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A rapid unravelling of mycobacterial activity and of their susceptibility to antibiotics

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Running title: Fast analysis of mycobacteria and their susceptibility

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Abstract

The development of antibiotic-resistant bacteria is a worldwide health-related emergency that calls for new tools to study the bacterial metabolism and to obtain fast diagnoses. Indeed, the conventional analysis timescale is too long and affects our ability to fight infections. Slowly growing bacteria represent a bigger challenge, since their analysis may require up to months. Among these bacteria, *Mycobacterium tuberculosis*, the causative agent of tuberculosis has caused, only in 2016 more than 10 million new cases and 1.7 million deaths. We employed a particularly powerful nanomechanical oscillator, the nanomotion sensor, to characterize rapidly and in real time a tuberculous and a non-tuberculous bacterial species, *Bacillus Calmette-Guérin* and *Mycobacterium abscessus* exposed to different antibiotics.

Here, we show how high speed and high sensitivity detectors, the nanomotion sensors, can provide a rapid and reliable analysis of different mycobacterial species, obtaining qualitative and quantitative information on their response to different drugs.

This is the first application of the technique to tackle the urgent medical issue of mycobacterial infections, evaluating the dynamic response of bacteria to different antimicrobial families and the role of the replication rate in the resulting nanomotion pattern. In addition to a fast analysis, which could massively benefit patients and the overall healthcare system, we investigated the real-time response of the bacteria to extract unique information on the bacterial mechanisms triggered in response to the antibacterial pressure, with consequences both at the clinical and at the microbiological level.

Introduction

Providing fast and reliable antibiotic susceptibility data, which allows initiating prompt and appropriate therapy schedules, is a main task of the clinical laboratory. While molecular techniques (*i.e.* MALDI-TOF Mass Spectrometry (MS) or PCR-based gene amplification)(1, 2) have radically changed the time-frame for Gram positive (+) and negative (-) bacterial identification (ID), most of current methods for antimicrobial susceptibility testing (AST) are still based on fluorescence staining or phenotypic assays(1, 3) which may require days, or even weeks, depending on the bacterial species. In conventional clinical workflow, the sample (*e.g.* blood, spinal fluid, urine, feces, nasal or throat swabs) is harvested from patients, streaked on agar nutrient media and, after 12-24 hours incubation, transferred for further analysis. This unavoidable culture step results in isolation of microorganisms in liquid cultures containing up to 10^8 – 10^9 Colony Forming Units (CFU)/ml.(4) ID and culture-based AST are performed after this incubation time, and can last more than 24 hours.(5) Similar protocols are commonly used for slowly growing bacteria such as mycobacteria but, in these cases, the incubation time can be longer than a week (*e.g.* in the MGIT 960 system), and more than one month may be required to obtain ID and AST.(5) This long timeframe, under the pressure of life-threatening infections, often results in imprudent use or misuse of antibiotics. Early appropriate therapy will significantly reduce the spread of pathogenic bacteria into the population. This will lead to higher patient survival rate, lower distress and an optimized use of the limited resources of healthcare systems.(6, 7)

Many options are emerging to achieve a rapid, accurate and cost-effective pathogen characterization of bacterial response to drugs, ranging from molecular to rapid phenotypic techniques to plasmonic single cell assays.(8-11) The conventional AST molecular techniques rely mainly on the determination of the genetic fingerprint associated with resistance to a specific antibiotic, including real-time PCR (RT PCR), DNA microarrays, Next-generation sequencing (NGS), cell lysis-based approaches, whole-genome sequencing, and MALDI-TOF MS.(12-16) Even though the

aforementioned techniques can provide fast and high-throughput results, they still present profound drawbacks. For instance, their outcome is strongly dependent on specific drug-target genes, which can only be indicative of the actual antibiotic resistance, leaving a large gap for drug phenotypic response.

Novel single-cell techniques are have gained importance in the last decade in particular for cancer-related applications. Nevertheless, the analysis of bacterial susceptibility requires the concurrent evaluation of hundreds or thousands of bacteria to assess response at the population level. Thus, their application is mostly limited to the research laboratory and their low throughput hinders their transition to a clinical susceptibility test.

Most commercial phenotypic assays rely on bacterial replication to deliver a correct AST and the timescale for antibiogram determination may range from days, for rapidly replicating bacteria, to weeks or months, for slowly growing and fastidious germs. To shorten turnaround times and costs, several automated systems are now available, including, for instance, MicroScan WalkAway (Beckman Coulter), BD Phoenix (Dickinson Becton), Vitek-2 (bioMérieux), SIDECAR (Alifax).(17, 18) The outcome is a relatively rapid susceptible/resistant response, which is available to the clinician as soon as the second day from the first examination of the sample, with large advantages for treatment, but still too long for severe infections (*e.g.* meningitis, sepsis). (1, 3) In addition, most of these techniques provide no clue on the particular drug effect or on the counter mechanisms employed by bacteria to react to antibiotic compounds.

Among the alternatives to conventional microbiological assays, small and extremely sensitive nanomechanical oscillators(19) stand out as very promising candidates(8, 9, 20, 21). At first, such devices were employed in Atomic Force Microscopes (AFM) to study dynamic behaviour in cells(22) or proteins.(23, 24) Nowadays they are increasingly used for the detection of very small masses(25) or for nano-stress sensing in molecular biology(26) and their sensitivity and versatility

is exploited in lab-on-a-chip devices to measure bio-molecular interactions(27) or mass variations in biological systems, including bacteria.(28, 29) Two major factors limit most of the available nanomechanical systems: (i) they require air or vacuum conditions for the measurements; (ii) they need bacteria to replicate directly on the sensors, to determine cell viability through mass change or local stress alterations. This reflects on the time required to perform analysis, and on the range of information that can be obtained, based on life or death assessments.

