In Vivo Effect of Flucloxacillin in Experimental Endocarditis Caused by *mecC*-positive *Staphylococcus aureus* Showing Temperature-dependent Susceptibility In Vitro

Running title: β-lactam treatment of *mecC* experimental endocarditis

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21 **ABSTRACT**

22 Methicillin-resistant *Staphylococcus aureus* (MRSA) carrying the *mecC* gene (*mecC*-
23 MRSA) exhibited at 37°C MICs of oxacillin close to those of methicillin-susceptible *S.*
24 *aureus* (MSSA). We investigated whether at this temperature, *mecC*-MRSA respond to
25 flucloxacillin treatment, like MSSA, using a rat model of endocarditis. Flucloxacillin (human-
26 like kinetics of 2 g intravenously every 6 h) cured 80-100% of aortic vegetations infected
27 with five different *mecC*-MRSA. These results suggest that *mecC*-MRSA infections may
28 successfully respond to treatment with β-lactams.

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TEXT

Methicillin-resistant *Staphylococcus aureus* (MRSA), isolated for the first time in the 1960s (1), developed resistance against methicillin and other β-lactams antibiotics through the acquisition of *mecA*, a gene encoding for the penicillin binding protein 2a (PBP2a) (2). In 2011, a novel *mecA* homologue, *mecC* (3), was identified in bovine and human MRSA isolates (4-8). Although the incidence of *mecC*-MRSA is low (8-10), these strains cause severe infections in humans (11, 12).

Most *mecC*-MRSA isolates exhibit MICs of oxacillin below or slightly above the MIC breakpoint of a susceptible strain (≤2 mg/liter) and no clear distinction from methicillin-susceptible *S. aureus* (MSSA) can be made (13, 14). Furthermore, the activity of the *mecC* product, namely PBP2c (15), appears to be thermosensitive, with a decline at 37°C (16). This raises the question as to whether *mecC*-MRSA infections should be considered like *mecA*-MRSA and treated with vancomycin (17), or like MSSA, generally treated with β-lactams, such as flucloxacillin (18). In this study, we investigated the in vivo activity of flucloxacillin on *mecC*-MRSA using a rat model of endocarditis.

Five *mecC*-MRSA, two of animal origin (NCTC 13552 and 1100) and three human isolates: the strain 820, from an infected knee (6), and the urine and screening strains S090 and S129 (19). *S. aureus* ATCC 29213 (MSSA) and Mu50 (*mecA*-MRSA) were used as controls. MICs of oxacillin and cefoxitin were determined according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (20). MICs were interpreted according to CLSI after incubation at 30°C and 37°C for 24 h. Population analysis profiles (PAPs) were performed onto agar plates containing two-fold serial dilutions of oxacillin, and on oxacillin-free plates, as described (21). Colony numbers were enumerated after 48 h incubation at

30°C and 37°C. Oxacillin-induced killing was assessed by time-kill assays using strains NCTC 13552 and S129. Flasks containing Mueller-Hinton broth supplemented with 2% NaCl were inoculated with 10^6 CFU/ml of bacteria, exposed to oxacillin (10 mg/liter) and incubated at 30°C or 37°C. The concentration of oxacillin approximate trough levels in the serum of rats and was either below or above the MIC for the organisms when tested at a temperature of 30°C or 37°C, respectively (sees Results).

The production of catheter-induced aortic vegetations and the installation of the infusion-pump device to deliver flucloxacillin were performed in rats as described (22, 23). Endocarditis was induced 24 h after catheterization by intravenous (i.v.) challenge of the animals with 10^5 CFU of each test *mecC*-MRSA and the *mecA*-MRSA Mu50. Therapy with flucloxacillin (human-like kinetics of 2 g i.v. every 6 h) was started 12 h later and lasted for three days. Control rats were sacrificed at the onset of treatment and treated rats 8 h after the end of the last antibiotic dose. A group of 3 to 4 control animals was also sacrificed at the same time than treated animals. After the sacrifice, aortic valve vegetations were removed and processed as described (24) for colony counts. All protocols for animal studies were reviewed and approved by the Cantonal Committee on Animal Experiments of the State of Vaud, Switzerland (Permit Number: 879.9).

