

1 Nanomotion technology for testing azithromycin 2 susceptibility of *Salmonella enterica*

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12 project on antimicrobial resistance part of the JPI-AMR action. The ERADIAMR consortium is composed of the
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23 **Running Head: Nanomotion-based AST for azithromycin in *S.enterica***

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27 ABSTRACT

28 Azithromycin is used to treat invasive salmonellosis, despite conflicting effective
29 concentrations *in vitro* and *in vivo*. Resistance of *Salmonella enterica* to azithromycin is
30 increasing. We demonstrate that nanomotion technology can be used for rapid phenotypic
31 testing of *Salmonella*'s susceptibility to azithromycin. Nanomotion changes under various
32 culture conditions correlated with susceptibility measured by MIC determination, CFU
33 counting, and fluorescent reporter-based estimates of intrabacterial azithromycin
34 accumulation.

35 MAIN

36 Invasive salmonellosis caused by *Salmonella enterica* subspecies is a major threat to human
37 health affecting >20 million people yearly (1, 2). Antimicrobial resistance to traditional drugs,
38 such as beta-lactams and fluoroquinolones, has emerged in all invasive *S. enterica* serovars
39 (1). The macrolide azithromycin (AZI) has been effectively used to treat *Salmonella* infections
40 resistant to other drug classes (3–5). AZI remains effective *in vivo*, despite recommended
41 doses achieving peak serum concentrations in the range of 0.4 µg/ml (6): 20-fold lower than

42 the MICs for most clinical strains (8 µg/ml) (7). Resistance to AZI is increasing (8, 9),
43 underlining the need for rapid susceptibility testing. Nanomotion technology can be used as
44 a rapid phenotypic antimicrobial susceptibility test (AST) (10–14). The technology is based on
45 measuring oscillations caused by metabolically active organisms attached to a
46 nanomechanical sensor, a cantilever (11, 15). The classification into resistant/susceptible
47 categories is based on machine learning algorithms for specific strain-drug combinations.
48 The susceptibility phenotype can already be detected two hours after blood culture
49 positivity (14). The technology has been successfully applied in various bacterial species, and
50 two clinical studies have been concluded (NANO-RAST (16), NCT05002413) and PHENOTECH-
51 1 (14), NCT05613322).

52 Prior to this study, nanomotion had not been used to determine susceptibility to AZI or any
53 other macrolide. We recorded nanomotion of *S. enterica* under various experimental
54 conditions affecting its susceptibility to AZI. We used neutral and acidic media, and two
55 different incubation temperatures. In the early stages of development, nanomotion was
56 measured at ambient room temperature (RT). The current setup uses 37°C for all ASTs to
57 mimic physiological conditions in humans and to decrease the time to results (14).

58 Based on MIC values, *Salmonella* is up to 4 times more sensitive to AZI at RT compared to
59 37°C in different growth media (Fig. S1A; Table S1). This effect cannot be fully explained by
60 the differences in growth rates (Fig. S1B). Nanomotion was recorded for AZI-susceptible *S.*
61 *enterica* serovar Typhimurium SL1344 (17–19) (wild-type; wt) during AZI treatment and
62 subsequent recovery in fresh drug-free medium at both RT and 37°C (Fig. 1 & S2). Before
63 the addition of the antibiotic, nanomotion variance over time increased, indicating the
64 presence and physiological activity of live bacteria on the cantilever. In the untreated
65 sample, the signal continued to increase during the measurement (Fig. 1A). When AZI was
66 added at concentrations exceeding the MIC, the nanomotion signal slope decreased. After
67 removal of AZI nanomotion started to increase again in fresh drug-free medium at 37°C,
68 indicating recovery (Fig. 1C; S2). However, no recovery was observed when the experiments
69 were conducted at RT (Fig. 1B; S2), except after treatment with 16 µg/ml AZI (Fig. S2). In all
70 cases, bacteria remained on the cantilever at the end of the experiment (Fig. S3).

