ACS OMEGA

http://pubs.acs.org/journal/acsodf

Article

Fast Antibiotic Susceptibility Testing via Raman Microspectrometry on Single Bacteria: An MRSA Case Study

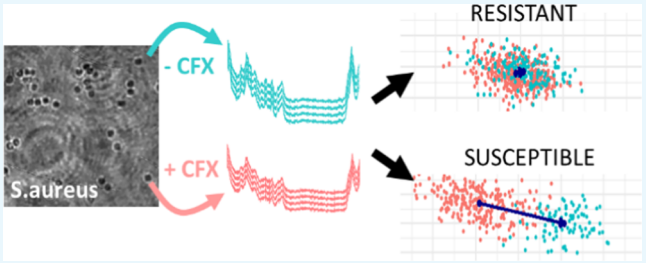
Armelle Novelli Rousseau,* Nicolas Faure,* Fabian Rol, Zohreh Sedaghat, Joël Le Galudec, Frédéric Mallard, and Quentin Josso

Cite This: *ACS Omega* 2021, 6, 16273−16279

Read Online

ACCESS | Metrics & More | Article Recommendations | Supporting Information

ABSTRACT: Despite recent advances in molecular diagnostics, ultrafast determination of the antibiotic susceptibility phenotype of pathogenic microorganisms is still a major challenge of in vitro diagnostics (IVD) of infectious diseases. Raman microspectroscopy has been proposed as a means to achieve this goal. Previous studies have shown that susceptibility phenotyping could be done through Raman analysis of microbial cells, either in large clusters or down to the single-cell level in the case of Gram-negative rods. Gram-positive cocci such as *Staphylococcus aureus* pose several challenges due to their size and their different metabolic and chemical characteristics. Using a tailored automated single-cell Raman spectrometer and a previously proposed sample preparation protocol, we acquired and analyzed 9429 *S. aureus* single cells belonging to three cefoxitin-resistant strains and two susceptible strains during their incubation in the presence of various concentrations of cefoxitin. We observed an effect on *S. aureus* spectra that is weaker than what was detected on previous bacteria/drug combinations, with a higher cell-to-cell response variability and an important impact of incubation conditions on the phenotypic resistance of a given strain. Overall, the proposed protocol was able to correlate strains' phenotype with a specific modification of the spectra using majority votes. We, hence, confirm that our previous results on single-cell Raman antibiotic susceptibility testing can be extended to the *S. aureus* case and further clarify potential limitations and development requirements of this approach in the move toward industrial applications.



INTRODUCTION

By allowing targeted instead of empirical antibiotic therapy, ultrafast prediction of antimicrobial susceptibility within minutes to hours after sampling an infected patient is expected to revolutionize therapeutic efficacy and to help optimizing antibiotic stewardship,[1,2] with strong positive impacts on the efficiency and costs of healthcare systems.[3−5]

Molecular detection of resistance-associated genes is clearly a part of the solution.[6−8] Indeed, molecular diagnostics meets the huge challenges of being directly applicable to raw samples, ultrafast (<1 h), while addressing sets of genes with proven clinical relevance in a number of cases. Such solutions have reached their industrial maturity in highly automated and point-of-care compatible formats (e.g., BioFire's FilmArray or Cepheid's GeneXpert). In the near future, sequencing-based solutions are expected to further improve the accuracy and exhaustiveness of molecular AST predictions.[9,10]

However, the accuracy of phenotype prediction based on the detection of genetic information alone has some limitations, depending on both antibiotics and bacterial species that are considered. Cases of resistance gene mutations or partial coverage of genetic variability may generate false results that may lead to wrong therapeutic decisions. Horizontal resistance gene transfer and samples with mixed microbial populations may lead to false-positive resistance detection. Importantly, this situation is known to evolve over time,[11−13] which is a real pain point for the industrial development of a routine AST solution due to the highly regulated context of in vitro diagnostics (IVD). Thus, a direct sample testing solution that would unambiguously correlate with the microbial phenotype and provide clinically actionable results within hours is highly desirable.[14]

To tackle this challenge, a number of nonmolecular methods to detect microbial phenotypes in the presence or the absence of antibiotics have been proposed.[15−21] These range from imaging of early division cycles to the detection of metabolic alterations using a wealth of analytical methods (optical spectroscopy, electrochemistry, mechanical vibration, hydrodynamics, redox markers, and cytometry, to mention a few; for more detailed information, see van Belkum et al.[22]).

Received: January 11, 2021
Accepted: April 5, 2021
Published: June 15, 2021

Among the new and rapid methods, Raman microspectrometry is a particularly attractive option.[23] The microbial Raman information is known for long to provide identification information down to the strain level.[24−28] More recently, it was used to probe the differences between resistant and susceptible phenotypes of microbial cell clusters in the presence of antimicrobials.[29−34] This was also demonstrated for smaller groups of single microbial cells, even down to single cells.[29,35−38] Cell-to-cell variability in a microbial strain population was not measured in these studies, which may lead to incorrect interpretation. Our group and others have applied this approach to large populations of single cells and provided an unprecedented vision of cell-to-cell response variability in the presence of antibiotics. Using *Escherichia coli* as a model organism, we showed that individual cells shift between distinct Raman signatures in an antimicrobial dose-dependent manner, with a massive population transition at antibiotic concentrations around the minimal inhibitory concentration (MIC).[39]

Although the sum of the results mentioned above clearly opens the way to ultrafast determination of resistance phenotype in a quantitative manner, their scope and modalities are far from an industrial IVD application for routine testing. Even before considering constraints linked to commercial deployment, a future Raman-based diagnostic system should at least have the ability to address an extended panel of microorganisms and drugs, which requires both a versatile sample preparation procedure and the biological demonstration of performances on this extended panel, in a simple-to-use (hence automated) format.