MICs are shown in Table 1. All the *mecC*-MRSA isolates were formally resistant to oxacillin at 30°C and susceptible at 37°C (threshold: ≤2 mg/liter). All but the S820 isolate were resistant to cefoxitin (threshold: ≥8 mg/liter) at both 30°C and 37°C. Fig. 1 shows that upon exposure to oxacillin at 30°C, *mecC*-MRSA exhibited subpopulations of resistant bacteria at concentrations up to 32 mg/liter. However, these oxacillin-resistant subpopulations virtually disappeared at 37°C. Moreover, with the exception of strain NCTC

13552, where resistant subpopulations emerged on oxacillin 8 mg/liter plates, the remaining four tested *mecC*-MRSA displayed a profile similar to that of MSSA ATCC 29213 (growth at up to 4 mg/liter oxacillin). The *mecA*-MRSA Mu50 displayed homogenous resistance to oxacillin, as reported previously (25). The rate of killing by oxacillin of the selected isolates NCTC 13552 and S129 was also affected by temperature (Fig. 2). After 24 h incubation at 30°C, oxacillin did not display any antibacterial activity (Fig. 2A). In contrast, after 24 h incubation at 37°C, oxacillin caused a 2-$\log_{10}$ CFU/ml decrease in cells viability (Fig. 2B). The results of experimental endocarditis are shown in Table 2. Vegetations of control animals sacrificed at the start of treatment were all infected and contained high (median, 8.5 $\log_{10}$ CFU/g) bacterial counts. The peak (30 min), trough (6 h) (mean ± standard deviation) and the area under the curve values of flucloxacillin in rat serum were 174 ± 50 mg/liter, 16 ± 5 mg/liter and 213 mg.h/liter, respectively. The half-life was of ca. 1h. These values were close to those reported in humans, i.e., 125-154 mg/liter, 14 mg/liter, 178.6 mg.h/liter and 1 h, respectively (26, 27). Flucloxacillin sterilized 80-100% of vegetations infected with *mecC*–MRSA strains, and reduced the vegetation counts by > 6 log10 CFU/g as compared to that of controls (P< 0.005). Bacteria recovered from vegetations showed unchanged oxacillin susceptibility. All the vegetations from control animals sacrificed at the end of treatment were heavily infected (median, 9.8 $\log_{10}$ CFU/g; data not shown). Flucloxacillin was totally ineffective against the *mecA*-MRSA Mu50 isolate (0/6 [0%] sterile valves).

In the current study we observed a clear effect of the temperature on the activity of oxacillin against *mecC*-MRSA in vitro. Indeed, all five tested *mecC*-MRSA isolates showed resistance to oxacillin in assays performed at 30°C but were susceptible at 37°C. These

results are in agreement with previous studies with the NCTC 13552 (formerly LGA251) strain (16). Temperature is known to impact in vitro on the susceptibility of heterogeneous *mecA*-MRSA to oxacillin (28-30), possibly due to PBP2a down-regulation (31) or enzymatic inactivation at ≥37°C (2). Our results indicate that thermosensitive heterogeneous expression of oxacillin resistance is also present in *mecC*-MRSA isolates. However, in contrast with *mecA*-MRSA, where resistant subpopulations grew on plates containing >1000 mg/liter oxacillin at 37°C (29), *mecC*-MRSA showed no subpopulations emerging on plates containing >32 mg/liter oxacillin. Furthermore, rats with endocarditis induced by *mecC*-MRSA were successfully treated with human-like kinetics of flucloxacillin. We attribute these positive outcomes to the body temperature (≥37°C) present in animals with endocarditis (32). Indeed, at such temperature the MICs of oxacillin for the tested isolates were ≤2 mg/liter, and plasma concentrations of flucloxacillin were above the MIC of the organisms throughout the dosing interval.

These data suggest considering β-lactams as an option for the treatment of *mecC*-MRSA infections. However, due to the presence of heterogeneous oxacillin-resistant subpopulations, albeit at low frequency, caution is advised in the use of these drugs. Indeed, in an analogous situation, resistant subpopulations in *mecA*-MRSA negatively impacted β-lactam therapy of MRSA infections (33).

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124 All authors declare they have no competing interests.

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241 Table 1. MICs of oxacillin and cefoxitin. MICs were determined at the indicated
242 temperatures in Mueller-Hinton broth supplemented with 2% NaCl. MICs were
243 determined at least three times independently.
244

	MICs (mg/liter)			
	Oxacillin		Cefoxitin	
Strain	30°C	37°C	30°C	37°C
NCTC 13552	8-16	0.5-1	64	8
1100	16	0.5-2	32	8
820	4	0.5-1	8	4
S090	16	1	32	16
S129	16-32	0.5-2	64	16-32

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249 Table 2. Outcome of 3-day treatment with flucloxacillin of experimental endocarditis caused

250 by *mecC*-MRSA isolates.

