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Role of Acidic pH in the Susceptibility of Intraphagocytic Methicillin-Resistant *Staphylococcus aureus* Strains to Meropenem and Cloxacillin▽

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Early studies showed that methicillin-resistant *Staphylococcus aureus* (MRSA) strains are susceptible to β-lactams when they are exposed to pH ≤ 5.5 in broth. Because *S. aureus* survives in the phagolysosomes of macrophages, where the pH may be acidic, we have examined the susceptibility of MRSA ATCC 33591 phagocytized by human THP-1 macrophages to meropenem (MEM) and cloxacillin (CLX). Using a pharmacodynamic model assessing key pharmacological (50% effective concentration and maximal efficacy) and microbiological (static concentration) descriptors of antibiotic activity, we show that intraphagocytic MRSA strains are as sensitive to MEM and CLX as methicillin-susceptible *S. aureus* (MSSA; ATCC 25923). This observation was replicated in broth if the pH was brought to 5.5 and was confirmed with clinical strains. Electron microscopy showed that both the MRSA and the MSSA strains localized and multiplied in membrane-bounded structures (phagolysosomes) in the absence of β-lactams. Incubation of the infected macrophages with ammonium chloride (to raise the phagolysosomal pH) made MRSA insensitive to MEM and CLX. No difference was seen in *mec*, *mecA*, *mecI*, *mecR1*, *femA*, and *femB* expression (reversed transcription-PCR) or in PBP 2a content (immunodetection) in MRSA grown in broth at pH 5.5 compared with that in MRSA grown in broth at 7.4. The level of [^{14}C]benzylpenicillin binding to cell walls prepared from a non-β-lactamase-producing MRSA clinical isolate was two times lower than that to cell walls prepared from MSSA ATCC 25923 at pH 7.4, but the levels increased to similar values for both strains at pH 5.5. These data suggest that the restoration of susceptibility of intraphagocytic of MRSA to MEM and CLX is due to the acidic pH prevailing in phagolysosomes and is mediated by an enhanced binding to penicillin-binding proteins.

Staphylococcus aureus causes a wide range of severe and often life-threatening infections, such as endocarditis, osteomyelitis, and complicated skin and skin structure infections. Often considered an extracellular organism, *S. aureus* is capable of surviving within phagocytic and nonphagocytic cells (4, 11), which is probably an important determinant in the recurrent and relapsing character of these infections. There is substantial evidence that the intracellular milieu may modulate both the pharmacological properties of the antibiotics and the response of the bacteria (20). Yet, this factor is not taken into account in the assessment of bacterial susceptibility to drugs in the routine clinical microbiology. In the course of a study on the activities of β-lactams against *S. aureus* phagocytosed by human THP-1 macrophages (10), we noted that methicillin-resistant *S. aureus* (MRSA) and methicillin-sensitive *S. aureus* (MSSA) strains showed similar susceptibilities to meropenem (10a), suggesting a restoration of susceptibility of MRSA to β-lactams in the intracellular milieu. We present here a detailed account of this finding and extend it to cloxacillin, taken as a typical β-lactamase-resistant penicillin. Our studies suggest that the restoration of the susceptibilities to β-lactams is due to the acidic pH prevailing in the vacuoles where *S. aureus* sojourns and thrives.

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MATERIALS AND METHODS

Bacterial strains and growth conditions. All key experiments were performed with one MSSA strain (MSSA strain ATCC 25923; American Type Culture Collection [ATCC], Manassas, VA) and one MRSA β-lactamase-producing strain (MRSA strain ATCC 33591). Additional surveys were made with recent Belgian MRSA isolates (three hospital-acquired strains [obtained during this study by Y.G.] and three community-acquired strains [two strains obtained during this study by Y.G. and one strain (NRS 192) obtained from the Network on Antimicrobial Resistance in *Staphylococcus aureus*, Focus Technologies, Inc., Herndon, VA]).

For [^{14}C]benzylpenicillin binding studies, we used a non-penicillinase-producing MRSA strain (strain 459, clinical isolate, which was checked for the absence of production of penicillinase with the BBL Dryslide Nitrocefin reagent [Becton, Dickinson and Co., Cockeysville, MD] and for the presence of *mecA* by PCR amplification [see below]). All bacteria were grown in Mueller-Hinton broth supplemented with 2% NaCl (wt/vol) for the MRSA strains.

Cell cultures. All experiments were performed with THP-1 cells (ATCC TIB-202), a human myelomonocytic cell line displaying macrophage-like activity, exactly as described previously (10).

Susceptibility testing and 24-h dose-response curve studies in broth. Bacteria in exponential phase of growth were harvested and resuspended at a final density of 10^6 CFU/ml in broth adjusted to pH 7.4, 6.0, or 5.5. MIC determinations and 24-h dose-response curve studies were performed as described previously (10). We checked that the final pH of the broths at the end of the 24-h incubation period was close to the original one at all antibiotic concentrations equal to the MIC or above.