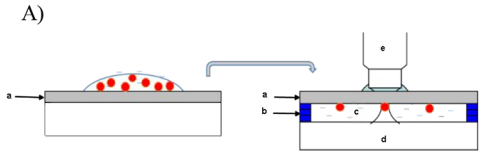
We already addressed some of these concerns in previous studies.[40] First, we described optimized mild electrostatic capture conditions to immobilize a wide variety of microorganisms onto glass coverslips, where they can be incubated in physiological conditions and keep their ability to grow. Second, we demonstrated the fully automated, high-throughput localization and Raman probing of immobilized single cells, giving access to large sets of highly informative single-cell Raman spectra with unprecedented speed and accuracy.

As for the applicability of Raman to testing microbial antibiotic susceptibility on a wider panel of microorganisms, we noted that published demonstrations generally use a few strains in the Gram-negative rod group. Here, we wanted to extend our previous demonstration to a significantly different kind of cells. We chose methicillin-resistant *Staphylococcus aureus* (MRSA) because it is a Gram-positive microorganism, with metabolic and chemical characteristics that are known to be different from Gram-negative microorganisms, and a coccus with a relatively small size (often <1 μm), a situation that is challenging for microspectrometry.[41] We document here the intensity and variability of single-cell spectra for a reduced set of susceptible and resistant *S. aureus* strains in the presence of cefoxitin and discuss our actual ability to use the Raman information to predict their resistant/susceptible phenotypes.

METHODS

Experimental Design and Data Processing Strains and Culture Conditions. Two susceptible *S. aureus* strains (SA44 and SA86 strains) and three MRSA strains (two *mecA* positive, SA127 and SA128, and one *mecC* positive, SA126) were selected (see the Supporting Information, SI1 section, and Table S1). As in most methicillin-resistant tests,[42,43] we used cefoxitin in all our susceptibility tests on *S. aureus*. For susceptible strains SA44 and SA86, cefoxitin concentrations were set to 4 times their MIC values (8 and 16 μg/mL, respectively) to create conditions with a clear effect on bacterial viability. For resistant strains (SA126, SA127, and SA128), 8 μg/mL cefoxitin was used, below their reported reference MIC (16, 16, and >256 μg/mL, respectively), to prevent an effect on viability (see the Supporting Information, Table S1). For comparison purposes, we also tested two *E. coli* strains (one susceptible EC10, one resistant EC21, see the Supporting Information, Table S2) that were used in our previous studies[39] in the presence of gentamicin, using our new experimental setting and procedure.

Raman Measurement. Detailed information about the successive steps of our protocol is provided in the Supporting Information, SI2−SI6 sections. Briefly, for each test, bacteria from a single colony were cultured in a liquid MHB medium for around 2 h at 37 °C. Bacteria were then recovered in a saline phosphate buffer at 2×10^7 CFU/mL. A drop of this suspension (300 μL) was deposited onto an aminosilane-functionalized glass coverslip (Schott Nexterion (Müllheim, Germany) ref 1666121) to immobilize bacteria on the surface. After washing, the coverslip was used to close a fluidic chamber made of a glass slide and a Gene Frame spacer. This chamber was filled with a 10% MHB growth medium with or without antibiotics depending on the testing condition. This assembly (see Figure 1) was set up on the Raman microspectrometer



B)

Strains	SA 44	SA 86	SA 127	SA 128	SA 126
Phenotype	S	S	R	R	R
Number of spectra / Replicates	2002 /4	1314 /3	2093 /2	2015 /3	2005 /3

Figure 1. (A) Scheme of the experimental setup: *S. aureus* bacteria were immobilized on an aminosilane-functionalized glass coverslip (a) by incubation in phosphate buffer 20 mM, pH 7.2, and NaCl 50 mM solution at a final concentration of 2×10^7 CFU/mL (300 μL). After washing, the coverslip was used to close a fluidic chamber made of a Gene Frame (Thermo Scientific) (b) and a glass slide (d). The chamber was filled with MHB 10% with or without an antibiotic. (c) Assembly was set up on the Raman microspectrometer stage (d). (B) Table view of the *S. aureus* strains used in this study and experiments performed.

stage. Raman spectrum acquisitions were performed automatically on single bacteria over a period of 4 h. In one given experiment, a sample without antibiotics was systematically tested in parallel with samples containing antibiotics. The effect of antibiotics on cell viability was tested in parallel in a liquid broth format, starting from the same inoculum and reagents used for Raman experiments. Each experiment was replicated independently on different dates.

Raman acquisitions were performed as described in the Supporting Information, SI6 section.