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	No. sterile vegetations/total no. of vegetations (%) - Median [range] log_{10} CFU/g of vegetation	
	Treatment group	
Strains	Control	Flucloxacillin[a]
NCTC 13552	0/5 (0%) - 8.5 [6.7-8-9]	10/11 (91%)[b] - 2.0 [2.0-2.7][c]
1100	0/5 (0%) - 8.8 [8.1-9.1]	9/9 (100%)[b] - 2.0 [2.0-2.0]
820	0/5 (0%) - 7.7 [6.4-9.0]	6/8 (75%)[b] - 2.0 [2.0-4.1][c]
S090	0/5 (0%) - 8.0 [7.6-9.4]	9/10 (90%)[b] - 2.0 [7.0-3.2][c]
S129	0/5 (0%) - 8.7 [8.3-9.1]	8/10 (80%)[b] - 2.0 [2.0-6.0][c]

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254 [a] simulation in rats of human pharmacokinetics following 2 g i.v. every 6 h.

255 [b] $P < 0.05$ compared to untreated controls by Fisher's exact test.

256 [c] $P < 0.005$ compared to untreated controls by Mann-Whitney test.

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Figure legends

Figure 1. Phenotypic expression of oxacillin resistance in *mecC*-MRSA strains assessed by population analysis. Large inocula (ca. 10^9 CFU) of the test strains were spread onto Mueller-Hinton agar plates supplemented with 2% NaCl and containing increasing concentrations of oxacillin and on oxacillin-free plates. The plates were incubated at 30°C (A) or 37°C (B) for 48 h and then the CFU were enumerated. Each assay was performed in two to three independent occasions.

Figure 2. Killing curves of *mecC*-MRSA isolates NCTC 13552 and S129 by oxacillin (OXA) at a concentration of 10 mg/liter, simulating antibiotic levels achieved at trough in rat or human serum. The flasks containing the test strains not exposed (controls) or exposed to OXA were incubated at 30°C (A) or 37°C (B) for 24 h. Results are representative of two independent experiments.