Cell infection, assessment of intracellular activities of antibiotics, and morphological studies. Infection of THP-1 cells, assessment of intracellular activity, and electron microscopy studies were performed as described earlier for *S.*

aureus ATCC 25923 (10), except that the concentration of gentamicin added to the culture medium of the controls (to which no β-lactam was added) to prevent the extracellular growth of bacteria was reduced to 0.5× MIC to better minimize its influence on the intracellular bacterial growth (see reference 1 for a full description of the gentamicin concentration effects). As reported previously (1), small colony variants were only very infrequently observed under the conditions of our experiments.

RT-PCR studies. Reverse transcription-PCR (RT-PCR) was used for the semiquantitative detection of *mecA*, *mecI*, *mecR1*, *femA*, and *femB* by using 16S rRNA as the housekeeping gene and by following a previously established procedure (9) and published primers (7, 14, 21), but with the following modifications. The bacteria were grown in broth adjusted to pH 7.4 or 5.5, collected in mid-exponential phase of growth (optical density at 600, ~0.3), and lysed with lysostaphin (100 mg/liter) and lysozyme (3 g/liter). Total RNA was extracted with an RNeasy Mini kit (QIAGEN, Hilden, Germany), the contaminating DNA was eliminated with RQ1 RNase-free DNase I (Promega Corporation, Madison, WI), and the RNA was further extracted with RNeasy mini columns. The PCRs were carried out in a Gene Cycler thermal cycler (Bio-Rad Laboratories, Hercules, CA) with 30 cycles to ensure amplification in the exponential range.

Immunoassay of PBP 2a. We used the MRSA latex screening test (Oxoid Ltd., Hants, United Kingdom), according to the method of Zhao et al. (23), and bacteria grown at pH 7.4 or 5.5; but the bacteria were tested at the pH (pH 7.0) of the commercial kit. Pilot experiments showed that the time for agglutination (20 to 180 s; detected with the naked eye) was inversely proportional ($R^2 \geq 0.912$) to the $\log_{10}$ of the bacterial density for both MRSA ATCC 33591 and MRSA strain 459 (5×10^9 to 2.5×10^8 CFU/ml) for bacteria grown at pH 7.4 or 5.5.

Whole-cell-wall binding of [^{14}C]benzylpenicillin. The assay for the whole-cell-wall binding of [^{14}C]benzylpenicillin was performed with strain 459 by a previously published procedure (2), with the following modifications. The bacteria were grown at pH 7.4 or 5.5 for 5 h and, thereafter, were exposed to [^{14}C]benzylpenicillin (50 mg/liter) for 30 min either at pH 5.5 or at pH 7.4, which generated four different conditions: pH 7.4 (culture) and pH 7.4 (binding), pH 7.4 (culture) and pH 5.5 (binding), pH 5.5 (culture) and pH 7.4 (binding), and pH 5.5 (culture) and pH 5.5 (binding). The bacteria were collected by centrifugation, washed four times with phosphate-buffered saline, and lysed by three successive freeze-thaw cycles (5 min at −80°C, followed by 5 min at 37°C.). The amount of bound radioactivity was measured by scintillation counting and was expressed by reference to the protein content in the sample.

Antibiotics and main reagents. Penicillin G, oxacillin (potency, 93%), and cloxacillin (potency, 88.4%) were purchased from Sigma-Aldrich (St. Louis, MO); and [^{14}C]benzylpenicillin (specific activity, 59 mCi/mmol) was purchased from Amersham Biosciences, Little Chalfont, United Kingdom (now GE Healthcare UK Ltd.). Meropenem, gentamicin, and ertapenem were obtained as the corresponding branded products distributed for clinical use in Belgium: meropenem was obtained as Meronem from AstraZeneca Pharmaceuticals (Brussels, Belgium); gentamicin was obtained as Geomycine from GlaxoSmithKline s.a. (Rixensart, Belgium), and ertapenem was made available to us as Invanz by Merck Sharp & Dohme Ltd. (Hoddesdon, United Kingdom). Cell culture or microbiology media were from Invitrogen (Life Science Technologies, Paisley, United Kingdom) or Becton Dickinson. Unless stated otherwise, all other reagents were obtained from Merck KgaA (Darmstadt, Germany) or Sigma-Aldrich.

Statistical analyses. Curve-fitting analyses were performed by using GraphPad Prism software (version 4.02) for Windows (GraphPad Prism Software, San Diego, CA). Analysis of variance (ANOVA) was performed with GraphPad Instat software, version 3.06 (GraphPad Prism software); and analysis of covariance was performed with XLStat (version 7.5.2; Addinsoft SARL, Paris, France).