Data Treatment and Analysis. Raw single-cell spectra were preprocessed to remove the background signal (mostly fluorescence) and glass signal (see details in the Supporting

https://doi.org/10.1021/acsomega.1c00170
ACS Omega 2021, 6, 16273−16279

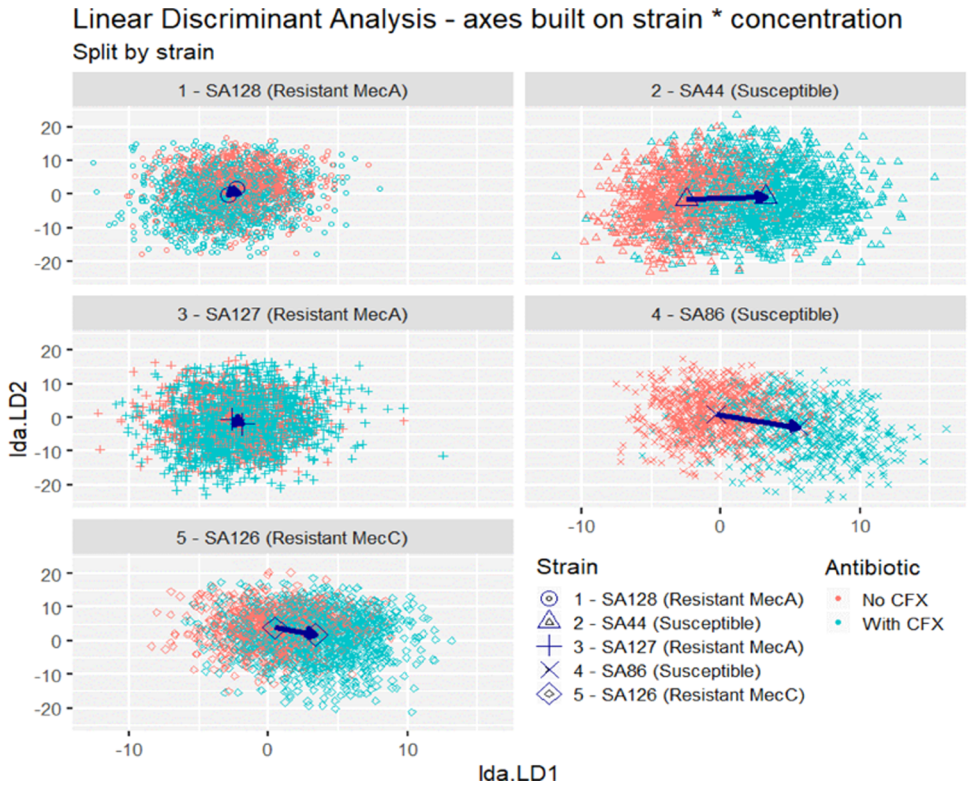


Figure 2. Response of resistant strains (SA128, SA127, and SA126) versus susceptible strains (SA44 and SA86) to cefoxitin: penalized linear discriminant analysis (LDA) axes built on classes corresponding to strain and antibiotic presence/absence combinations, therefore 10 classes. The spectra corresponding to several experiments are lumped in the same class and are not separated in this figure. Each graph corresponds to a strain. The color corresponds to the presence/absence of the antibiotic. The blue arrows are traced between the average spectra with no antibiotic and with an antibiotic (tip of the arrow). Therefore, the length of the blue arrow is a measure of the spectral response of the strain to the antibiotic.

Information, SI7 section) to reduce the contribution of nonbiological and nonspecific signals.[44] The overall database contains 9429 spectra of single bacteria after data treatment.

Linear discriminant analysis (LDA) was preferred to principal component analysis (PCA) for visualization purposes to determine the effect of specific test conditions or combinations thereof (species, resistance) on the spectra. Each of these conditions is referred to as a class.

In brief, LDA is a supervised method that provides a representation of the spectra in a low dimension (two-dimensional (2D) in our case). This representation is constructed to maximize the ratio between the interclass and intraclass variance, therefore allowing a good separation between the classes. In our particular case, LDA has been regularized to avoid artifacts linked to the fact that the data have a dimensionality (number of channels per spectrum) comparable to the number of instances (number of spectra). See the Supporting Information for more details on LDA and its implementation.

LDA can be used as a predictor to discriminate between bacterial phenotypes. Explicit and easily interpretable rules could be carved out from it, for instance, based on the distance between centroids in LDA visualization. Nonetheless, to keep consistency with our 2018 study,[39] we preferred to evaluate the discriminative capability of the system through a ridge-regularized SVM with a linear kernel. The SVM classifier was trained on a set of strains and one strain was left aside for testing.

RESULTS AND DISCUSSION

An important first step in the perspective of routine use in clinical microbiology diagnostics is to show that Raman-based resistance phenotype determination can be applied to very different microorganisms.

In the present study, we consider *S. aureus* cells, which are known to have a small size - a challenging situation for single-cell Raman spectra acquisition - and a significantly different overall chemical composition compared to the *E. coli* strains that we have studied so far.

To document Raman spectra for a statistically relevant number of single cells, in conditions where their metabolism is best preserved, we used the automated system described by Douet et al.[40] We have shown that this system allows for the acquisition of highly informative spectra on very large numbers of cells (>65 000 Raman spectra of single cells) and in a growth-compatible liquid environment. We also wanted to determine if this system would catch the potentially faint Raman differences caused by metabolic adaptations in the presence of antibiotics in the *S. aureus*/cefoxitin case.