71 We hypothesized that the bacteria might have been killed or their recovery delayed. Delay in
72 post-treatment recovery after an antibiotic is removed from the extracellular environment is
73 known as the postantibiotic effect (PAE), and it impacts antibiotic dosing (20, 21). Colony
74 counts after treatment indicated that AZI killed less than one log of *S. enterica* when plates
75 were incubated at 37°C during recovery. However, during RT recovery, the same
76 concentrations of AZI killed at least one log more irrespective of the treatment temperature
77 (Fig. 1D). The enhanced post-treatment killing by AZI at a lower temperature may reflect
78 slower dissociation of the drug from the ribosome, which is known to increase the
79 bactericidal activity of macrolides (22).

80 The slope of the nanomotion variance during drug exposure is a proxy for estimating drug
81 susceptibility (14). To test whether detection of AZI resistance in *Salmonella* is feasible with
82 nanomotion, we determined the slope of the variance at different AZI concentrations for wt
83 SL1344 and a resistant mutant *acrB* R717Q, which harbors a clinically relevant mutation that

84 increases AZI efflux in the acrAB-TolC efflux pump and has an MIC of 32 μ g/ml (8, 23–25).
85 We also determined the slope of the variance for wt strain at acidic pH, a condition
86 encountered by intracellular *Salmonella* in acidic vacuoles (26) which increases AZI's MIC
87 above 1024 μ g/ml (Table S1) (18). We used the rolling regression method for slope
88 estimation, which demonstrated better reliability and robustness compared to the methods
89 employed in previous studies (Supplementary materials and methods, Figure S4).

90 Remarkable differences in nanomotion arise between the strains at AZI concentrations near
91 the MIC value of the wt at neutral pH. The drug slope values of the resistant mutant begin
92 decreasing at higher AZI concentrations than the wt (Fig. 2A). A comparable difference is
93 seen in the wt between neutral and acidic pH (Fig. 2B), indicating that nanomotion can be
94 used to detect AZI susceptibility.

95 Drug slopes started to decline at AZI concentrations several-fold below the MIC, indicating
96 an effect on the bacteria (Fig 2). To verify these sub-MIC effects of AZI, we used a fluorescent
97 reporter in which the translational attenuation-based regulatory leader region (*ermCL*) is
98 fused to GFP (Fig. 3A) instead of the native *ermC* methyltransferase that confers macrolide
99 resistance (27, 28). Macrolides stall the ribosome during ErmCL translation, which opens the
100 mRNA secondary structure allowing translation initiation of the downstream gene (28). AZI
101 induced GFP expression in bacteria containing the reporter plasmid in a concentration-
102 dependent manner (Fig. 3B) at these same sub-MIC concentrations where drug slopes began
103 to decline. Maximum reporter induction was observed at or slightly above the MIC at pH
104 7.4, however little to no induction was seen at concentrations \leq 1 μ g/ml (Fig.-s 3B, S5-S6),
105 which is in good agreement with the nanomotion data (Fig. 2). In accordance with the lower
106 MIC at RT, the signal peaked at 4X lower concentrations at RT than it did at 37°C (Fig. 3B, S6).
107 However, the induction levels remained significantly lower at RT, reflecting slower
108 translation processes. At pH 5.5, GFP induction began at significantly higher concentrations
109 compared to pH 7.4 (Fig.-s 3B, S5-S6), supporting the notion that the pH-dependence of AZI
110 sensitivity is due to differences in antibiotic accumulation within the cell. AZI did not induce
111 GFP at RT at pH 5.5 (Fig. 3B, S6).

112 In summary, we show that nanomotion technology can be used for rapid detection of AZI
113 susceptibility. MIC values obtained using the standard dilution method, CFU counting results,
114 and *ermCL*-dependent GFP induction by AZI were all consistent with the physiological
115 responses recorded by nanomotion. Additionally, we found that nanomotion is effective for
116 detecting PAE and assessing bactericidal activity. Our study highlights the importance of
117 assay conditions, which significantly affected AZI efficacy and readout of the test.