Very recently, we have introduced a new way of exploiting the capabilities of cantilever nanosensors: the nanomotion sensor (NMS)(30, 31). By exploiting the intimate link between life and motion, measuring the fluctuations of flexible cantilevers that act as solid support for microorganisms, we can monitor in real time the metabolism of living organisms.(32) The sensor's fluctuations strongly depend on the microorganisms' metabolic activities, which combine energy consumption, cell vibration and movement. The sensitivity of the technique allows detecting the energy consumption of few ATP molecules, as demonstrated in previous works using Finite Elements Modelling or studying conformational changes in quaternary protein structures. (31, 33) Thus, the NMS can been used to evaluate the fluctuations of a very limited number of viable specimens (single mammalian cells or tens of bacteria).(32, 34-37)

By measuring the fluctuations as a function of time and of external conditions, the NMS delivers a unique insight on microorganisms response to environmental, chemical or physical stimuli.(32) The amplitude and the low frequency of these fluctuations (<1 kHz) help circumventing the major limitations of current nanomechanical sensors: the NMS can be used under various conditions (*i.e.* buffer solutions or growing media), and the viable specimens are attached on both sides of the sensor, reducing complexity and cost of each experiment. In addition, due to the time resolution of the NMS, these experiments allow investigating in real-time and with sensitivity in the Angstrom-to-micron range(33) the evolving metabolism of the adsorbed bacteria, long before a single replication(30, 31), suggesting that the analysis timescales are only marginally dependent on

bacterial duplication rates, as demonstrated by studying *B. pertussis*, which replicates in 48 hours (38). In addition, the sensitivity of these sensors allows extracting a measurable signal from groups of less than 100 bacterial cells, leading to a drastic reduction of incubation times. Overall, the outcome is a quantitative evaluation of the real bacterial response to the applied stimuli, on a time-scale (hours) comparable to the fastest molecular analyses.(39) Furthermore, the real-time monitoring of a nanomotion susceptibility test (N-AST) reveals a different kind of information on the bacterial behaviour compared to conventional phenotypic and genetic studies, with the potential to improve therapeutic interventions, which are paramount for clinical decisions and, hopefully, therapeutic outcomes, as demonstrated in previous works on rapidly growing bacteria.(40) For this reason, coupling the NMS with other conventional techniques could help to better unravel which molecular processes arise to account for specific observed motion-change behaviour.

Results

We exploited the capabilities of the NMS to study slowly growing *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) and the fast growing nontuberculous mycobacterium (NTM) *Mycobacterium abscessus.*(41) In particular, the former species belongs to the *M. tuberculosis* complex (MTC), which includes *M. tuberculosis* (Mtb), *M. africanum*, representing a growing medical emergency in both developing and developed regions.(42) By studying their nanomotion, we characterized the interaction between these two species and three antibacterial drugs, and were able to determine in a matter of hours their susceptibility, calculating their minimum inhibitory concentration (MIC) and minimum bactericidal concentrations (MBC). Furthermore, we exploited the real-time analysis of the NMS to evaluate the peculiar responses of these bacteria to the different antibacterial agents. We chose to work with a relatively large number of bacteria on the sensor (approximately between 100 and 1000 cells) to ensure a good population-level analysis of

the susceptibility and to average out the possible presence of outliers or single naturally resistant microorganisms.

In preliminary experiments, we determined whether NMS preparation protocols influenced the viability of BCG and *M. abscessus* in the MGIT medium. Overall, we kept the bacteria for at least 200 min in the analysis chamber by monitoring the oscillations over time. As shown in Figure 1, the variance of the nanomotion signals remained constant over time, indicating that the bacteria were viable for the entire control experiment.

BCG

We performed a series of experiments involving exposure of BCG to RIF or INH, two first line anti-TB drugs inhibiting the DNA-dependent RNA polymerase, and specific enzymes implicated in cell wall synthesis, respectively.(42, 43) We selected this species because it belongs to the MTC, is not dangerous for humans, and can be safely handled in a Biosafety Level 2 laboratory, constituting a safe NMS testing ground for the study of more dangerous mycobacteria, including Mtb.

When exposed to bactericidal doses of these agents, the BCG produced a sharp reduction of the sensor's fluctuations, underlining the drug's activity. In a typical experiment, the outcome of the exposure to a bactericidal dose of INH (2 μg/ml) could be determined in less than 30 min (Figure 2). On the other hand, after exposure to a high dose of RIF (0.7 μg/ml in Figure 3), we observed a reduction in the sensor's movement, demonstrating a slower bactericidal effect and confirming bacterial death which required almost 2 hours.

The subsequent step in the analysis of the response of BCG to these antibacterial agents consisted in a series of dose-dependence experiments. The results, summarized in Figure 4a for INH and 4b for RIF, indicate for each experiment the relative reduction of the nanomotion fluctuations. In both cases, the linear fit of the sigmoid curve was used to determine the MIC and MBC values. The

obtained values were MIC 0.09+-0.03 μg/ml and MBC 0.17+-0.03 μg/ml for INH; MIC 0.15+-0.07 μg/ml and MBC 0.4+-0.07 μg/ml for RIF. The MICs of BCG Pasteur determined by NMS differed by about 1-fold dilution from those determined by conventional proportion methods (0.2 *versus* 0.09 μg/ml for INH, and 0.063 *versus* 0.15 μg/ml for RIF, respectively).(44-46) Such discrepancy between the conventionally measured MIC and the N-AST is something that has been also highlighted in previous experiments involving rapidly growing bacteria.(31, 39) This difference can be interpreted by invoking many factors, such as growth conditions, measurement geometry or temperature. It must be noted that conventional and NMS assays monitor different metabolic parameters. In most conventional analyses, the MIC is identified by the bacterial ability to replicate, while for the NMS, this concentration is associated to the reduction of the sensor's fluctuations associated to alterations in the bacterial membrane elasticity(36, 37) or to their internal metabolic activity. Indeed, while the information content is the similar, the concentration at which one or the other phenomenon occurs can be different.