As a test case, we challenged the effect of cefoxitin on two susceptible and three resistant strains with two different resistance genes (see characteristics in the Supporting Information, Table S1). The use of our new instrument allowed us to acquire spectra at a high rate (400 spectra/h) and have refined documentation of cell-to-cell variability. The resulting spectra were processed as described in the Supporting Information, SI7 section. The differences between mean spectra of resistant or susceptible strains in the presence or

https://doi.org/10.1021/acsomega.1c00170
ACS Omega 2021, 6, 16273−16279

Table 1. Susceptible (S)/Resistant (R) Classification Performances for Each Strain Tested

Strain	Exp #	Nb of Spectra	Nb of Correctly predicted spectra	% correctly predicted spectra	Whole experiment majority vote result	Ground truth	Nb of image fields analyzed	Nb of correct by-field majority votes	% correctly classified fields
SA44	1	306	195	63.7 %	S	S	14	12	85,70%
	2	398	231	58.0 %	S	S	12	10	83,30%
	3	263	121	46.0 %	R	S	8	3	37,50%
	4	231	131	56.7 %	S	S	9	5	55,60%
SA86	5	311	185	59.5 %	S	S	11	8	72,70%
	6	139	74	53.2 %	S	S	6	2	33,30%
	7	150	97	64.7 %	S	S	5	4	80%
SA127	8	786	680	86.5 %	R	R	17	17	100%
	9	475	439	92.4 %	R	R	16	16	100%
SA128	10	166	149	89.8 %	R	R	7	7	100%
	11	562	407	72.4 %	R	R	21	21	100%
	12	393	355	90.3 %	R	R	11	11	100%
SA126	13	568	385	67.8 %	R	R	17	17	100%
	14	586	245	41.8 %	S	R	22	8	36,40%
	15	151	80	53,00%	R	R	9	4	44,40%

the absence of cefoxitin are illustrated in Figure S3 in the Supporting Information.

We built up an LDA representation on a 10 class problem (five strains/two antibiotic conditions). The first two axes are plotted in Figure 2. We showed in a separate repeatability experiment (susceptible SA44 and resistant SA128 strain/see the Supporting information, SI10 section, and Figure S4) that interexperiment reproducibility was good enough for us to lump several repeated experiments on each of the plots of Figure 2 to increase the representativeness (see the Supporting Information, Table S1 for the number of replicates).

As expected, we observe that single-cell spectra can be separated according to their class, despite some overlaps, in this reduced-dimensionality representation, between conditions. In particular, the susceptible strains (SA44 and SA86) exhibit a clear response to cefoxitin, basically oriented along the first LDA axis, while the spectral modification in *mecA*-positive resistant populations (SA128 and SA127) show much less variation.

We checked if this faint response could be due to a reduced ability of our new measurement setting to catch the Raman information related to the cells' response to antibiotics. To do so, we repeated the same experiment on the *E. coli*/gentamicin model used in our 2018 study.[39] The susceptible EC10 and the resistant EC21, which were known to show very clear antibiotic-related Raman modifications, were used (see the Supporting Information, Table S2). Mean spectra and classification results are shown in the Supporting Information, Figure S5 and clearly evidence a strong response of the EC10 gentamicin-susceptible strain, while the resistant strain EC21 remains unaffected. This confirms that our new setting is able to catch the spectral modifications associated with antibiotic susceptibility at least as well as the 2018 one.

Not only spectral modifications in *S. aureus* in the presence of cefoxitin were faint, but the *mecC*-positive resistant strain SA126 exhibited an intermediate behavior, suggesting that the antibiotic effect itself could be partial. We checked this hypothesis by challenging the viability of microorganisms, in the same incubation conditions as for Raman experiments, by plating and colony-counting (see the Supporting Information, SI3 section). As expected, the viability of susceptible strains decreased in the presence of antibiotics. The decrease was moderate due to both the short incubation time in the presence of the antibiotics and the nutrient-poor medium used for the incubation, in which growth and bacterial metabolism are inherently slowed down. On the contrary, the resistant SA127 and SA128 strains grew in the presence of antibiotics, similar to the control condition without antibiotics. Strikingly, the SA126 population did not grow in the presence of cefoxitin as much as in the control condition. So, it appears that the cefoxitin concentration used in our experiment was unexpectedly close to the effective MIC of SA126 in our experiment and that the Raman effect observed can be correlated to the actual growth behavior of *S. aureus* cells.

In the end, the modification of single-cell Raman spectra in the presence of antibiotics correlates with the longer-term growth, or growth inhibition, of a cell population among all *S. aureus* strains tested in this study. This is very consistent with the observations and interpretation of Lopatkin et al.,[45] suggesting that the metabolic state of microorganisms is a good predictor of the antibiotic effect. Raman spectra, as a representation of this metabolic state, would also be a good predictor of the fate of a bacterial population in the presence of antibiotics. Interestingly, we observed that spectral changes, when occurring, were detectable within the first 30 min of incubation in the presence of antibiotics and did not change significantly more over time (see results in the Supporting Information, SI11 section). The same observation was noted in our previous work in the *E. coli*/gentamicin case.[39] This would be in favor of a very rapid orientation of cells toward division or death, which is also consistent with observations by Collins's group.[46,47]

We tested if, despite the faint Raman modifications at the single-cell level, our data would allow us to predict the antibiotic resistance/susceptibility phenotype at the population/strain level. To do so, we applied a machine-learning approach with a leave-one-strain-out strategy to our data (see

https://doi.org/10.1021/acsomega.1c00170
ACS Omega 2021, 6, 16273−16279

the Supporting Information, SI9 section for details). The classification results are shown in Table 1.