RESULTS

Intracellular susceptibilities of MSSA and MRSA to meropenem and cloxacillin. In a first series of experiments, we compared the susceptibilities of intraphagocytic MSSA ATCC 25923 and MRSA ATCC 33591 to meropenem and cloxacillin after 24 h of incubation over a wide range of extracellular concentrations to obtain a pharmacological description of the bacterial response to these antibiotics (1). As shown in Fig. 1, we observed clear-cut, concentration-dependent effects on

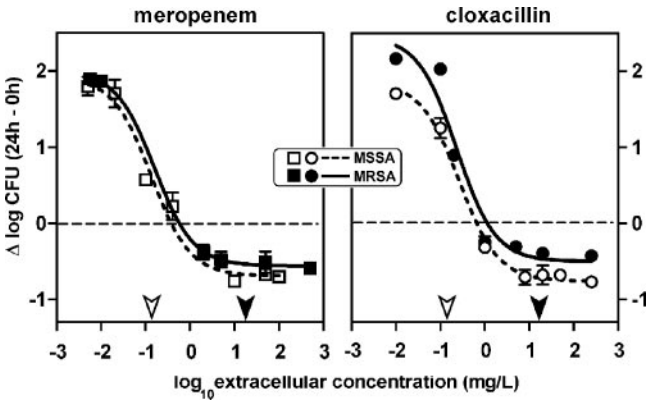

FIG. 1. Concentration killing effects of meropenem (squares; left panel) and cloxacillin (circles; right panel) toward MSSA strain ATCC 25923 (open symbols and dotted line) and MRSA strain ATCC 33591 (closed symbols and continuous line) after phagocytosis by THP-1 macrophages. Cells were incubated with the antibiotics for 24 h at the concentrations (total drug) indicated on the abscissa. All values are the means ± standard deviations of three independent determinations (standard deviation bars that are not visible are smaller than the size of the symbols). The arrows along the abscissa point to the MIC of the organisms determined in broth at pH 7.4 (open arrows, MSSA strain ATCC 25923; closed arrows, MRSA ATCC 33591).

which a model based on the Hill equation could be fitted by nonlinear regression. This allowed determining the typical pharmacological descriptors of relative potency (50% effective concentration [EC_{50}]) and apparent maximal efficacy (E_{max}) of each drug for each condition, together with an estimation of their corresponding apparent static concentrations (Table 1). This analysis showed that there was no significant difference in the responses of intracellular MSSA ATCC 25923 and MRSA ATCC 33591 to these two antibiotics, whatever criterion was used, even though there was, as anticipated, a large difference in susceptibility when the MICs were measured in broth at pH 7.4 (values shown by arrows in the graphs of Fig. 1). This suggested that MSSA ATCC 25923 and MRSA ATCC 33591 had very similar susceptibilities to meropenem and cloxacillin in the intracellular milieu.

Morphological studies. Previous studies have shown that MSSA strain ATCC 25923 phagocytosed by THP-1 cells sojourns and multiplies in membrane-bounded vacuoles belonging to the phagolysosomal apparatus (1, 10). Electron microscopy was therefore used to compare the intracellular localization of MRSA ATCC 33591 to that of MSSA ATCC 25923. In both cases, intracellular bacteria were systematically seen enclosed in membrane-bound structures (Fig. 2), consistent with a phagolysosomal localization.

Studies of MSSA and MRSA susceptibility to meropenem and cloxacillin at acidic pH in broth. Because phagolysosomes are known to be acidic (12), we examined the extent to which the acidic pH would restore the susceptibility of MRSA ATCC 33591 to β-lactams in broth. Figure 3 shows that while acidic pH only modestly decreased the MICs of meropenem and cloxacillin against MSSA ATCC 25923, it drastically reduced those of the same antibiotics against MRSA ATCC 33591, bringing them to values close to the MICs of MSSA ATCC 25923 at pH 5 to 5.5. In parallel, we examined the effect of pH on bacterial growth and observed that pH 5.5 allowed reliable

TABLE 1. Pertinent regression parameters,[a] statistical analysis, and calculation of the static concentrations from the dose-response curves shown in Fig. 1 and 3

Condition	Antibiotic[b]	Strain[c]	Regression parameter values (95% confidence intervals)		Statistical analysis[d]		Static concn[e] (mg/liter)[f]
			E_{max}[g] (mg/liter)[f]	EC_{50}[h] (mg/liter)[f]	R^2	All data	
THP-1 macrophages	MEM	MSSA	−0.69 (−0.95 to −0.42) a-A	0.14 (0.01 to 0.08) a-A	0.980	a-A	0.38
	MEM	MRSA	−0.56 (−1.21 to −0.18) a-A	0.16 (0.05 to 0.49) a-A	0.999	a-A	0.56
	CLX	MSSA	−0.76 (−0.94 to −0.58) a-A	0.27 (0.03 to 0.14) a-A	0.995	a-A	0.67
	CLX	MRSA	−0.50 (−0.95 to 0.08) a-A	0.23 (0.01 to 0.14) a-A	0.953	a-A	0.80
Broth pH 7.4	MEM	MSSA	−3.91 (−4.90 to −2.93) a-B	0.29 (0.07 to 1.28) a-A	0.984	a-B	0.18
	MEM	MRSA	NF[i]	NF	0.990		22.9
	CLX	MSSA	−2.70 (−3.48 to −1.93) b-C	0.35 (0.13 to 0.90) a-A	0.969	b-B	0.35
	CLX	MRSA	−0.39 (−1.09 to 0.30) c-A	16.9 (8.19 to 34.92) b-B	0.968	c-B	86.12
Broth pH 5.5	MEM	MSSA	−4.02 (−4.51 to −3.54) a-B	0.20 (0.08 to 0.48) a-A	0.988	a-B	0.10
	MEM	MRSA	−3.60 (−4.15 to −3.04) a,b-B,C	0.33 (0.17 to 0.63) a-A	0.980	a-B	0.25
	CLX	MSSA	−2.99 (−4.02 to −1.97) b,c-C,D	0.22 (0.08 to 0.57) a-A	0.950	b-B	0.18
	CLX	MRSA	−2.57 (−2.93 to −2.19) c-D	0.07 (0.03 to 0.14) a-A	0.983	b-C	0.10