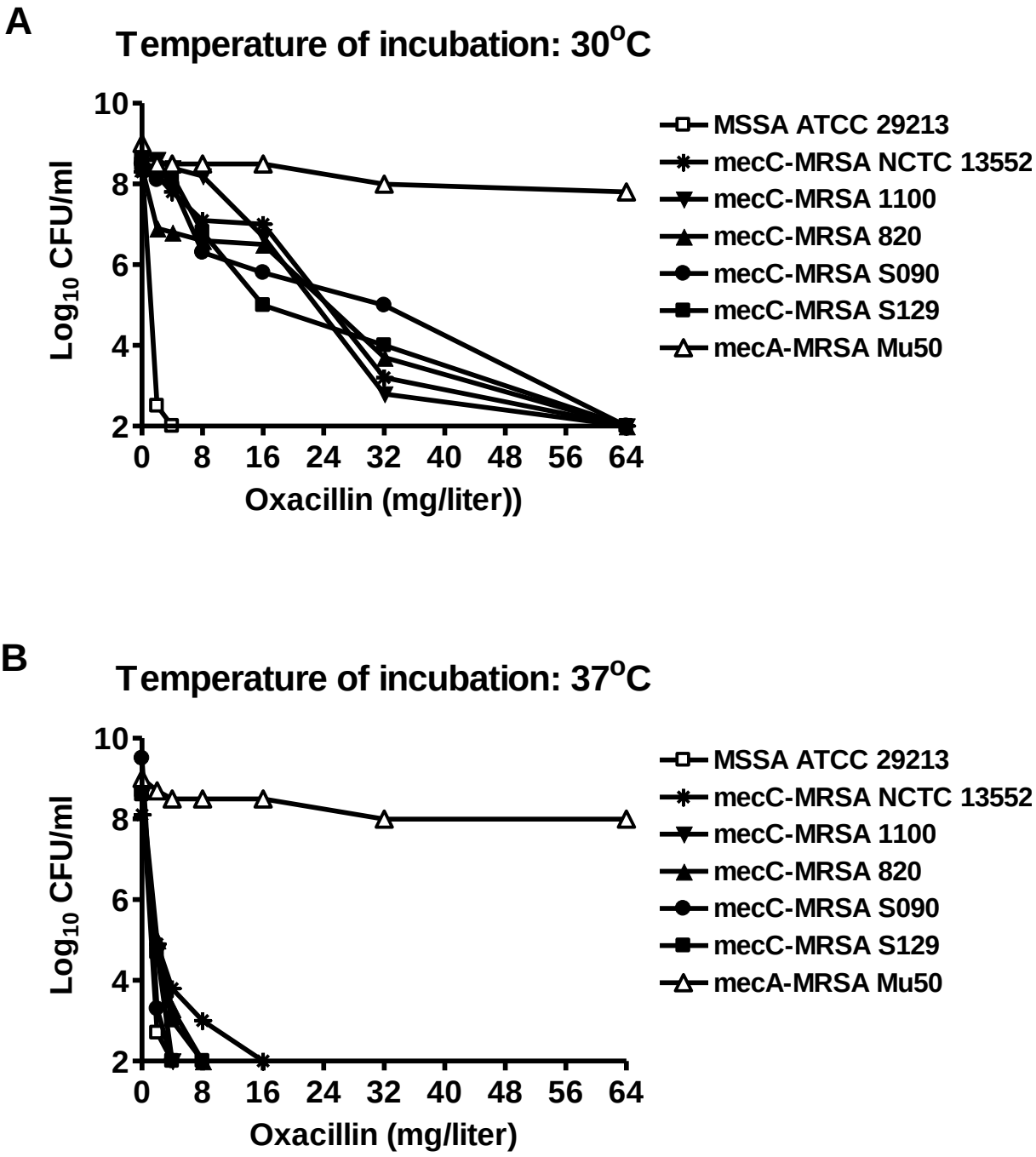

Figure 1

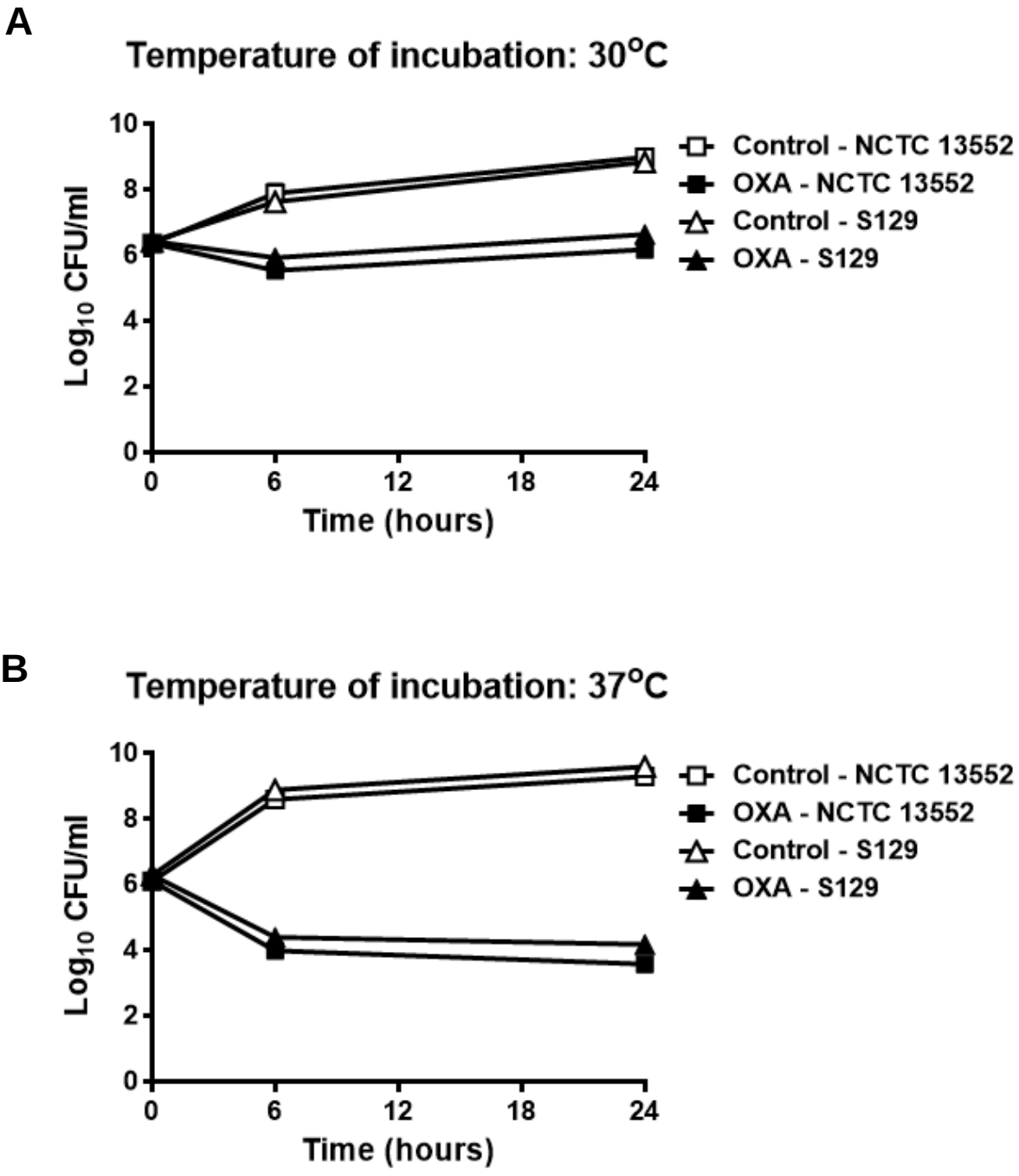

Figure 2