As expected, at the single-cell level, the variability is too high to allow for accurate predictions in all cases. The prediction is at least better than random in most cases, with very good (86−92%) accuracy for some cases but also very difficult cases (41.8% for one of the SA126 experiment for which the antibiotic effect on viability was partial).

We carried out a majority vote on the predictions associated with all of the bacteria of a microscope field, which corresponds to typically 30 bacteria. This allows us to evaluate whether lumping a moderate number of predictions (which corresponds to a few minutes of acquisition) is sufficient to yield an accurate phenotypic prediction. We found that the percentage of correct prediction at the field level reached 100% in the best cases (on resistant strains SA127 and SA128 noticeably) but that field-level majority vote did not yield substantial improvement in other cases. The same tendency was found with a majority vote at the full experiment level, with correct prediction in 13/15 cases

These limits in prediction accuracy may be due to the fact that the number of strains included in the study was limited. For instance, only two susceptible strains were included, which means that in the cross-validation design, only one example of the resistant strain was provided to the classifier during training. This may explain the relatively poor behavior of the classifier on susceptible strains. We believe that increasing the number of strains in the study will allow the classifier to capture more inter-strain variability and eventually improve its performance.

CONCLUSIONS

Overall, our results confirm that single-cell Raman spectra can be acquired rapidly over large cell populations and provide a refined vision of their spectral variability. This is expected to help tackling challenging bacterial susceptibility/resistance diagnostics cases by rapidly and easily probing the spectral response over thousands of cells rather than an averaged response. As acquisitions can be performed in a liquid environment, bacteria keep their ability to grow, which is an important asset to facilitate future sample preparation procedures for direct sample testing. In some cases, e.g., the Gram-negative *E. coli* in the presence of gentamicin, we have shown that resistant and susceptible cell populations can be unambiguously separated based on their spectral changes in the presence of antibiotics. We show, here, that this conclusion can be extended to the Gram-positive *S. aureus*/cefoxitin case, however, with some care. Indeed, Raman characteristics do allow for the separation of *S. aureus* cell populations according to their resistance phenotypes. This was not documented before and was expected to be more challenging due to the smaller staphylococcal cell size. In a more quantitative approach, however, we show that cell-to-cell variability is higher in the *S. aureus*/cefoxitin case than the *E. coli*/gentamicin case, which affects the accuracy of phenotype prediction. This might prevent prediction to be done from small cell numbers, which would be a clear restriction for direct testing of samples with low bacterial cell counts. We suggest that a part of this variability might be reduced by optimizing the testing conditions (growth conditions, media, and antibiotic concentration). Improving test automation and throughput is thus necessary but not sufficient. Optimized testing procedures are expected to be required for virtually each new bug−drug combination on the road toward extended-panel Raman-based AST.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.1c00170.

Materials and methods, control experiments, data analysis, and results (PDF)

AUTHOR INFORMATION

Corresponding Authors

Armelle Novelli Rousseau − *bioMérieux, R&D Microbiology, 38024 Grenoble, France;* orcid.org/0000-0003-1407-3895; Email: armelle.novelli-rousseau@biomerieux.com

Nicolas Faure − *bioMérieux, R&D Microbiology, 38024 Grenoble, France*; Email: nicolas.faure@biomerieux.com

Authors

Fabian Rol − *Bioaster, 69007 Lyon, France*

Zohreh Sedaghat − *Bioaster, 69007 Lyon, France*

Joël Le Galudec − *bioMérieux, R&D Microbiology, 38024 Grenoble, France*

Frédéric Mallard − *bioMérieux, R&D Microbiology, 38024 Grenoble, France*

Quentin Josso − *bioMérieux, R&D Microbiology, 69280 Marcy-l'Etoile, France;* orcid.org/0000-0002-8980-9505

Complete contact information is available at: https://pubs.acs.org/10.1021/acsomega.1c00170

Author Contributions

A.N.R. and N.F. contributed equally. A.N.R. designed and performed the experiments, analyzed results, and contributed to the writing of the manuscript. N.F. developed analytical tools, treated data, performed statistical analysis, analyzed results, and contributed to the writing of the manuscript. A.N.R. and N.F. contributed equally to this work. F.R. and Z.S. contributed to instrument and software optimization. J.L.G. contributed to the development of analytical tools and data treatment. F.M. contributed to project definition, result discussion, and writing of the manuscript. Q.J. contributed to project definition, results analysis, results discussion, and contributed to the writing of the manuscript. All authors contributed extensively to the work presented in this manuscript.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

This work has received, through BIOASTER investment, funding from the French Government through the Investissements d'Avenir program (Grant No. ANR-10-AIRT-03). The authors wish to thank their collaborators, in particular, Pr. Alex van Belkum and Dr. Sylvain Orenga from bioMérieux for helpful reading and comments on the manuscript.