[a] The regression parameters are based on the Hill equation (by using a slope factor of 1).
[b] MEM, meropenem; CLX, cloxacillin.
[c] MSSA, strain ATCC 25923; MRSA, strain ATCC 33591.
[d] Statistical analysis is as follows: values (E_{max} or EC_{50}) or rows (all data) with different letters are significantly different from each other within the pertinent comparison group ($P < 0.05$; lowercase letters, comparisons within each condition [THP-1 macrophages, broth pH 7.4, or broth pH 5.5]; uppercase letters, comparison throughout all conditions). E_{max} and EC_{50}, one-way ANOVA (with Tukey's test for multiple comparisons) for values of the corresponding parameters; all data, analysis of covariance (with Tukey's test for multiple comparisons) for all experimental data.
[e] Concentration (mg/liter) resulting in no apparent bacterial growth (the number of CFU was identical to that in the original inoculum), as determined by graphical interpolation.
[f] Total drug.
[g] CFU decrease (in $\log_{10}$ units) at 24 h from the corresponding original inoculum, as extrapolated for antibiotic concentration at infinity; the counts for samples yielding less than 5 counts were considered below the detection level.
[h] Concentration (mg/liter) causing a reduction of the inoculum halfway between the initial (E_0) and the maximal (E_{max}) values, as obtained from the Hill equation (by using a slope factor of 1).
[i] NF, the Hill equation could not be fitted to the data.

reproducible growth without a marked difference with that at neutral pH (24-h $\log_{10}$ CFU increases at pH 5.5 versus pH 7.4, 2.49 ± 0.01 and 2.85 ± 0.03, respectively, for MSSA ATCC 25923 and 2.78 ± 0.05 and 3.13 ± 0.02, respectively, for MRSA ATCC 33591).

We therefore examined the full dose-response curves of both strains for meropenem and cloxacillin in broth at pH 5.5 versus those at pH 7.4, and the results are shown in Fig. 4, with the pertinent pharmacological descriptors and the apparent static concentrations for each condition presented in Table 1. As anticipated, meropenem and cloxacillin were considerably less active against MRSA ATCC 33591 than against MSSA ATCC 25923 at pH 7.4 (based on the determination of the E_{max} and EC_{50} values and of the apparent static concentrations). Yet, there was no significant difference between the responses of the two strains (based on the same criteria) when the assays were performed at pH 5.5 (cloxacillin, however, showed a slightly weaker E_{max} compared to that of meropenem under all conditions).

Observations with additional strains of clinical interest. Three hospital-acquired MRSA isolates and three community-acquired MRSA isolates were tested for their susceptibilities to meropenem and cloxacillin (i) at acidic pH in broth and (ii) after phagocytosis by THP-1 macrophages. The results were very similar to those described for MRSA ATCC 33591 in Fig. 1 and 2.

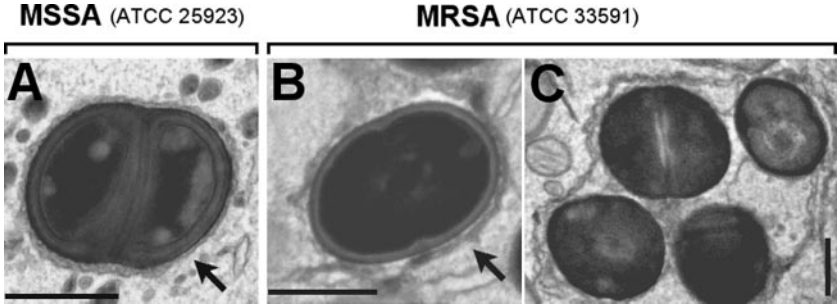

FIG. 2. Morphology of *S. aureus* in THP-1 macrophages. Cells were allowed to phagocytize the bacteria for 1 h and thereafter were incubated for 24 h (in the presence of gentamicin at 0.5× MIC) to prevent the extracellular growth of bacteria and ensuing cell death due to acidification of the medium). (A and B) Arrows point to the membrane surrounding the bacterial profiles; (C) evidence of multiplication of MRSA in a membrane-bounded structure. Bars, 0.5 μm.

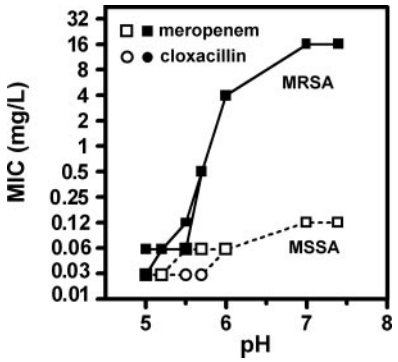

FIG. 3. Influence of pH on the MICs of meropenem and cloxacillin for MRSA ATCC 25923 and MRSA ATCC 33591 as determined in broth. Each datum point corresponds to three determinations with identical results. Symbols for one antibiotic that are not visible are overlapped by the corresponding symbols of the other antibiotic.

