Supplemental material

Making antimicrobial susceptibility testing more physiologically relevant with bicarbonate?

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Supplemental tables

Table S1. Previously published papers on the “bicarbonate effect”.

| Organism | Antibiotic | Comments on pH | Reference |
|----------|------------|----------------|-----------|
| Various Gram-positive Gram-and negative bacteria | Macrolides: • Azithromycin • Erythromycin | Media (CA-MHB and DMEM) adjusted to pH 7.4 with 100 mM Tris; pH after incubation not determined or discussed | (1) |
| *E. coli*  *S. aureus* | Macrolides: • Dirithromycin • Erythromycin | CA-MHB medium adjusted to pH 7.4 with HCl; pH after incubation not determined or discussed | (2) |
| *S. aureus* MRSA | β-lactams: • Oxacillin • Cefazolin | CA-MHB medium adjusted to pH 7.3 with 100 mM Tris; pH after incubation not determined or discussed | (3–7) |
| *P. aeruginosa*  *K. pneumoniae*  *E. coli, E. cloacae* | Macrolide: • Azithromycin | pH of CA-MHB medium not adjusted | (8) |
| *S. aureus* MRSA  *S. pneumoniae*  *P. aeruginosa* | Macrolide: • Azithromycin | pH of CA-MHB medium not adjusted | (9) |
| Various Gram-positive Gram-and negative bacteria | Colistin | In some experiments pH of CA-MHB medium adjusted with HCl; pH after incubation not determined | (10) |
| *S. aureus*  *P. aeruginosa*  *E. coli*  *S. agalactiae*  *E. faecalis*  *H. influenza* | no antibiotics | Medium (artificial sputum) without bicarbonate was incubated in ambient air, and with bicarbonate (25 and 100 mM) was incubated in 5% or 20% CO₂ to control pH | (11) (12) |
### Table S2. Previously published papers on the „pH effect“ on macrolide antibiotics.

| Organism | pH effect | Proposed explanation | Reference |
|----------|-----------|----------------------|-----------|
| *H. pylori* | Macrolides were more active at alkaline pH and less active at acidic pH | | (13) |
| *H. influenza; E. faealis; S. agalactiae; S. aureus; S. epidermidis* | AZI and 3 other macrolides are more active at alkaline pH and less active at acidic pH. CO₂ incubation will lower the pH of the test medium enough to diminish the potency of macrolides | | (14) |
| *S. aureus; S. pyogenes; E. faecalis; E. coli; K. pneumoniae; several anaerobes* | MIC values for AZI and ERY decrease at higher pH. CO₂ incubation will increase MIC due to lower pH better crossing of the bacterial membrane (unionized AZI) and better binding to the ribosome | | (15) |
| *E. coli* and *S. aureus* | MIC values of erythromycin and roxithromycin for *E. coli* and *S. aureus* were reduced 9-fold and 11-fold, respectively by increasing the pH from 7.2 to 8. Bactericidal activity increased with increasing pH. | | (16) |
| *Salmonella Typhi* from blood cultures of patients | AZI’s MIC decreased stepwise when pH increased with phosphate-citrate and Tris buffers. Increased transport of AZI into the cells and better access to ribosome at alkaline pH | | (17) |
Table S3. MIC values of azithromycin for *Salmonella enterica* serovar Typhimurium wild-type (wt) and its *acrB* R717Q mutant (mut)