REFERENCES

(1) van Belkum, A.; Bachmann, T. T.; Ludke, G.; Lisby, J. G.; Kahlmeter, G.; Mohess, A.; Becker, K.; Hays, J. P.; Woodford, N.; Mitsakakis, K.; Moran-Gilad, J.; Vila, J.; Peter, H.; Rex, J. H.; Dunne, W. M., Jr.; the JPIAMR AMR-RDT Working Group on Antimicrobial Resistance and Rapid Diagnostic Testing. Developmental roadmap for

antimicrobial susceptibility testing systems. *Nat. Rev. Microbiol.* **2019**, *17*, 51−62.
(2) Reller, L. B.; Weinstein, M.; Jorgensen, J. H.; Ferraro, M. J. Antimicrobial susceptibility testing: a review of general principles and contemporary practices. *Clin. Infect. Dis.* **2009**, *49*, 1749−1755.
(3) Moloney, E.; Lee, K. W.; Craig, D.; Allen, A. J.; Graziadio, S.; Power, M.; Steeds, C. A PCR-based diagnostic testing strategy to identify carbapenemase-producing Enterobacteriaceae carriers upon admission to UK hospitals: early economic modelling to assess costs and consequences. *Diagn. Progn. Res.* **2019**, *3*, No. 8.
(4) Wabe, N.; Li, L.; Lindeman, R.; Yimsung, R.; Dahm, M. R.; Clezy, K.; McLennan, S.; Westbrook, J.; Georgiou, A. The impact of rapid molecular diagnostic testing for respiratory viruses on outcomes for emergency department patients. *Med. J. Aust.* **2019**, *210*, 316−320.
(5) Wabe, N.; Li, L.; Lindeman, R.; Yimsung, R.; Dahm, M. R.; McLennan, S.; Clezy, K.; Westbrook, J. I.; Georgiou, A. Impact of Rapid Molecular Diagnostic Testing of Respiratory Viruses on Outcomes of Adults Hospitalized with Respiratory Illness: a Multicenter Quasi-experimental Study. *J. Clin. Microbiol.* **2019**, *57* (4), No. e01727-18.
(6) Rood, I. G. H.; Li, Q. Review: Molecular detection of extended spectrum-beta-lactamase- and carbapenemase-producing Enterobacteriaceae in a clinical setting. *Diagn. Microbiol. Infect. Dis* **2017**, *89*, 245−250.
(7) van Belkum, A.; Rochas, O. Laboratory-Based and Point-of-Care Testing for MSSA/MRSA Detection in the Age of Whole Genome Sequencing. *Front. Microbiol.* **2018**, *9*, No. 1437.
(8) MacVane, S. H.; Nolte, F. S. Benefits of Adding a Rapid PCR-Based Blood Culture Identification Panel to an Established Antimicrobial Stewardship Program. *J. Clin. Microbiol.* **2016**, *54*, 2455−63.
(9) Su, M.; Satola, S. W.; Read, T. D. Genome-Based Prediction of Bacterial Antibiotic Resistance. *J. Clin. Microbiol.* **2019**, *57* (3), No. e01405-18.
(10) Boolchandani, M.; D'Souza, A. W.; Dantas, G. Sequencing-based methods and resources to study antimicrobial resistance. *Nat. Rev. Genet.* **2019**, *20*, 356−370.
(11) Knopp, M.; Andersson, D. I. Predictable Phenotypes of Antibiotic Resistance Mutations. *mBio* **2018**, *9* (3), No. e00770-18.
(12) Wistrand-Yuen, E.; Knopp, M.; Hjort, K.; Koskiniemi, S.; Berg, O. G.; Andersson, D. I. Evolution of high-level resistance during low-level antibiotic exposure. *Nat. Commun.* **2018**, *9*, No. 1599.
(13) Balaban, N. Q.; Helaine, S.; Lewis, K.; Ackermann, M.; Aldridge, B.; Andersson, D. I.; Brynildsen, M. P.; Bumann, D.; Camilli, A.; Collins, J. J.; Dehio, C.; Fortune, S.; Ghigo, J. M.; Hardt, W. D.; Harms, A.; Heinemann, M.; Hung, D. T.; Jenal, U.; Levin, B. R.; Michiels, J.; Storz, G.; Tan, M. W.; Tenson, T.; Van Melderen, L.; Zinkernagel, A. Definitions and guidelines for research on antibiotic persistence. *Nat. Rev. Microbiol.* **2019**, *17*, 441−448.
(14) Smith, K. P.; Kirby, J. E. Rapid Susceptibility Testing Methods. *Clin. Lab. Med.* **2019**, 333−344.
(15) Leonard, H.; Colodner, R.; Halachmi, S.; Segal, E. Recent Advances in the Race to Design a Rapid Diagnostic Test for Antimicrobial Resistance. *ACS Sens.* **2018**, *3*, 2202−2217.
(16) Tannert, A.; Grohs, R.; Popp, J.; Neugebauer, U. Phenotypic antibiotic susceptibility testing of pathogenic bacteria using photonic readout methods: recent achievements and impact. *Appl. Microbiol. Biotechnol.* **2019**, *103*, 549−566.
(17) van Belkum, A.; Dunne, W. M. Next-Generation Antimicrobial Susceptibility Testing. *J. Clin. Microbiol.* **2013**, *51*, 2018−2024.
(18) Khan, Z. A.; Siddiqui, M. F.; Park, S. Current and Emerging Methods of Antibiotic Susceptibility Testing. *Diagnostics* **2019**, *9*, No. 49.
(19) Pulido, M. R.; García-Quintanilla, M.; Martín-Peña, R.; Cisneros, J. M.; McConnell, M. J. Progress on the development of rapid methods for antimicrobial susceptibility testing. *J. Antimicrob. Chemother.* **2013**, *68*, 2710−2717.