In addition to these quantitative susceptibility results, performing a real-time analysis on antibiotics susceptibility allowed us to evaluate how the drug pressure influenced the investigated microorganisms, including their peculiar response patterns and typical timescales. For instance, INH exposure, even if using sub-MIC concentrations, caused an immediate response of BCG, which was registered as a fluctuation intensity increase that lasted 10-15 min before a rapid decay of the movements. After few tens of minutes, if the concentration was not bactericidal (*i.e.* 0.025 μg/ml in Figure 5a), the variance of the fluctuations recovered their intensity and, returned to values comparable to those measured before the antibiotic injection. This entire response pattern did not last more than 20 minutes. On the other hand, if the drug concentration was higher than the MBC (*e.g.* 1 μg/ml, Figure 6), the response was more complex. After an initial rise of the oscillations, the movements rapidly decreased to lower values for up to 25 min, followed by few seconds of wide fluctuations. This biphasic pattern repeated itself several times for more than 1 h, until the

fluctuations stabilized to low values, indicating the death of the BCG. A possible interpretation of this pattern is related to BCG clumping: these bacteria exploit their waxy coating to form cell aggregates not completely dissolved during sample preparation procedures. In such clumps, external bacteria are expected to be metabolically more active than internal ones, partially shielding them from some environmental attacks. In this view, the bactericidal antibiotics could kill, at first, the cells of the external layer, and then the internal bacteria would be activated, resulting in the movement-stasis pattern we observed and measured. Clumping is an already known defense mechanism in microbiology and can be found in many different species, such as *Candida albicans*, or in self-aggregation in *Escherichia coli* or in flocculation in *Saccharomyces cerevisiae* (47-50) but it has never been reported in this way for BCG.

While we identified these peculiar movements in INH-exposed BCG, these were never seen in RIF experiments (Figure 5b), suggesting that this behavior is strictly dependent on the response mechanism of BCG to INH. Indeed, the response against 0.07 μg/ml of RIF did not involve a strong initial reaction to the drug, with fluctuations lasting from 1 to 2 h. In some cases, after minutes from drug exposure, we measured a temporary reduction of movements, which lasted up to 30 min, followed by full recovery of the nanomotion fluctuations. Furthermore, in RIF-exposed BCG, we never identified the oscillating patterns seen for INH. A possible interpretation of the different reported behavior respect to the two different drugs could rely on their different time-scale of the effect. While INH affects the cell wall synthesis, its effect is quite fast and the shielding of the clumping could produce a visible transitional oscillation. On the other hand, since RIF targets RNA polymerase consequently blocking protein translation, its longer timescale could cover the aforementioned effect.

M. abscessus

To investigate the response of drugs against *M. abscessus*, we used the protein synthesis inhibitor, amikacin (AK), because this organism is known to be resistant to INH and RIF. In the presence of a bactericidal concentration of the drug (10 μg/ml in Figure 7a), *M. abscessus* did not show an immediate response, without alterations of the fluctuations for the first 30 min, followed by a sharp decrease of the movements between 50 and 70 minutes, after which the fluctuations did not recover. When non-bactericidal doses were used (1 μg/ml in Figure 7b), a lag phase of approximately 10-25 min was observed, followed by a full recovery of the nanomotion signal. This trend is in line with the NMS response of *Staphylococcus aureus* exposed to cefoxitin(31) or *E. coli* to ceftriaxone(39). It is worth noting that the response time of *M. abscessus* to AK (50-70 minutes) is longer than in the BCG-INH case (20-30 min) and comparable with the BCG-RIF timescales (80-100 min). This can be attributed to the different mechanisms of action of AK compared to INH. Indeed, INH inhibits the cell wall synthesis and is expected to have a more rapid effect than RIF and AK, which inhibit the protein synthesis. Remarkably, the response times of the BCG and *M. abscessus* are similar to the typical timescales of the experiments involving rapidly replicating bacteria (between 15 and 60 minutes for both *E. coli* and *S. aureus*(31, 39)). This clearly demonstrates the nanomotion sensor's independence to the replication rate of the specimens under investigation. Furthermore, it also depicts the range of interesting new information that the technique can provide.

As done for BCG, we performed a series of dose-dependence measurements, in order to obtain quantitative susceptibility results for *M. abscessus* exposed to AK (Figure 4c). The linear fit for the sigmoid curve indicated a MIC of 1.7+-0.6 μg/ml and a MBC of 7.8+-0.6 μg/ml. These concentrations are in good agreement (within one dilution) with those present in literature for this reference *M. abscessus* strain (3.1 *versus* 1.7 μg/ml).(51, 52)

Discussion

M. tuberculosis and NTM are the causative agents of extremely dangerous infections, such as TB, which caused in 2016 more than 10.4 million new cases and 1.7 million deaths in developing and industrialized countries (WHO Global TB Report 2017). A fast diagnosis of these bacteria will allow a more specific, tailored treatment, more effective, better tolerated by the patient, and less likely to produce relapses. An early diagnosis of infections from slowly growing bacteria could be lifesaving and could significantly reduce the spread of harmful pathogens into the population, leading to higher survival rate, lower distress and an improved use of the limited resources of the healthcare system.(53, 54)

This work shows how the NMS can be used to obtain a rapid and reliable investigation of the MTC and NTM, with possible impact in the early clinical and diagnostics fields. Indeed, while the clinical application of the NMS has already been demonstrated on *E. coli*,(39) its importance to the study of slowly growing germs can be safely suggested. In addition, the speed of the nanomotion analyses can allow a rapid screening of innovative molecules and antitubercular agents, to accelerate and reduce the costs for drug discovery and drug development.(55, 56) Furthermore, the NMS can highlight the specific behavior of slowly growing bacteria during antibiotic treatment, evidencing cooperative vibrations and activity reduction, which could not be determined using other phenotypic techniques. This provides a better overview of the metabolism of slowly growing bacteria in models more closely mimicking *in vivo* microenvironments and of their dynamic responses to external stimuli, which is of great importance in medical and pharmacological TB research. These results illustrate how N-AST can become a reliable and rapid investigation tool for slowly growing bacterial species, providing also a new insight into the behavior of these bacteria during antibiotic treatment.