| Buffer | Atmosphere | Bicarbonate addition | Medium pH after 18h | Strain | MIC in μg/mL Range (Geometric mean ± SD) |
|--------|------------|----------------------|---------------------|--------|----------------------------------------|
|        | Ambient    | -                    | 7.11 ± 0.13         | wt     | 4...8 (5.0 ± 2.0)                       |
|        |            |                      |                     | mut    | 32 (32.0 ± 0.0)                         |
|        |            | +                    | 8.77 ± 0.21         | wt     | 0.5...1 (0.8 ± 0.2)                     |
|        |            |                      |                     | mut    | 1...4 (2.1 ± 1.3)                       |
|        | Unbuffered | -                    | 6.47 ± 0.03         | wt     | 32...128 (58.0 ± 45.3)                  |
|        |            |                      |                     | mut    | 521...1024 (724.1 ± 295.6)              |
|        |            | +                    | 7.33 ± 0.0137       | wt     | 1...4 (1.8 ± 1.3)                       |
|        |            |                      |                     | mut    | 4-16 (8.8 ± 6.4)                        |
|        | 5% CO₂     | +                    | 7.32 ± 0.02         | wt     | 2 (2.0 ± 0.0)                           |
|        |            |                      |                     | mut    | 16 (16.0 ± 0.0)                         |
|        | 10% CO₂    | +                    | 6.8 ± 0.022         | wt     | 32 (32.0 ± 0.0)                         |
|        |            |                      |                     | mut    | 128 (128.0 ± 0.0)                       |
|        | Tris       | -                    | 6.9 ± 0.022         | wt     | 2...4 (2.2 ± 0.8)                       |
|        |            |                      |                     | mut    | 8...16 (10.1 ± 4.1)                     |
|        |            | +                    | 7.39 ± 0.003        | wt     | 2...4 (2.2 ± 0.8)                       |
|        |            |                      |                     | mut    | 8...16 (10.1 ± 4.1)                     |
|        | 5% CO₂     | -                    | 6.59 ± 0.21         | wt     | 32...64 (53.8 ± 16.0)                   |
|        |            |                      |                     | mut    | 256 (256.0 ± 0.0)                       |
|        |            | +                    | 7.1 ± 0.011         | wt     | 4...8 (4.8 ± 2.0)                       |
|                | pH               |        |        |        |        |
|----------------|------------------|--------|--------|--------|--------|
| **Hepes**      |                  |        |        |        |        |
| **Ambient**    |                  |        |        |        |        |
|                | -                | 6.83 ± 0.003 | wt | 8...16 (11.3 ± 4.6) | mut | 64 (64.0 ± 0.0) |
|                | +                | 7.23 ± 0.013 | wt | 2...4 (3.4 ± 1.0) | mut | 16 (16.0 ± 0.0) |
| **5% CO₂**     |                  |        |        |        |        |
|                | -                | 6.73 ± 0.011 | wt | 16...32 (19.0 ± 7.4) | mut | 64...256 (128.0 ± 53.4) |
|                | +                | 7.03 ± 0.009 | wt | 4...8 (4.8 ± 2.4) | mut | 16...32 (26.9 ± 7.4) |
| **MOPS**       |                  |        |        |        |        |
| **ambient**    |                  |        |        |        |        |
|                | -                | 6.7 ± 0.029 | wt | 16...32 (22.6 ± 9.2) | mut | 128 (128.0 ± 0.0) |
|                | +                | 7.1 ± 0.009 | wt | 4...8 (5.7 ± 2.3) | mut | 32 (32.0 ± 0.0) |
| **5% CO₂**     |                  |        |        |        |        |
|                | -                | 6.66 ± 0.004 | wt | 16...32 (26.9 ± 8) | mut | 128...256 (181.0 ± 73.9) |
|                | +                | 6.94 ± 0.009 | wt | 8 (8.0 ± 0.0) | mut | 32 (32.0 ± 0.0) |
| **Phosphate**  | **pH 6**         |        |        |        |        |
| **ambient**    |                  |        |        |        |        |
|                | -                | 6.17 ± 0.0062 | wt | 128...256 (190.2 ± 68.4) | mut | 512...1024 (724.1 ± 295.0) |
| Phosphate pH 6.5 | wt   | 16...32 (20.2 ± 9.2) |
|----------------|------|---------------------|
|                | mut  | 128 (128.0 ± 0.0)   |
| Phosphate pH 7  | wt   | 4 (4.0 ± 0.0)       |
|                | mut  | 16...32 (20.2 ± 9.24) |
| Phosphate pH 7.5 | wt    | 1 (1.0 ± 0.0)     |
|                 | mut  | 4...8 (5.7 ± 2.3)   |
| Phosphate pH 8  | wt   | 0.5 (0.5 ± 0.0)    |
|                 | mut  | 2 (2.0 ± 0.0)      |
| Phosphate pH 8.5 | wt   | 0.25...0.5 (0.3 ± 0.1) |
|                 | mut  | 1 (1.0 ± 0.0)      |
**Figure S1.** Phenol Red calibration curve for pH in MHB medium without added bicarbonate in ambient air based on ratiometric absorption at 415 and 560 nm. Because of low absorption of phenol red at 415 nm, ratios for pH > 8.0 are not reliable for determining pH. pH was adjusted to a desired value with HCl or NaOH. Arithmetic means of three technical replicates are shown.
Figure S2. pH kinetics in MHB medium in ambient air with or without *Salmonella* cells and with or without addition of 25 mM sodium bicarbonate. Two independent experiments for each conditions with three or more biological replicates (arithmetic means and SDs) are shown.
Supplemental Text