(20) Behera, B.; Vishnu, G. K. A.; Chatterjee, S.; Sitaramgupta V, V. S. N.; Sreekumar, N.; Nagabhushan, A.; Rajendran, N.; Prathik, B. H.; Pandya, H. J. Emerging technologies for antibiotic susceptibility testing. *Biosens. Bioelectron.* **2019**, *142*, No. 111552.
(21) Ellington, M. J.; Ekelund, O.; Aarestrup, F. M.; Canton, R.; Doumith, M.; Giske, C.; Grundman, H.; Hasman, H.; Holden, M. T. G.; Hopkins, K. L.; Iredell, J.; Kahlmeter, G.; Köser, C. U.; MacGowan, A.; Mevius, D.; Mulvey, M.; Naas, T.; Peto, T.; Rolain, J. M.; Samuelsen, Ø.; Woodford, N. The role of whole genome sequencing in antimicrobial susceptibility testing of bacteria: report from the EUCAST Subcommittee. *Clin. Microbiol. Infect.* **2017**, *23*, 2−22.
(22) van Belkum, A.; Burnham, C. D.; Rossen, J. W. A.; Mallard, F.; Rochas, O.; Dunne, W. M., Jr. Innovative and rapid antimicrobial susceptibility testing systems. *Nat. Rev. Microbiol.* **2020**, 299−311.
(23) Lorenz, B.; Wichmann, C.; Stöckel, S.; Rösch, P.; Popp, J. Cultivation-Free Raman Spectroscopic Investigations of Bacteria. *Trends Microbiol.* **2017**, *25*, 413−424.
(24) Maquelin, K.; Kirschner, C.; Choo-Smith, L. P.; van den Braak, N.; Endtz, H. P.; Naumann, D.; Puppels, G. J. Identification of medically relevant microorganisms by vibrational spectroscopy. *J. Microbiol. Methods* **2002**, *51*, 255−71.
(25) Harz, M.; Rosch, P.; Popp, J. Vibrational spectroscopy–a powerful tool for the rapid identification of microbial cells at the single-cell level. *Cytometry, Part A* **2009**, *75*, 104−13.
(26) Neugebauer, U.; Rosch, P.; Popp, J. Raman spectroscopy towards clinical application: drug monitoring and pathogen identification. *Int. J. Antimicrob. Agents* **2015**, *46*, S35−S39.
(27) Pahlow, S.; Meisel, S.; Cialla-May, D.; Weber, K.; Rosch, P.; Popp, J. Isolation and identification of bacteria by means of Raman spectroscopy. *Adv. Drug Delivery Rev.* **2015**, *89*, 105−20.
(28) Jarvis, R. M.; Goodacre, R. Characterisation and identification of bacteria using SERS. *Chem. Soc. Rev.* **2008**, *37*, 931−6.
(29) Teng, L.; Wang, X.; Wang, X.; Gou, H.; Ren, L.; Wang, T.; Wang, Y.; Ji, Y.; Huang, W. E.; Xu, J. Label-free, rapid and quantitative phenotyping of stress response in *E. coli* via ramanome. *Sci. Rep.* **2016**, *6*, No. 34359.
(30) Neugebauer, U.; Rosch, P.; Popp, J. Raman spectroscopy towards clinical application: drug monitoring and pathogen identification. *Int. J. Antimicrob. Agents* **2015**, *46*, S35−9.
(31) Athamneh, A. I. M.; Alajlouni, R. A.; Wallace, R. S.; Seleem, M. N.; Senger, R. S. Phenotypic Profiling of Antibiotic Response Signatures in *Escherichia coli* Using Raman Spectroscopy. *Antimicrob. Agents Chemother.* **2014**, *58*, 1302−1314.
(32) Dekter, H. E.; Orelio, C. C.; Morsink, M. C.; Tektas, S.; Vis, B.; te Witt, R.; van Leeuwen, W. B. Antimicrobial susceptibility testing of Gram-positive and -negative bacterial isolates directly from spiked blood culture media with Raman spectroscopy. *Eur. J. Clin. Microbiol. Infect. Dis.* **2017**, *36*, 81−89.
(33) Kirchhoff, J.; Glaser, U.; Bohnert, J. A.; Pletz, M. W.; Popp, J.; Neugebauer, U. Simple Ciprofloxacin Resistance Test and Determination of Minimal Inhibitory Concentration within 2 h Using Raman Spectroscopy. *Anal. Chem.* **2018**, *90*, 1811−1818.
(34) Schröder, U.-C.; Beleites, C.; Assmann, C.; Glaser, U.; Hubner, U.; Pfister, W.; Fritzsche, W.; Popp, J.; Neugebauer, U. Detection of vancomycin resistances in enterococci within 3 (1/2) hours. *Sci. Rep.* **2015**, *5*, No. 8217.
(35) Moritz, T. J.; Taylor, D. S.; Polage, C. R.; Krol, D. M.; Lane, S. M.; Chan, J. W. Effect of Cefazolin Treatment on the Nonresonant Raman Signatures of the Metabolic State of Individual *Escherichia coli* Cells. *Anal. Chem.* **2010**, *82*, 2703−2710.
(36) Münchberg, U.; Rösch, P.; Bauer, M.; Popp, J. Raman spectroscopic identification of single bacterial cells under antibiotic influence. *Anal. Bioanal. Chem.* **2014**, *406*, 3041−3050.
(37) Ježek, J.; Pilát, Z.; Bernatová, S.; Kirchhoff, J.; Tannert, A.; Neugebauer, U.; Samek, O.; Zemánek, P. In *Laser Tweezers Raman Spectroscopy of E. coli under Antibiotic Stress in Microfluidic Chips*, 21st Czech-Polish-Slovak Optical Conference on Wave and Quantum Aspects of Contemporary Optics; SPIE, 2018.
(38) Zhou, K.; Tang, X.; Wang, L.; Guo, Z.; Xiao, S.; Wang, Q.; Zhuo, C. An Emerging Clone (ST457) of Acinetobacter baumannii