The possibility to combine NMS and molecular biology assays would deliver a thorough comprehension of the bacterial resistance mechanisms, which could provide invaluable information

to produce newer, more targeted drugs and anti-mycobacterial agents, to fight some of the deadlier diseases of our times.

Materials and methods

Substrates, enzymes and reagents

Chemicals, phosphate buffered saline (PBS), glutaraldehyde and antibiotics rifampicin (RIF), isoniazid (INH) and amikacin (AK), all with analytical grade, were obtained from Sigma-Aldrich (St Louis, MO). MGIT 960 (MGIT) tubes (Becton-Dickinson Microbiology Systems, Sparks, MD) were used to obtain the bacterial culture medium.

Bacterial preparation and isolation

Mycobacterium bovis BCG [American Type Culture Collection (ATCC) 27291 (Pasteur strain)] cells, frozen at -70°C, were thawed, and grown in Löwenstein Jensen slants at 37°C for 3 weeks. Few colonies were transferred in tubes containing 500 µl of MGIT medium. Since the BCG tends to form clumps that are difficult to disaggregate, before transferring the bacteria-rich medium to the nanomotion experiments we vortexed the tubes to ensure that any large bacterial clump was dissolved or reduced. Still, as shown in Figure 8, small BCG clumps were present on the sensors. The MIC of BCG Pasteur was determined using the proportion method. The characterizations showed that, in good agreement with the values present in literature, the MICs were 0.2 µg/ml for INH(45) and 0.063 µg/ml for RIF(46), respectively, with an uncertainty of one dilution.

Mycobacterium abscessus [ATCC 19977] cells frozen at -70°C were thawed, and the specimens were grown in MGIT medium at 37°C for 3 days under continuous agitation. 1 ml of the bacteria-

rich medium was centrifuged (5000 rpm for 5 min), the pellet was re-suspended in PBS and transferred directly to nanomotion experiments. The susceptibility values present in literature for ATCC 19977 towards amikacin detail a MIC of 3.1 μg/ml, with an uncertainty of one dilution.(52)

Nanomotion experiments

The nanoscale movements of the microorganisms induced dynamic deflections of the NMS, which we monitored in real-time, using the laser-based signal transduction typically used in most AFMs. We collected these fluctuations in a time-dependent chart of the vertical movements of the sensor, which appeared as a coloured noise signal, superposition of a large number of vibrations.. Overall, a typical experiment lasted more than 2 hours divided in data chunks of at least 30 minutes. The control experiments were carried out for at least 4 hours. In each experiment, the sensors were followed in real-time using optical cameras.

We used a commercial Nanowizard III microscope (JPK Instruments, Berlin, DE) and custom homemade devices developed in the Laboratories of Living Matter Physics at the EPFL (LPMV-EPFL). The sensors chosen for the JPK experiments were the ONP-10 tipless AFM cantilevers from Bruker (Bruker Nano Inc., MA, USA). For the homemade devices, we employed the SD-qp-CONT tipless cantilevers (Nano and More Gmbh, DE). For all experiments, we used sensors with spring constant in the range of 0.08+-0.03 N/m, which demonstrated a good sensitivity coupled with geometrical properties that ensured space for an adequate number of bacteria (as controlled through optical images throughout each experiment). The data from the Nanowizard III microscope were collected using the JPK software using a 10 kHz acquisition rate, while the data from the experiments on the custom devices were collected using a USB-4431 DAQ card (National Instruments, USA), using a 20 kHz acquisition rate. Remarkably, as a demonstration of the solidity

303 of the analysis technique, we were able to compare the results obtained with the two different
304 instrumentations, which exhibited similar sensitivity and noise levels.

305 To enhance bacterial adhesion, as established and detailed in many previous works, we chemically
306 treated the sensors. In literature, there are examples of fibronectin, poly-lysine, polyethylene imine,
307 APTES, glutaraldehyde, concanavalin or even collagen used for this purpose.(15, 40, 57-60) In
308 comparison with the specimens analyzed in some previous works,(61) the thick, hydrophobic and
309 waxy cell walls of mycobacteria required new immobilization strategies and the use of specific
310 culture media and drugs. In the present work, we exposed the sensors to 0.5% glutaraldehyde for
311 15 min, followed by rinsing in ultrapure water and air-drying. This immobilization protocol allowed
312 mycobacteria to adhere on the sensors with little influence on their metabolic activity (as
313 demonstrated by the control experiments depicted in Figure 1)

314 We equipped both instruments with closed custom analysis chambers, made of plastic or
315 polydimethylsiloxane (PDMS), which allowed performing experiments in liquid while changing the
316 medium with very low noise(62).

317 For the subsequent immobilization of the living bacteria on the sensors, we strictly followed a
318 protocol that ensured a remarkable adhesion of the cells on the sensors. Since the growth media
319 often contain complex molecules that could passivate the chemical functionalization on the sensor,
320 we washed twice in PBS an aliquot of MGIT-containing bacteria (by centrifuging cells at 3000xg
321 for 5 min, and re-suspending the pellet in 100 µl of PBS with a final OD that ranged from 10^6 to 10^8
322 CFU). In order to let the bacteria adhere on the sensor we deposited a droplet (typically 20 µl) of
323 the bacteria-rich PBS on the sensor and incubated at room temperature for about 15 min. This
324 protocol allows the formation of a roughly uniform distribution of bacteria on the sensor surface,
325 with an innate variability in their number and spatial distribution. Thereafter, we mounted the
326 sensor on the holder and inserted it into the analysis chamber, carrying 2 ml of MGIT medium for

the analyses. As shown in the optical images of the sensors during the experiments (Figure 8), an approximate estimate of 100 to 1000 cells adhered to the NMS, while more complex procedures must be carried out to obtain a quantitative determination.(31) It is worth highlighting that the immobilization protocol produces a variability in the absolute value of the oscillations which are different for each experiment, but the strong attachment ensures that the number and position of the bacteria is well-preserved throughout each single analysis.