Influence of ammonium chloride on the intracellular activities of meropenem and cloxacillin. Ammonium chloride raises the pH of phagolysosomes of macrophages because of its proton-shuttling properties (17). Since the previous experiments identified acidic pH as a potential determinant for restoring the susceptibility of intraphagocytic MRSA ATCC 33591 to meropenem and cloxacillin, we compared the activities of both antibiotics against this strain in THP-1 macrophages in the absence and in the presence of 10 mM ammonium chloride, using fixed concentrations (meropenem, 50 mg/liter; cloxacillin, 8 mg/liter) that ensured maximal efficacy (based on the data in Fig. 1) and that are clinically meaningful (corresponding to the maximum concentration in the serum [C_{max}; the concentration of total drug] of humans). Figure 5 shows that ammonium chloride (i) caused an increased intracellular growth of MRSA ATCC 33591 in control cultures (incubated with gentamicin (0.5× MIC) only) and (ii) made meropenem and cloxacillin essentially inactive.

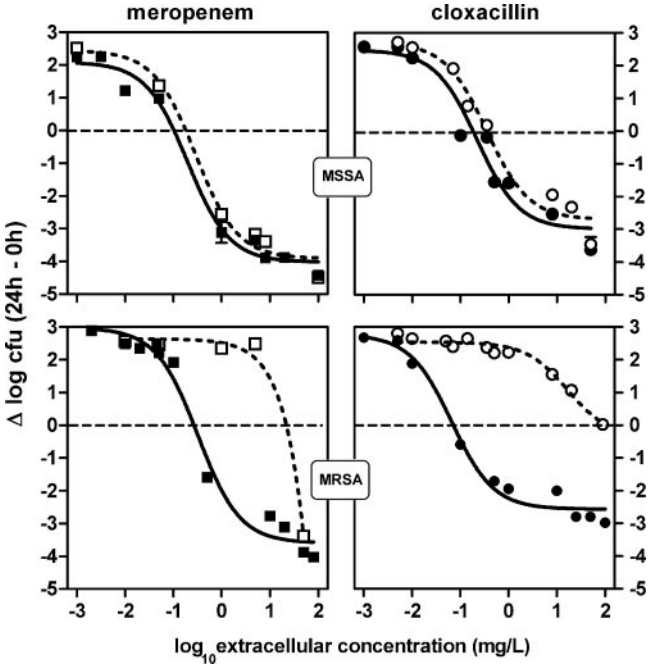

FIG. 4. Concentration killing effects of meropenem (squares; left panels) and cloxacillin (circles; right panels) toward MSSA strain ATCC 25923 (upper panels) and MRSA strain ATCC 33591 (lower panels) in broth at an initial pH of 7.4 (open symbols and dotted line) or 5.5 (closed symbols and continuous line). The bacteria were incubated with the antibiotics for 24 h at the concentrations (total drug) indicated on the abscissa. All values are the means ± standard deviations of three independent determinations (standard deviation bars that are not visible are smaller than the size of the symbols).

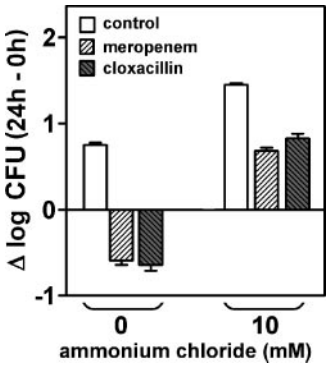

FIG. 5. Influence of ammonium chloride on the intracellular growth of methicillin-resistant *S. aureus* (ATCC 33591) and on the activities of meropenem and cloxacillin in THP-1 macrophages. Ammonium chloride was added after phagocytosis simultaneously with the antibiotics, and the cells were further incubated for 24 h before collection. Open bars, control (cells incubated for 24 h with gentamicin at 0.5× MIC to prevent the extracellular growth of bacteria and the ensuing cell death due to acidification of the medium); striped white bars, meropenem (50 mg/liter [total drug; corresponding to the human total drug C_{max}]); striped gray bars, cloxacillin (8 mg/liter [total drug; corresponding to the human total drug C_{max}). All values are the means ± standard deviations of three independent determinations.

Influence of acidic pH on the expression of *mecA* and its regulatory and accessory genes and PBP 2a immunodetection in MRSA. RT-PCR failed to reveal significant differences in the expression of *mecA* in MRSA ATCC 33591 grown in broth at pH 7.4 or 5.5, in the absence or the presence of ertapenem (used as an inducer; ratios of *mecA*/16S rRNA, 0.21 ± 0.04 at pH 7.4 and 0.19 ± 0.05 at pH 5.5 without ertapenem; 0.79 ± 0.04 at pH 7.4 and 0.67 ± 0.08 at pH 5.5 with ertapenem [0.5× MIC; 5 h]). No influence of pH was noted for the expression of *mecR1*, *femA*, and *femB* in bacteria grown at pH 5.5 or 7.4 with or without inducer. The expression of *mecI* could not be detected with RNA at amounts of up to 200 ng and 35 amplifications cycles. Finally, there was no difference in the time of agglutination of anti-PBP 2a monoclonal antibody-coated latex particles (tested at different bacterial densities to check for the consistency of the results) between MRSA ATCC 33491 and MRSA strain 459 when they were grown at pH 7.4 or 5.5 in the presence of meropenem as an inducer (0.5× MIC; 24 h).