Materials and methods

**Bacterial strains:** A prototrophic hisG\(^+\) variant of *Salmonella enterica* serovar Typhimurium SL1344 (18, 19) was used as wild-type. An acrB R717Q point mutation associated with reduced azithromycin susceptibility (20) was introduced with a dual-negative selection genome editing method (21). Both strains are resistant to >1 mg/mL streptomycin due to episomal *aph3* and *aph6* genes.

**Antibiotic solution:** Azithromycin dihydrate (Fluorochem, UK) 15 mg/ml stock solution was prepared in 96% ethanol, and stored at 4°C for up to 7 days. The antimicrobial activity of the azithromycin solution was verified using the quality control strain *Staphylococcus aureus* ATCC 29213 (DSM2569) yielding MIC values of 1-2 ug/mL (6 biological replicates and 3 different media preparations/batches). These values were consistent with the expected range of 0.5-2 ug/mL (http://www.eucast.org).

**Buffers & bicarbonate:** 1 M buffer stock solutions of Tris(hydroxymethyl)aminomethane hydrochloride (Tris, Sigma), 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes, Sigma) or 3-Morpholinopropanesulfonic acid (MOPS, Fluka BioChemika) were adjusted to pH 7.4 or 8.1 with HCl or NaOH. To prepare 0.5 M phosphate buffer stock solutions, 0.5 M monobasic sodium phosphate (AppliChem) and 0.5 M dibasic sodium phosphate (AppliChem) were mixed until the desired pH was reached. The buffers and 1 M stock solutions of sodium bicarbonate (Sigma, USA) were sterilized by filtration through 0.22 μm filters and stored at 4°C.

**Media:** Mueller Hinton II Broth (Cation-Adjusted) (BD\(^®\), USA) was used as growth medium. 2X concentrated stock medium was prepared in a Microjet autoclave or by boiling for 1 minute in a microwave oven and filter sterilized in order to assure uniform media composition between experiments. Medium stock was stored at 4°C and used within 2 days. 2X stock medium was filled up to volume with water after addition of bicarbonate (25 mM final concentration) and/or buffer solution (100 mM final concentration), and pH adjustment to 7.2. For the calibration curve, the pH was adjusted with HCl or NaOH to a desired value. Modified media were prepared directly before experiments.

**pH measurement with phenol red:** Phenol red (0.5% phenol red solution, Sigma) was used at a final concentration of 15 mg/L. Absorbance at 415 nm, 560 nm, and 600 nm (22) was measured with BioTek SynergyMx (BioTek Instruments, Inc., USA) at 37°C in ambient air. Absorbance data were first normalized according to absorbance at 600 nm, then normalized absorbance of media without phenol red was
subtracted, and the 415 nm to 560 nm absorbance ratio was calculated. The results were converted to pH using a calibration curve (Figure S1) and equation

\[ \text{pH} = -0.9464 \times \ln(\text{absorbance ratio}) + 7.007 \]

**Susceptibility testing:** MICs were determined using a standard protocol (23) with 2-fold antibiotic serial dilutions on a 96-well plate in a 150 µl final volume. A standard lid enabling gas exchange with the surrounding atmosphere was used. All incubations were done at 37°C. Plates were incubated statically in ambient air or in a 5% CO₂ incubator. Results were recorded after 18 h by visual inspection. **Inoculum:** Strains were streaked from a -80°C glycerol stock onto low-salt lysogeny broth agar containing 90 µg/mL streptomycin and incubated overnight. A single colony was picked for an overnight culture in 3 mL MHB containing 90 µg/ml streptomycin and incubated aerobically. Streptomycin was routinely added in pre-cultures to reduce risks of contamination. On the next day, the culture was 100-fold diluted with fresh MHB without streptomycin and grown until OD₆₀₀ 0.5 to 0.8. The cultures were then diluted to OD 0.1 in unmodified MHB medium, followed by further dilution in various media to an initial density of 3 – 7 x 10⁵ CFU/mL, which was verified by plating.
Supplementary References

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