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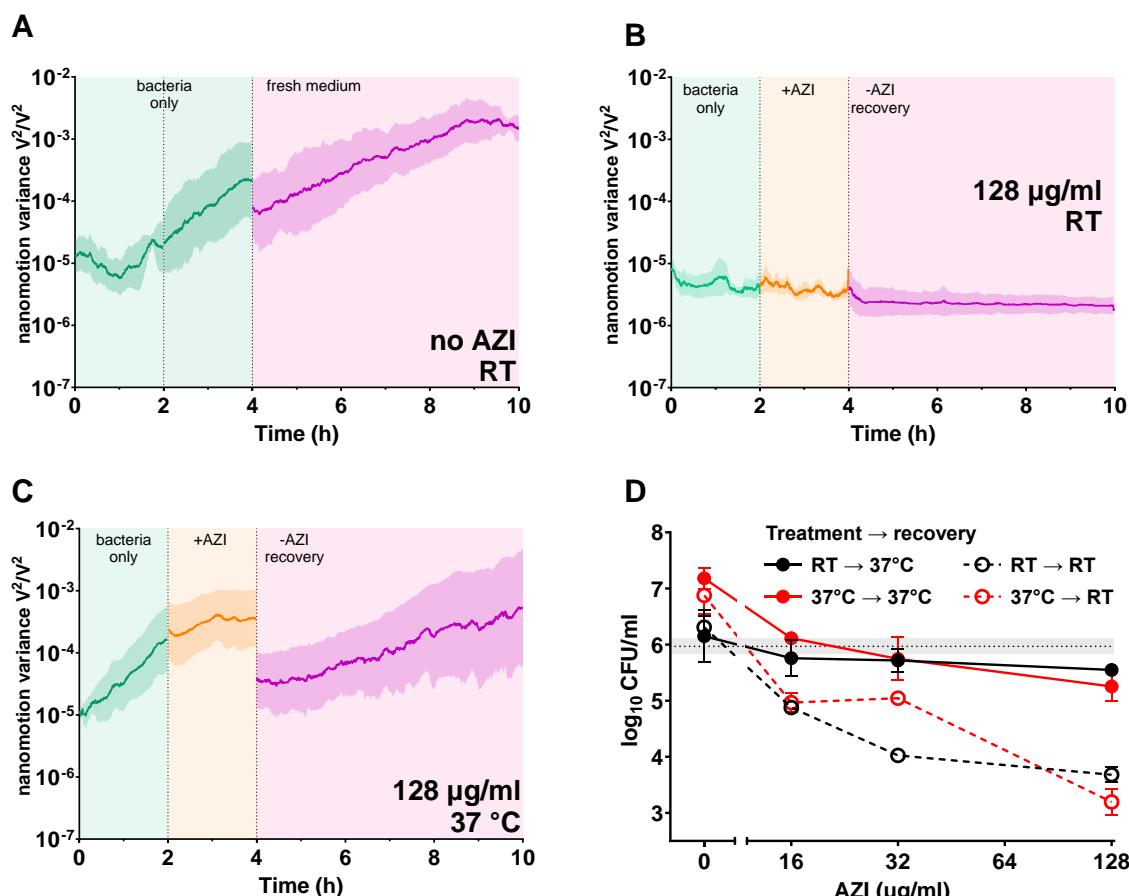
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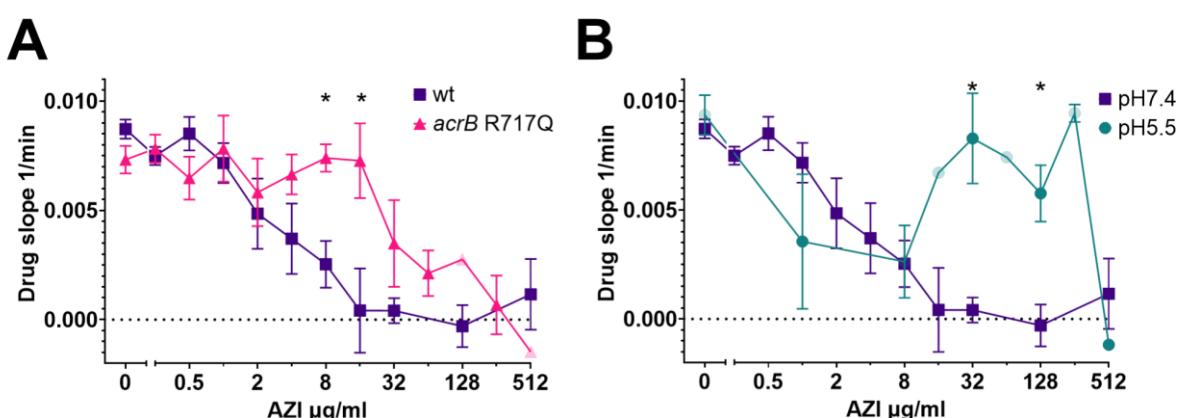
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217 **Figures**