Influence of pH on [^{14}C]benzylpenicillin binding to MSSA ATCC 25923 and MRSA. Experiments for determination of the influence of pH on [^{14}C]benzylpenicillin binding were performed with a clinical isolate (MRSA strain 459) rather than with MRSA ATCC 33591 because the latter produces an active penicillinase and no radiolabeled β-lactamase-resistant β-lactam was available to us. We first checked that the susceptibility of MRSA strain 459 to β-lactams was significantly increased

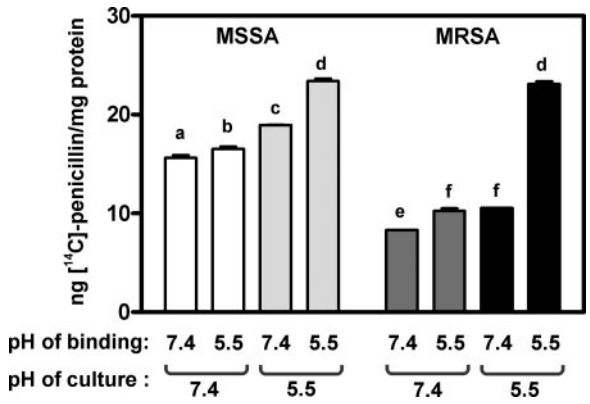

FIG. 6. Binding of [^{14}C]benzylpenicillin to MSSA strain ATCC 25923 (open and light gray bars) and MRSA strain 459 (β-lactamase negative; dark gray and closed bars) after 5 h of growth in broth at an initial pH of 7.4 (open or dark gray bars) or 5.5 (light gray or closed bars). For each culture condition, binding was made at pH 7.4 or 5.5, as indicated on the abscissa. All values are the means ± standard deviations of three independent determinations (standard deviation bars that are not visible are smaller than the size of the frame of the corresponding bar). Statistical analysis (ANOVA), bars with different letters are significantly different from all others ($P < 0.01$).

when it was tested in broth at pH 5.5 compared with that in broth at pH 7.4 (MICs, 0.03 mg/liter and 0.25 mg/liter, respectively, for penicillin G; 0.03 mg/liter and 0.125 mg/liter, respectively, for cloxacillin; and 0.03 mg/liter and 1 to 2 mg/liter, respectively, for oxacillin). Figure 6 shows that binding of [^{14}C]benzylpenicillin for MRSA strain 459 was only half of the level observed for MSSA ATCC 25923 when the bacteria had been grown and tested at pH 7.4; for MSSA, binding increased in a stepwise fashion if (i) the bacteria were grown at pH 7.4 but binding was performed at pH 5.5, (ii) the bacteria were grown at pH 5.5 and binding was performed at pH 7.4, and (iii) the bacteria were grown at pH 5.5 and binding was performed at pH 5.5. For MRSA, a small increase in binding was seen for bacteria grown at pH 7.4 and tested at pH 5.5 or grown at pH 5.5 and tested at pH 5.5, but a twofold increase in binding was seen if the bacteria were grown and tested at pH 5.5. The level of binding was then not significantly different from what was observed for MSSA grown and tested under the same conditions.

DISCUSSION

Restoration of the susceptibility of MRSA to β-lactams by acidic pH was described for a large number of laboratory and clinical isolates in the early 1970s (18) but was not considered of clinical importance by its discoverers. Indeed, they saw this effect in a reproducible fashion only at pHs lower than 5.5 (by using agar dilution methods) and were "not aware of this degree of acidity occurring frequently in infected foci" (18). The present study confirms this original observation and extends it to recent hospital- as well as community-acquired MRSA isolates. The present study also gives the observation a larger significance by showing that (i) full restoration of susceptibility can be obtained in a reproducible fashion at pH 5.5 or lower when assays are performed in pH-adjusted broths and (ii) the vacuoles in which *S. aureus* survives and thrives in macrophages may represent foci of infection with a degree of acidity sufficient to cause such a restoration of activity. We base the latter conclusion on four complementary pieces of evidence.

First, whereas MRSA ATCC 33591 and MSSA ATCC 25923 showed the expected differences in susceptibility to meropenem and cloxacillin when they were tested in broth at pH 7.4, they could not be distinguished from one another when they were exposed to these antibiotics at pH 5.5 in broth, based on both pharmacological criteria (shape of dose-response curves, E_{max}, EC_{50}) and microbiological criteria (static concentration, MIC). Second, MRSA ATCC 33591 and MSSA ATCC 25923 also could not be distinguished by their responses to meropenem or cloxacillin when they were challenged with these antibiotics after phagocytosis by THP-1 macrophages and by using the same pharmacological and microbiological criteria used for broth. Third, both strains clearly develop in phagolysosomes after phagocytosis by THP-1 macrophages (based on electron microscopic studies), and we know that the pH of phagolysosomes of macrophages is about 5 (12). Fourth, addition of ammonium chloride, which is known to raise the pH of phagolysosomes of macrophages (17), makes intraphagocytic MRSA ATCC 33591 insensitive to meropenem and cloxacillin.

