364F in PBP3 were detected previously in MRLM strains (9). Some AA substitutions in GdpP (D105N and P392S) are also present in MRSA CC5 reference strains (N315, Mu3, and Mu50). *Tn*, insertion of a putative IS*30* family transposase; –, absence of mutations or amino acid substitutions.

[b]Stop codon.

[c]Absence of amino acid.

Accession number(s). The *pbp1*, *pbp2*, *pbp3*, *pbp4*, *yjbH*, and *gdpP* sequences generated in this study were deposited in GenBank under accession numbers MF070915 to MF071106 (see the supplemental material).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.00091-18.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

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We have no conflicts to declare.

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