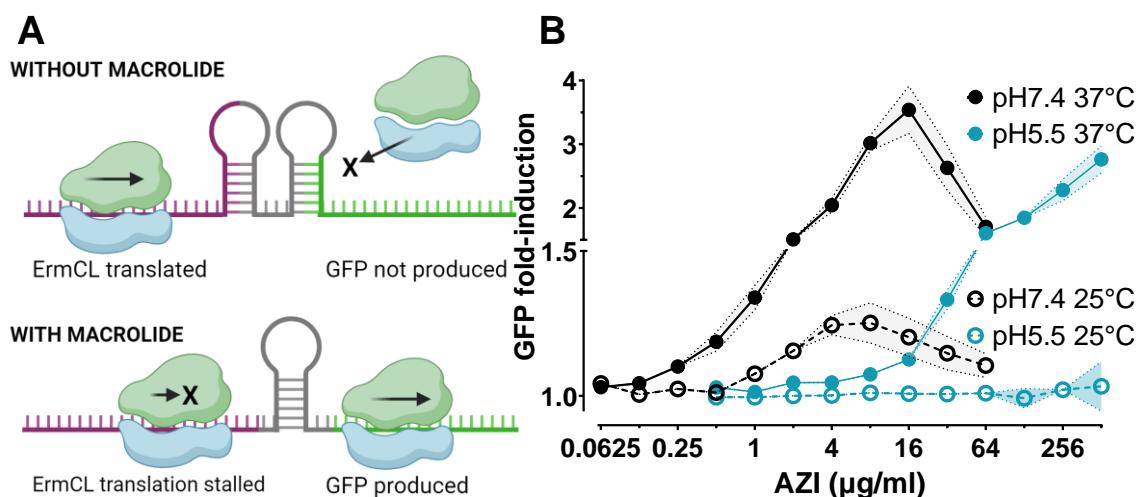
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219 Figure 1. Variance over time of the nanomotion signal measurements of wt *Salmonella*
220 without AZI (A) or with 128 µg/ml AZI for 2 hours and subsequent recovery in fresh medium
221 at room temperature (RT) (B) or at 37°C (C). Green indicates bacterial nanomotion variance
222 signal before adding the drug, orange is during drug treatment and pink is the recovery in
223 fresh medium after removing the drug. Means ± SEM (N ≥ 3 biological replicates) shown for
224 nanomotion data. D. Recovery of wt *Salmonella* colonies on LB-agar after 2h of treatment
225 with AZI at indicated temperature at pH 7.4. Grey dotted line indicates the initial inoculum.
226 Means ± SD (N ≥ 3 biological replicates).



227

228 Figure 2. The drug-phase slope of nanomotion variance reflects the response to AZI in
229 *Salmonella* and can be used to estimate susceptibility to the drug. A. The slope of the
230 variance during the drug phase of the AZI-resistant *acrB* R717Q mutant and susceptible wild-
231 type (wt) strain SL1344 at neutral pH. B. Drug slope of the wt strain at two different pH
232 values. RT data; means \pm SEM ($N \geq 3$); transparent data points shown, where $N < 3$. *Indicates
233 p-value < 0.05 of the difference between the groups at the indicated concentration.