Clonal Complex 92 With Enhanced Virulence and Increasing Endemicity in South China. *Clin. Infect. Dis.* **2018**, *67*, S179−S188.
(39) Novelli-Rousseau, A.; Espagnon, I.; Filiputti, D.; Gal, O.; Douet, A.; Mallard, F.; Josso, Q. Culture-free Antibiotic-susceptibility Determination From Single-bacterium Raman Spectra. *Sci. Rep.* **2018**, *8*, No. 3957.
(40) Douet, A.; Josso, Q.; Marchant, A.; Dutertre, B.; Filiputti, D.; Novelli Rousseau, A.; Espagnon, I.; Kloster-Landsberg, M.; Mallard, F.; Perraut, F. In *Fast Raman Single Bacteria Identification: Toward a Routine In-Vitro Diagnostic*, Biophotonics: Photonic Solutions for Better Health Care V, 2016, 98871W.
(41) Monteiro, J. M.; Fernandes, P. B.; Vaz, F.; Pereira, A. R.; Tavares, A. C.; Ferreira, M. T.; Pereira, P. M.; Veiga, H.; Kuru, E.; VanNieuwenhze, M. S.; Brun, Y. V.; Filipe, S. R.; Pinho, M. G. Cell shape dynamics during the staphylococcal cell cycle. *Nat. Commun.* **2015**, *6*, No. 8055.
(42) *Performance Standards for Antimicrobial Susceptibility Testing CLSI supplement M100*, 29th ed.; Clinical and Laboratory Standards Institute: Wayne, PA, 2019.
(43) *European Committee on Antimicrobial Susceptibility Testing EUCAST Guidelines for Detection of Resistance Mechanisms and Specific Resistances of Clinical and/or Epidemiological Importance; European Society of Clinical Microbiology and Infectious Diseases*, 2017.
(44) Espagnon, I.; Ostrovskii, D.; Mathey, R.; Dupoy, M. G.; Joly, P.; Novelli-Rousseau, A.; Pinston, F.; Gal, O.; Mallard, F.; Leroux, D. F. Direct identification of clinically relevant bacterial and yeast microcolonies and macrocolonies on solid culture media by Raman spectroscopy. *J. Biomed. Opt.* **2014**, *19*, No. 027004.
(45) Lopatkin, A. J.; Stokes, J. M.; Zheng, E. J.; Yang, J. H.; Takahashi, M. K.; You, L.; Collins, J. J. Bacterial metabolic state more accurately predicts antibiotic lethality than growth rate. *Nat. Microbiol.* **2019**, *4*, 2109−2117.
(46) Dwyer, D. J.; Belenky, P. A.; Yang, J. H.; MacDonald, I. C.; Martell, J. D.; Takahashi, N.; Chan, C. T.; Lobritz, M. A.; Braff, D.; Schwarz, E. G.; Ye, J. D.; Pati, M.; Vercruysse, M.; Ralifo, P. S.; Allison, K. R.; Khalil, A. S.; Ting, A. Y.; Walker, G. C.; Collins, J. J. Antibiotics induce redox-related physiological alterations as part of their lethality. *Proc. Natl. Acad. Sci. U.S.A.* **2014**, *111*, E2100−E2109.
(47) Lobritz, M. A.; Belenky, P.; Porter, C. B.; Gutierrez, A.; Yang, J. H.; Schwarz, E. G.; Dwyer, D. J.; Khalil, A. S.; Collins, J. J. Antibiotic efficacy is linked to bacterial cellular respiration. *Proc. Natl. Acad. Sci. U.S.A.* **2015**, *112*, 8173−8180.

https://doi.org/10.1021/acsomega.1c00170
ACS Omega 2021, 6, 16273−16279