Twelve years after its original discovery, the restoration of susceptibility of MRSA grown at acidic pH to β-lactams was ascribed by Hartman and Tomasz to the absence of expression of PBP 2a, based on its lack of detection with ^{3}H-labeled benzylpenicillin, even at high concentrations (8). We show here that the growth of bacteria at pH 5.5 rather than at pH 7.4 (i) does not alter the level of expression of the gene encoding the gene corresponding to PBP 2a (*mecA*) or of its regulatory genes when expression is examined by RT-PCR (a similar conclusion was drawn for the MRSA COL strain by using microarray analysis [22]) and (ii) does not modify the bacterial content in immunodetectable PBP 2a. The observation of Hartman and Tomasz must, therefore, be reinterpreted as indicating not the absence of PBP 2a but the inability of PBP 2a to bind to penicillin when it is expressed in bacteria growing at acidic pH. We could also see that cell walls from MRSA strain 459 bound about half the amount of ^{14}C-labeled benzylpenicillin bound by cell walls from MSSA ATCC 25923 at pH 7.4 but that cell walls from both strains bound a larger and not significantly different amount at pH 5.5. A tentative, global interpretation of these data is, therefore, that acidic pH (i) makes PBP 2a unable to bind to β-lactams but improves the binding of penicillin to other targets (explaining the decrease in the MIC for MSSA at acidic pH) and (ii) also makes PBP 2a unable to compensate for the inactivation of the other PBPs, resulting in the similar susceptibilities of MRSA and MRSA to β-lactams. Thus, PBP 2a exposed to acidic pH may actually be an inactive enzyme. At pH 7.4, PBP 2a already shows very weak binding and a low acylation rate when it is exposed to oxacillin but maintains efficient peptidoglycan synthesis activity. Substantial conformational changes in PBP 2a are, however, required for these reactions to occur (6), and acidic pH may make such changes impossible or much too slow (recent data indicate that cephalosporins showing activity against MRSA act by facilitating this conformational change [5]). We also know that the full expression of methicillin resistance

requires (i) the transglycosylase function of PBP 2 to be maintained, together with the transpeptidase function of PBP 2a (15), and (ii) the capacity of PBP 2a to properly localize the other components necessary for cell wall synthesis, including PBP 2, at the site of division of *S. aureus* (16). It is therefore also possible that acidic pH prevents PBP 2 from functioning in conjunction with PBP 2a and/or perturbs its recruitment. These nonmutually exclusive hypotheses may now need to be tested by the use of appropriate biochemical and morphological approaches.

Pending more detailed mechanistic investigations, the present data may already be of significance for the present therapy of MRSA infections and for an improved evaluation of presently available as well as novel antistaphylococcal agents. First, our observations with MRSA ATCC 33591 can probably be generalized to most clinical strains, since the restoration of susceptibility to methicillin by acidic pH was considered to be a general property of all MRSA isolates in its original description (8) and has been confirmed here with recent isolates. Second, our observations may trigger further in vitro and animal studies to delineate the therapeutic interest of including a β-lactam in the treatment of MRSA infections in situations where intracellular forms are suspected to play an important role the intracellular persistence of *S. aureus*, causing relapses and recurrences (19, 20). While it is not certain that intracellular *S. aureus* will always be confined within acidic phagolysosomes, restoration of the susceptibility of MRSA to β-lactams could also take place in other environments, such as the skin surface (3, 13), the vagina, or the urinary tract, which are all habitats where the pH may reach a sufficiently low value. Finally, the present data show that the study of the actual susceptibility of intracellular bacteria to antibiotics is critical to obtaining a comprehensive view of their therapeutic potential, since this susceptibility cannot simply be deduced from the cellular accumulation and disposition properties of the drug.