As detailed in previous works, to generate dose-dependence curves we compared several parallel experiments in which the bacteria were exposed to different concentrations of antibiotic drugs. To obtain these graphs we focused on the relative reduction of the fluctuations compared to the maximum variance measured before the exposure to the chosen drug concentration. In this way we were able to define a comparative measure of the bacterial response and to compare and average the many experiments we have performed. Each experiment was performed at least in triplicate, lasted at least 2 hours and comprised three steps: i) preparation of viable bacteria in medium (BCG or *M. abscessus* in MGIT medium); ii) exposure to the desired drug concentration (INH, RIF or AK); iii) exposure to a high bactericidal drug concentration.(31) The concentration dependence curve was then fitted with a sigmoid curve interpolation, bearing a similar information content of a conventional antibiogram. By performing a linear fitting of the central part of the sigmoid, we calculated two concentration values which can be associated to the minimum inhibitory concentration (MIC - the drug concentration that inhibits visible bacterial growth) and the minimum bactericidal concentration (MBC - the concentration that ensures the death of 99.9% of the bacteria)(31). New AST assays, intended as substitute of conventional microbiological procedures should provide these two concentrations, and this is one of the main advantages of phenotypic assays compared to faster molecular counterparts. Indeed, just as for the conventional values, at drug concentrations below the NM-MIC, the fluctuations of the cantilever are largely unaffected, while at values higher than the NM-MBC the movements are largely reduced.

352

353 **Statistical analysis**

354 The nanomotion data is presented as acquired, with a simple linear fit to remove any long-term drift
355 from the traces. The variance calculations are presented as histograms +-SD calculated on the
356 corresponding fluctuations over the corresponding raw data, but the trends are indicative of at least
357 three independent sample preparations.

358 Each datapoint of the dose-dependence graphs was obtained from minimally three independent
359 experiments and the graphs were drawn following the procedures detailed elsewhere.(31) The error
360 bars represent the variability of the different experiments performed at each concentration.

361

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Data availability

All the presented data is available upon reasonable request. Figures 1, 2, 3, 5, 6 and 7 present raw data.

All data collection and analysis were performed using custom Labview code, which is freely available upon reasonable request.

Author contributions

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AAC

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382 GL, AM and LV performed the nanomotion experiments; SK and GD provided the nanomotion
383 sensor infrastructure; GL, SK and GD developed and optimized the methodology; GL and LV
384 developed the software; AM and LF provided and characterized the BCG; KB and RAF provided
385 and characterized the *M. abscessus*; GL, LV, SD and MG performed the data analysis; GL, LV,
386 AM, SD and MG wrote the manuscript; all authors read and commented on the manuscript.

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388 **Transparency declaration**

389 The authors declare no competing interests

References

1. Franck J, Arafah K, Elayed M, Bonnel D, Vergara D, Jacquet A, Vinatier D, Wisztorski M, Day R, Fournier I, Salzet M. 2009. MALDI Imaging Mass Spectrometry. Molecular & Cellular Proteomics 8:2023-2033.
2. Fournier P-E, Drancourt M, Colson P, Rolain J-M, Scola BL, Raoult D. 2013. Modern clinical microbiology: new challenges and solutions. Nat Rev Micro 11:574-585.
3. Goff DA, Jankowski C, Tenover FC. 2012. Using Rapid Diagnostic Tests to Optimize Antimicrobial Selection in Antimicrobial Stewardship Programs. Pharmacotherapy 32:677-687.
4. Steward CD, Raney PM, Morrell AK, Williams PP, McDougal LK, Jevitt L, McGowan JE, Jr., Tenover FC. 2005. Testing for induction of clindamycin resistance in erythromycin-resistant isolates of Staphylococcus aureus. J Clin Microbiol 43:1716-21.
5. Horvat RT. 2010. Review of Antibiogram Preparation and Susceptibility Testing Systems. Hospital Pharmacy 45:S6-S9.
6. Cosgrove SE. 2006. The relationship between antimicrobial resistance and patient outcomes: mortality, length of hospital stay, and health care costs. Clinical Infectious Diseases 42:S82-S89.
7. Seale AC, Gordon NC, Islam J, Peacock SJ, Scott JAG. 2017. AMR Surveillance in low and middle-income settings - A roadmap for participation in the Global Antimicrobial Surveillance System (GLASS). Wellcome Open Research 2:92.
8. Safavieh M, Pandya HJ, Venkataraman M, Thirumalaraju P, Kanakasabapathy MK, Singh A, Prabhakar D, Chug MK, Shafiee H. 2017. Rapid Real-Time Antimicrobial Susceptibility Testing with Electrical Sensing on Plastic Microchips with Printed Electrodes. ACS Applied Materials & Interfaces 9:12832-12840.
9. Syal K, Shen S, Yang Y, Wang S, Haydel SE, Tao N. 2017. Rapid Antibiotic Susceptibility Testing of Uropathogenic E. coli by Tracking Submicron Scale Motion of Single Bacterial Cells. ACS Sensors 2:1231-1239.
10. Liu C-Y, Han Y-Y, Shih P-H, Lian W-N, Wang H-H, Lin C-H, Hsueh P-R, Wang J-K, Wang Y-L. 2016. Rapid bacterial antibiotic susceptibility test based on simple surface-enhanced Raman spectroscopic biomarkers. Scientific Reports 6:23375.
11. Syal K, Iriya R, Yang Y, Yu H, Wang S, Haydel SE, Chen H-Y, Tao N. 2016. Antimicrobial Susceptibility Test with Plasmonic Imaging and Tracking of Single Bacterial Motions on Nanometer Scale. ACS Nano 10:845-852.
12. Schubert S, Kostrzewa M. 2015. Chapter 14 - MALDI-TOF Mass Spectrometry in the Clinical Microbiology Laboratory; Beyond Identification, p 501-524. *In* Andrew S, Yi-Wei T (ed), Methods in Microbiology, vol Volume 42. Academic Press.
13. Pulido MR, Garcia-Quintanilla M, Martin-Pena R, Cisneros JM, McConnell MJ. 2013. Progress on the development of rapid methods for antimicrobial susceptibility testing. J Antimicrob Chemother 68:2710-7.
14. Didelot X, Bowden R, Wilson DJ, Peto TE, Crook DW. 2012. Transforming clinical microbiology with bacterial genome sequencing. Nat Rev Genet 13:601-12.
15. Dinarelli S, Girasole M, Kasas S, Longo G. 2017. Nanotools and molecular techniques to rapidly identify and fight bacterial infections. J Microbiol Methods 138:72-81.
16. Mokrousov I, Chernyaeva E, Vyazovaya A, Sinkov V, Zhuravlev V, Narvskaya O. 2016. Next-Generation Sequencing of Mycobacterium tuberculosis. Emerging infectious diseases 22:1127-1129.
17. Mittman SA, Huard RC, Della-Latta P, Whittier S. 2009. Comparison of BD Phoenix to Vitek 2, MicroScan MICroSTREP, and Etest for Antimicrobial Susceptibility Testing of Streptococcus pneumoniae. Journal of Clinical Microbiology 47:3557-3561.