234

235 Figure 3. ErmCL-based reporter system was used to estimate AZI's intracellular
236 concentrations and effects on translation. A. Schematic representation of the reporter
237 system. B. GFP induction of wt *Salmonella* after 2 h treatment with AZI. Flow cytometry data.
238 Means \pm SD ($N=3$).

239

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248 Author contributions: M.H. - investigation, methodology, data curation, formal analysis,
249 visualization, writing - original draft; T.M., I.K., M.P.V. - investigation; G.C. - data curation,
250 formal analysis, software; M.P., D.C., A.S., N.K., T.T. - conceptualization, funding acquisition,
251 project administration, supervision. All authors contributed to the review & editing of the
252 manuscript. Resistell AG has developed the patented (WO2023174728A1) methodology for
253 nanomotion detection.

254 Conflicts of interest

255 G.C., M.P.V., D.C., and A.S. are employees of Resistell AG and declare competing financial
256 interests.

Figures

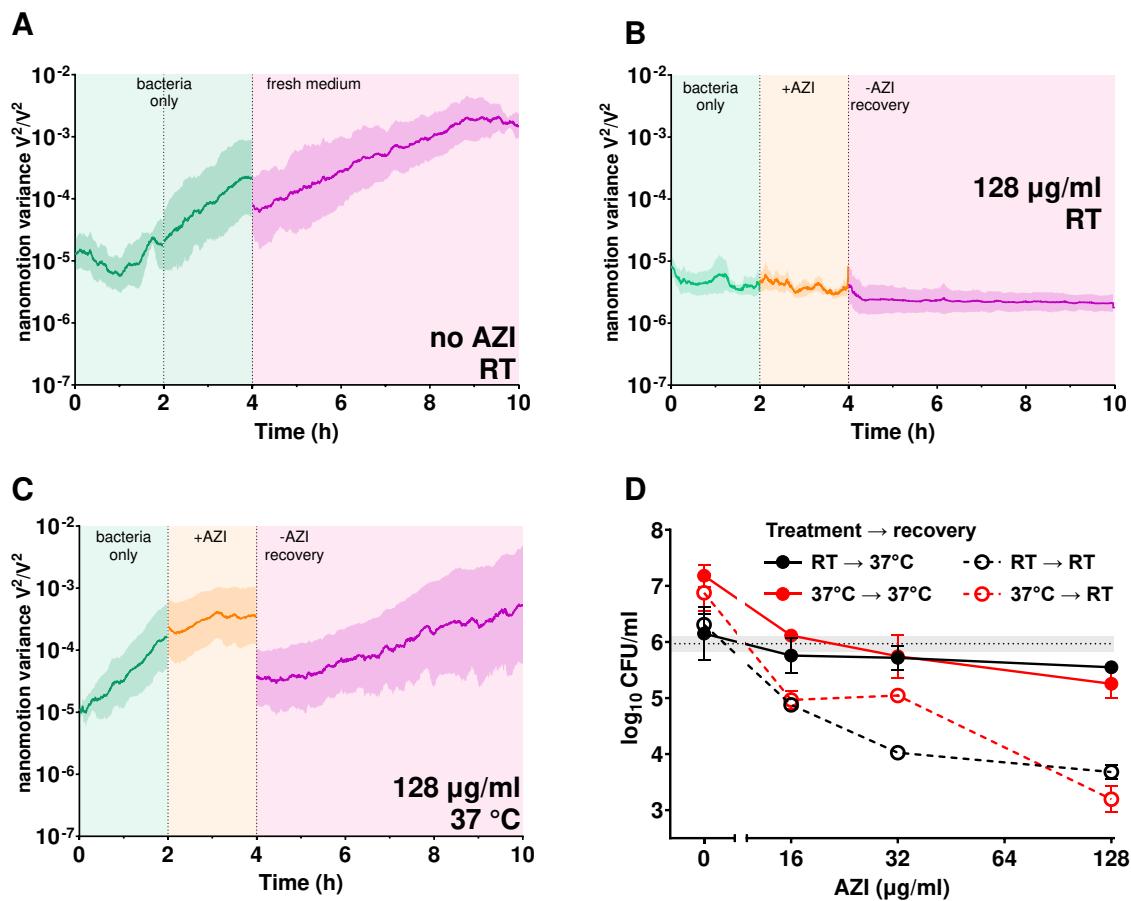


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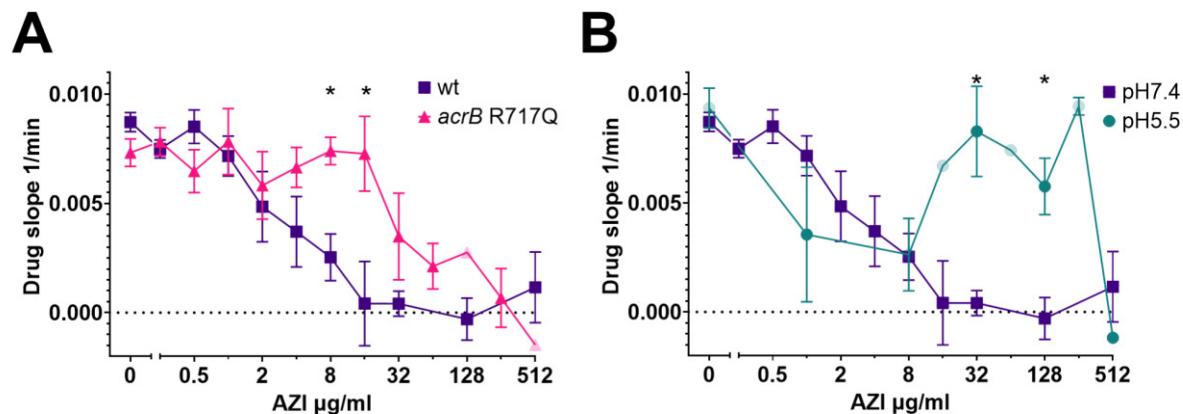


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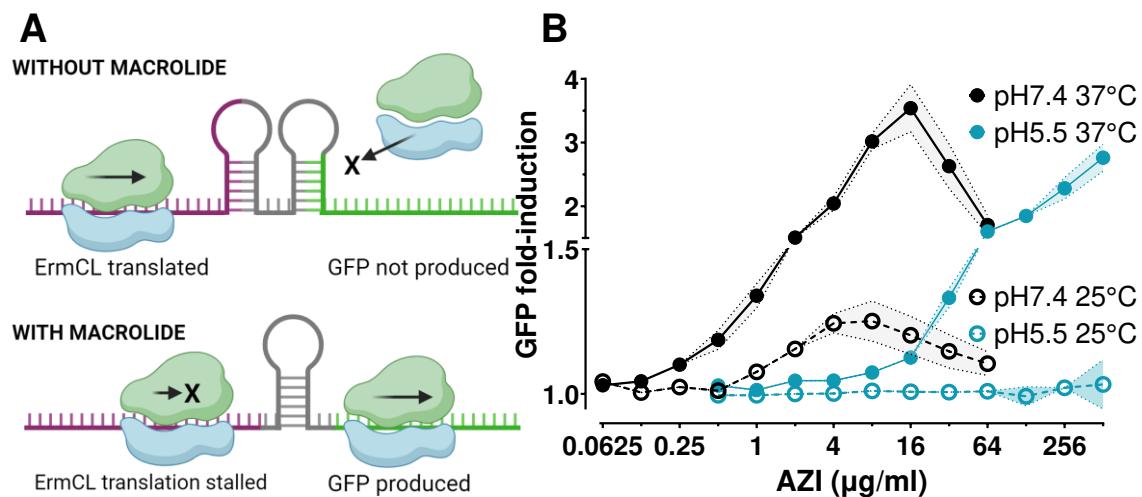


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