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REFERENCES

1. **Barcia-Macay, M., C. Seral, M. P. Mingeot-Leclercq, P. M. Tulkens, and F. Van Bambeke.** 2006. Pharmacodynamic evaluation of the intracellular activity of antibiotics against *Staphylococcus aureus* in a model of THP-1 macrophages. Antimicrob. Agents Chemother. **50:**841–851.
2. **Chambers, H. F., and C. Miick.** 1992. Characterization of penicillin-binding protein 2 of *Staphylococcus aureus*: deacylation reaction and identification of two penicillin-binding peptides. Antimicrob. Agents Chemother. **36:**656–661.
3. **Dikstein, S., and A. Zlotogorski.** 1994. Measurement of skin pH. Acta Dermatol. Venereol. Suppl. (Stockholm) **185:**18–20.
4. **Foster, T. J.** 2005. Immune evasion by staphylococci. Nat. Rev. Microbiol. **3:**948–958.
5. **Fuda, C., D. Hesek, M. Lee, W. Heilmayer, R. Novak, S. B. Vakulenko, and S. Mobashery.** 2006. Mechanistic basis for the action of new cephalosporin antibiotics effective against methicillin- and vancomycin-resistant Staphylococcus aureus. J. Biol. Chem. **281:**10035–10041.
6. **Fuda, C., M. Suvorov, S. B. Vakulenko, and S. Mobashery.** 2004. The basis for resistance to beta-lactam antibiotics by penicillin-binding protein 2a of methicillin-resistant Staphylococcus aureus. J. Biol. Chem. **279:**40802–40806.
7. **Hanssen, A. M., G. Kjeldsen, and J. U. Sollid.** 2004. Local variants of staphylococcal cassette chromosome *mec* in sporadic methicillin-resistant *Staphylococcus aureus* and methicillin-resistant coagulase-negative staphylococci: evidence of horizontal gene transfer? Antimicrob. Agents Chemother. **48:**285–296.
8. **Hartman, B. J., and A. Tomasz.** 1984. Low-affinity penicillin-binding protein associated with beta-lactam resistance in *Staphylococcus aureus*. J. Bacteriol. **158:**513–516.
9. **Kuwahara-Arai, K., N. Kondo, S. Hori, E. Tateda-Suzuki, and K. Hiramatsu.** 1996. Suppression of methicillin resistance in a *mecA*-containing pre-methicillin-resistant *Staphylococcus aureus* strain is caused by the *mecI*-mediated repression of PBP 2′ production. Antimicrob. Agents Chemother. **40:**2680–2685.
10. **Lemaire, S., F. Van Bambeke, M. P. Mingeot-Leclercq, and P. M. Tulkens.** 2005. Activity of three β-lactams (ertapenem, meropenem and ampicillin) against intraphagocytic Listeria monocytogenes and Staphylococcus aureus. J. Antimicrob. Chemother. **55:**897–904.

10a. **Lemaire, S., et al.** 2005. Carbapenems are active against intracellular MRSA, abstr. A-1832. Abstr. 45th Intersci. Conf. Antimicrob. Agents Chemother. American Society for Microbiology, Washington, DC.

11. **Lowy, F. D.** 2000. Is Staphylococcus aureus an intracellular pathogen? Trends Microbiol. **8:**341–343.
12. **Ohkuma, S., and B. Poole.** 1978. Fluorescence probe measurement of the intralysosomal pH in living cells and the perturbation of pH by various agents. Proc. Natl. Acad. Sci. USA **75:**3327–3331.
13. **Ohman, H., and A. Vahlquist.** 1994. In vivo studies concerning a pH gradient in human stratum corneum and upper epidermis. Acta Dermatol. Venereol. **74:**375–379.
14. **Perez-Roth, E., F. Claverie-Martin, J. Villar, and S. Mendez-Alvarez.** 2001. Multiplex PCR for simultaneous identification of *Staphylococcus aureus* and detection of methicillin and mupirocin resistance. J. Clin. Microbiol. **39:** 4037–4041.
15. **Pinho, M. G., H. de Lencastre, and A. Tomasz.** 2001. An acquired and a native penicillin-binding protein cooperate in building the cell wall of drug-resistant staphylococci. Proc. Natl. Acad. Sci. USA **98:**10886–10891.
16. **Pinho, M. G., and J. Errington.** 2005. Recruitment of penicillin-binding protein PBP2 to the division site of Staphylococcus aureus is dependent on its transpeptidation substrates. Mol. Microbiol. **55:**799–807.
17. **Poole, B., and S. Ohkuma.** 1981. Effect of weak bases on the intralysosomal pH in mouse peritoneal macrophages. J. Cell Biol. **90:**665–669.
18. **Sabath, L. D., S. J. Wallace, and D. A. Gerstein.** 1972. Suppression of intrinsic resistance to methicillin and other penicillins in *Staphylococcus aureus*. Antimicrob. Agents Chemother. **2:**350–355.
19. **Sinha, B., and M. Herrmann.** 2005. Mechanism and consequences of invasion of endothelial cells by Staphylococcus aureus. Thromb. Haemost. **94:** 266–277.
20. **Van Bambeke, F., M. Barcia-Macay, S. Lemaire, and P. M. Tulkens.** 2006. Cellular pharmacodynamics and pharmacokinetics of antibiotics: current views and perspectives. Curr. Opin. Drug Discov. Dev. **9:**218–230.
21. **Vannuffel, P., J. Gigi, H. Ezzedine, B. Vandercam, M. Delmee, G. Wauters, and J. L. Gala.** 1995. Specific detection of methicillin-resistant *Staphylococcus* species by multiplex PCR. J. Clin. Microbiol. **33:**2864–2867.
22. **Weinrick, B., P. M. Dunman, F. McAleese, E. Murphy, S. J. Projan, Y. Fang, and R. P. Novick.** 2004. Effect of mild acid on gene expression in *Staphylococcus aureus*. J. Bacteriol. **186:**8407–8423.
23. **Zhao, W. H., Z. Q. Hu, S. Okubo, Y. Hara, and T. Shimamura.** 2001. Mechanism of synergy between epigallocatechin gallate and beta-lactams against methicillin-resistant *Staphylococcus aureus*. Antimicrob. Agents Chemother. **45:**1737–1742.