18. Rhoads S, Marinelli L, Imperatrice CA, Nachamkin I. 1995. Comparison of MicroScan WalkAway system and Vitek system for identification of gram-negative bacteria. J Clin Microbiol 33:3044-6.
19. Boisen A, Dohn S, Keller SS, Schmid S, Tenje M. 2011. Cantilever-like micromechanical sensors. Reports on Progress in Physics 74:036101.
20. Domínguez CM, Kosaka PM, Sotillo A, Mingorance J, Tamayo J, Calleja M. 2015. Label-Free DNA-Based Detection of Mycobacterium tuberculosis and Rifampicin Resistance through Hydration Induced Stress in Microcantilevers. Analytical Chemistry 87:1494-1498.
21. Etayash H, Khan MF, Kaur K, Thundat T. 2016. Microfluidic cantilever detects bacteria and measures their susceptibility to antibiotics in small confined volumes. Nature Communications 7:12947.
22. Pelling AE, Sehati S, Gralla EB, Valentine JS, Gimzewski JK. 2004. Local nanomechanical motion of the cell wall of Saccharomyces cerevisiae. Science 305:1147-1150.
23. Radmacher M, Fritz M, Hansma HG, Hansma PK. 1994. Direct Observation of Enzyme-Activity with the Atomic-Force Microscope. Science 265:1577-1579.
24. Schneider SW, Egan ME, Jena BP, Guggino WB, Oberleithner H, Geibel JP. 1999. Continuous detection of extracellular ATP on living cells by using atomic force microscopy. PNAS 96:12180-12185.
25. Braun T, Ghatkesar MK, Backmann N, Grange W, Boulanger P, Letellier L, Lang H-P, Bietsch A, Gerber C, Hegner M. 2009. Quantitative time-resolved measurement of membrane protein-ligand interactions using microcantilever array sensors. Nature Nanotechnology 4:179-185.
26. Ndieyira JW, Watari M, Barrera AD, Zhou D, Vogtli M, Batchelor M, Cooper MA, Strunz T, Horton MA, Abell C, Rayment T, Aeppli G, McKendry RA. 2008. Nanomechanical detection of antibiotic mucopeptide binding in a model for superbug drug resistance. Nature Nanotechnology 3:691-696.
27. Berger R, Delamarche E, Lang HP, Gerber C, Gimzewski JK, Meyer E, Guntherodt HJ. 1997. Surface stress in the self-assembly of alkanethiols on gold. Science 276:2021-2024.
28. Burg TP, Godin M, Knudsen SM, Shen W, Carlson G, Foster JS, Babcock K, Manalis SR. 2007. Weighing of biomolecules, single cells and single nanoparticles in fluid. Nature 446:1066-1069.
29. Park K, Millet LJ, Kim N, Li H, Jin X, Popescu G, Aluru NR, Hsia KJ, Bashir R. 2010. Measurement of adherent cell mass and growth. Proceedings of the National Academy of Sciences 107:20691-20696.
30. Kasas S, Longo G, Alonso-Sarduy L, Dietler G. 2011. Nanoscale Motion Detector. Patent Switzerland patent PCT/IB2011054553.
31. Longo G, Alonso Sarduy L, Rio LM, Bizzini A, Trampuz A, Notz J, Dietler G, Kasas S. 2013. Rapid detection of bacterial resistance to antibiotics using AFM cantilevers as nanomechanical sensors. Nat Nano 8:522-526.
32. Kasas S, Ruggeri FS, Benadiba C, Maillard C, Stupar P, Tournu H, Dietler G, Longo G. 2015. Detecting nanoscale vibrations as signature of life. Proceedings of the National Academy of Sciences 112:378-381.
33. Alonso-Sarduy L, De Los Rios P, Benedetti F, Vobornik D, Dietler G, Kasas S, Longo G. 2014. Real-Time Monitoring of Protein Conformational Changes Using a Nano-Mechanical Sensor. PLoS ONE 9:e103674.
34. Ruggeri FS, Mahul-Mellier A-L, Kasas S, Lashuel HA, Longo G, Dietler G. 2017. Amyloid single-cell cytotoxicity assays by nanomotion detection. Cell Death Discov 3:17053.
35. Lissandrello C, Inci F, Francom M, Paul MR, Demirci U, Ekinci KL. 2014. Nanomechanical motion of Escherichia coli adhered to a surface. Appl Phys Lett 105:113701.

36. Wu S, Liu X, Zhou X, Liang XM, Gao D, Liu H, Zhao G, Zhang Q, Wu X. 2016. Quantification of cell viability and rapid screening anti-cancer drug utilizing nanomechanical fluctuation. Biosensors and Bioelectronics 77:164-173.
37. Yang F, Riedel R, Del Pino P, Pelaz B, Said AH, Soliman M, Pinnapireddy SR, Feliu N, Parak WJ, Bakowsky U, Hampp N. 2017. Real-time, label-free monitoring of cell viability based on cell adhesion measurements with an atomic force microscope. J Nanobiotechnology 15:23.
38. Ines VM, Petar S, Wojciech C, Massimiliano B, Giovanni D, Laura A, Elena VM, Osvaldo Y, Sandor K. 2018. Nanomotion Detection Method for Testing Antibiotic Resistance and Susceptibility of Slow-Growing Bacteria. Small 14:1702671.
39. Stupar P, Opota O, Longo G, Prod'hom G, Dietler G, Greub G, Kasas S. 2017. Nanomechanical sensor applied to blood culture pellets: a fast approach to determine the antibiotic susceptibility against agents of bloodstream infections. Clin Microbiol Infect 23:400-405.
40. Beaussart A, El-Kirat-Chatel S, Herman P, Alsteens D, Mahillon J, Hols P, Dufrene YF. 2013. Single-cell force spectroscopy of probiotic bacteria. Biophys J 104:1886-92.
41. Tortoli E. 2014. Microbiological features and clinical relevance of new species of the genus Mycobacterium. Clin Microbiol Rev 27:727-52.
42. Zumla AI, Gillespie SH, Hoelscher M, Philips PP, Cole ST, Abubakar I, McHugh TD, Schito M, Maeurer M, Nunn AJ. 2014. New antituberculosis drugs, regimens, and adjunct therapies: needs, advances, and future prospects. The Lancet infectious diseases 14:327-340.
43. Calvori C, Frontali L, Leoni L, Tecce G. 1965. Effect of Rifamycin on Protein Synthesis. Nature 207:417.
44. Marianelli C, Armas F, Boniotti MB, Mazzone P, Pacciarini ML, Di Marco Lo Presti V. 2015. Multiple drug-susceptibility screening in Mycobacterium bovis: new nucleotide polymorphisms in the embB gene among ethambutol susceptible strains. International Journal of Infectious Diseases 33:39-44.
45. Kolibab K, Derrick SC, Morris SL. 2011. Sensitivity to isoniazid of Mycobacterium bovis BCG strains and BCG disseminated disease isolates. Journal of clinical microbiology 49:2380-2381.
46. Rastogi N, Goh K, Berchel M, Bryskier A. 2000. Activity of rifapentine and its metabolite 25-O-desacetylrifapentine compared with rifampicin and rifabutin against Mycobacterium tuberculosis, Mycobacterium africanum, Mycobacterium bovis and M. bovis BCG. Journal of Antimicrobial Chemotherapy 46:565-570.
47. Aon MA, Roussel MR, Cortassa S, O'Rourke B, Murray DB, Beckmann M, Lloyd D. 2008. The Scale-Free Dynamics of Eukaryotic Cells. PLOS ONE 3:e3624.
48. Lloyd D, Cortassa S, O'Rourke B, Aon MA. 2012. What yeast and cardiomyocytes share: ultradian oscillatory redox mechanisms of cellular coherence and survival. Integr Biol (Camb) 4:65-74.
49. Kruse K, Julicher F. 2005. Oscillations in cell biology. Curr Opin Cell Biol 17:20-6.
50. Schembri MA, Christiansen G, Klemm P. 2001. FimH-mediated autoaggregation of Escherichia coli. Mol Microbiol 41:1419-30.
51. Maurer FP, Bruderer VL, Castelberg C, Ritter C, Scherbakov D, Bloemberg GV, Bottger EC. 2015. Aminoglycoside-modifying enzymes determine the innate susceptibility to aminoglycoside antibiotics in rapidly growing mycobacteria. J Antimicrob Chemother 70:1412-9.
52. Pryjma M, Burian J, Kuchinski K, Thompson CJ. 2017. Antagonism between Front-Line Antibiotics Clarithromycin and Amikacin in the Treatment of Mycobacterium abscessus Infections Is Mediated by the whiB7 Gene. Antimicrob Agents Chemother 61.

53. Ibrahim EH, Sherman G, Ward S, Fraser VJ, Kollef MH. 2000. The influence of inadequate antimicrobial treatment of bloodstream infections on patient outcomes in the ICU setting. Chest 118:146-155.
54. Premanandh J, Samara BS, Mazen AN. 2015. Race Against Antimicrobial Resistance Requires Coordinated Action – An Overview. Frontiers in Microbiology 6:1536.
55. Imperi F, Leoni L, Visca P. 2014. Antivirulence activity of azithromycin in Pseudomonas aeruginosa. Frontiers in Microbiology 5.
56. Rampioni G, Visca P, Leoni L, Imperi F. 2017. Drug repurposing for antivirulence therapy against opportunistic bacterial pathogens. Emerging Topics in Life Sciences doi:10.1042/etls20160018.
57. Formentin P, Catalan U, Pol L, Fernandez-Castillejo S, Sola R, Marsal LF. 2018. Collagen and fibronectin surface modification of nanoporous anodic alumina and macroporous silicon for endothelial cell cultures. J Biol Eng 12:21.
58. Kim YH, Baek NS, Han YH, Chung MA, Jung SD. 2011. Enhancement of neuronal cell adhesion by covalent binding of poly-D-lysine. J Neurosci Methods 202:38-44.
59. Lupoli F, Vannocci T, Longo G, Niccolai N, Pastore A. 2018. The role of oxidative stress in Friedreich's ataxia. FEBS Letters 592:718-727.
60. Dinarelli S, Girasole M, Longo G. 2018. FC_analysis: a tool for investigating atomic force microscopy maps of force curves. BMC Bioinformatics 19:258.
61. Aghayee S, Benadiba C, Notz J, Kasas S, Dietler G, Longo G. 2013. Combination of fluorescence microscopy and nanomotion detection to characterize bacteria. Journal of Molecular Recognition 26:590-595.
62. Kasas S, Radotic K, Longo G, Saha B, Alonso-Sarduy L, Dietler G, Roduit C. 2013. A universal fluid cell for the imaging of biological specimens in the atomic force microscope. Microsc Res Tech 76:357-363.

566 **Figure captions**

567

568 **Figure 1. Control experiments involving BCG and M. abscessus.** Typical data patterns
569 (performed minimally in triplicate) of BCG (panel a) and *M. abscessus* (panel b) in MGIT medium.
570 The fluctuations are present for more than 200 minutes.

571

572 **Figure 2. Nanomotion experiments on BCG exposed to an over-MIC dose of INH.** Top panel.
573 Typical 10 minutes segments of the sensor's fluctuations: before the exposure to INH (left);
574 immediately after the exposure to INH at 2 μg/ml (center); 20 minutes after the exposure to INH,
575 when the movement reduction has stabilized. Lower panel. Histogram of the corresponding
576 variance of the fluctuations. The graph is representative of minimally 5 independent experiments
577 which produced similar results. The error bars in the variance histogram represent +- SD of the
578 corresponding fluctuations over the shown 10-minute time-period.

579

580 **Figure 3. Nanomotion experiments on BCG exposed to an over-MIC dose of RIF.** Top panel.
581 Typical 20 minutes segments of the sensor's fluctuations: before the exposure to RIF (left);
582 immediately after the exposure to RIF at 0.7 μg/ml (center); 80 minutes after the exposure to RIF,
583 when the movement reduction has stabilized. Lower panel. Histogram of the corresponding
584 variance of the fluctuations. The graph is representative of 3 independent experiments which
585 produced similar results. The error bars in the variance histogram represent +- SD of the
586 corresponding fluctuations over the shown 20-minute time-period.

587

588 **Figure 4. Dose dependence experiments.** Panel a. Normalized variance calculated from the
589 deflections collected by exposing the BCG to different INH concentrations. Panel b. Normalized
590 variance calculated from the deflections collected by exposing the BCG to different RIF
591 concentrations. Panel c. Normalized variance calculated from the deflections collected by exposing
592 the *M. abscessus* to different AK concentrations. The concentration values can be well fitted with a
593 sigmoid function, which is comparable with the antibiogram plots, obtained using conventional
594 microbiological techniques. The MIC and the MBC of towards the bacterial species can be obtained
595 by calculating the tangent of the sigmoid fit at half height (black dashed line). Each data-point
596 represents the average of a minimum of 3 independent experiments performed using the same drug
597 concentration. The error bars represent the variability of the different experiments performed at the
598 same concentration. In each graph, the experiments involving sub-MIC drug concentrations are
599 represented as a single data-point, which summarizes all these experiments.

600

601 **Figure 5. Nanomotion experiments on BCG exposed to a sub-MIC dose of INH and RIF.** Panel
602 a: Top panel. Typical 20 minutes segments of the sensor's fluctuations: before the exposure to INH
603 (left); immediately after the exposure to INH at 0.025 μg/ml (center); 140 minutes after the
604 exposure to INH, when the movement has stabilized. Lower panel. Histogram of the corresponding
605 variance of the fluctuations. Panel b: Top panel. Typical 20 minutes segments of the sensor's
606 fluctuations: before the exposure to RIF (left); immediately after the exposure to RIF at 0.07 μg/ml
607 (centre); 95 minutes after the exposure to RIF, when the movement has stabilized. Lower panel.
608 Histogram of the corresponding variance of the fluctuations. Each graph is representative of
609 minimally 3 independent experiments which produced similar results. The error bars in the variance
610 histogram represent +- SD of the corresponding fluctuations over the shown 20-minute time-period.

Figure 6. Time-resolved analysis of the BCG response to INH. Typical data pattern of the response of BCG to a bactericidal dose of INH (1 μg/ml). Over a 140-minute period, the fluctuations increase and decrease in amplitude, highlighting the bacterial response to the antibiotic pressure. The graph is representative of 3 independent experiments in which this feature was evidenced.

Figure 7. Nanomotion experiments on *M. abscessus* exposed to AK. Panel a: Top panel. Typical 20 minutes segments of the sensor's fluctuations exposed to an over-MIC dose of AK: before the exposure to AK (left); immediately after the exposure to AK at 10 μg/ml (center); 50 minutes after the exposure to AK, when the movement has stabilized. Lower panel. Histogram of the corresponding variance of the fluctuations. Panel b: Top panel. Typical 20 minutes segments of the sensor's fluctuations exposed to a sub-MIC dose of AK: before the exposure to AK (left); 30 minutes after the exposure to AK at 1 μg/ml (center); 90 minutes after the exposure to AK, when the movement has recovered and stabilized. Lower panel. Histogram of the corresponding variance of the fluctuations. Each graph is representative of at least 3 independent experiments which produced similar results. The error bars in the variance histogram represent +- SD of the corresponding fluctuations over the shown 20-minute time-period.

629 **Figure 8. Optical images of bacteria-bearing sensors.** Typical optical images of rectangular (left
630 panels) and triangular sensors (right panels) bearing BCG (panel a) and *M. abscessus* (panel b). The
631 optical images show the BCG clumping. The scale bars indicate 50 μm.

632