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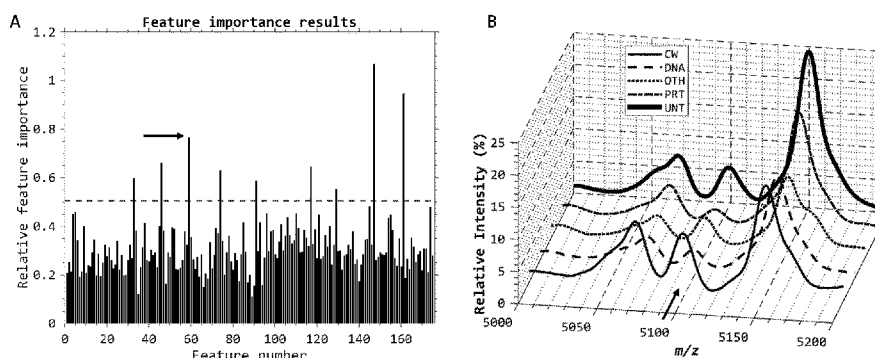


FIG. 3

(57) Abstract: The present invention provides a method for analyzing an action of a stressor on a cell sample, the method comprising the following steps: a) exposing the cell sample to the stressor, b) acquiring a sample mass spectrometry profile of the cell sample, c) determining one or more values of one or more features of the sample mass spectrometry profile, and d) classifying the stressor using a model-based comparison of the one or more values of the features of the sample mass spectrometry profile with one or more reference stressors.



PHENOTYPIC PROFILING BY MASS SPECTROMETRY AND MACHINE LEARNING

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TECHNICAL FIELD

The present invention relates to a method and an apparatus for analyzing an action of a stressor on a cell sample. The present invention also relates to a computer-readable storage medium storing program code, the program code comprising instructions for carrying out
10 such a method.

BACKGROUND

There are few analytical methods capable of producing such a fine-grained view of the metabolic and proteomic state of the cell as mass spectrometry. However, mass spectra originating
15 from complex, biological samples are relatively difficult to interpret, since limited information is available on specific mass peaks and the identity of the underlying biomolecules.

Mass spectra can be obtained using a variety of ionization methods and mass analysers,
20 whose requirements and capabilities vary over a great range. Notably, one method stands out in being economic with respect to sample preparation, throughput, workload for the operator, potential for automatization, and consumption of materials: MALDI-TOF MS. The advantages of this method have already led to its use in identification of microorganisms. It represents an economic method to obtain a fine-grained picture of the molecular composition
25 of an organism, resembling a fingerprint of its metabolic and proteomic state. Until now, analysis of mass spectral data of biological samples remained limited to subjective approaches such as visual, non-automated identification of individual peaks and simplistic unsupervised learning algorithms. The applications of MALDI-TOF MS are mainly in the typing of organisms and, more recently, preliminary attempts to determine the minimal inhibitory
30 concentrations of bacteria.

Thus, there is a need for an improved method for analyzing an action of a stressor on a cell sample.

SUMMARY OF THE INVENTION

The objective of the present invention is to provide a method and an apparatus for analyzing an action of a stressor on a cell sample, wherein the method and the device overcome one or more of the above-mentioned problems of the prior art.

A first aspect of the invention provides a method for analyzing an action of a stressor on a cell sample, the method comprising the following steps:

- a) exposing the cell sample to the stressor,
- b) acquiring a sample mass spectrometry profile of the cell sample,
- c) determining one or more values of one or more features of the sample mass spectrometry profile, and
- d) classifying the stressor using a model-based comparison of the one or more values of the features of the sample mass spectrometry profile with one or more reference stressors.

The method of the first aspect can apply state-of-the-art machine learning and computational data analysis methods to leverage the detailed information content of mass spectra. The method of the first aspect, in the following also referred to as “Phenotypic profiling by Mass Spectrometry and Machine Learning” (PhenoMS-ML) can be used to study and model the phenotypic response of cells, e.g. bacteria, to a variety of stressors, such as antibiotic drugs.

Classifying the stressor using a model-based comparison of the one or more values of the features of the sample mass spectrometry profile with one or more reference stressors may involve e.g. evaluating a Support Vector Machine that has been previously trained with reference stressors. Alternatively, it may involve comparing the value of a feature (e.g. a relative intensity at an m/z peak) with a value of this feature at a reference stressor on a reference cell sample. The model may be a set of rules, defined by logical and mathematical quantities and operations, in particular for SVM a set of hyperplanes, obtained from the model building process.

The mass-spectral data can be investigated by a variety of machine-learning methods. This can result in predictive models that are capable of identifying an antibiotic effect at concentrations well below the minimal inhibitory concentration and to determine the mechanism of action of unknown antibiotic compounds. Embodiments of the method are applicable to study the influence of stressors such as steroidal drugs, tubulin ligands and antifungal compounds on the mass-spectral profile of eukaryotic cells such as HeLa and fungi. Embodiments of the method of the first aspect require minor sample preparation and are amenable

to high-throughput screening. Furthermore, embodiments can employ wild-type cells without the need for reporter genes or (radio)labels, and provide a fine-grained phenotypic readout. It allows the pharmacological characterization of weakly active compounds that will be missed by traditional antibacterial or other phenotypic screening methods. The presented method as such has considerable potential in drug discovery, toxicology, plant protection and related settings where phenotypic responses of organisms to an external influence, such as a drug or pesticide, are studied.

In a first implementation of the method according to the first aspect, the model is determined by:

- e) exposing one or more reference cell samples to one or more reference stressors,
- f) acquiring one or more reference mass spectrometry profiles of the reference cell samples and a non-exposed cell sample which is not exposed to a reference stressor, and
- g) selecting features on said reference mass spectrometry profiles, and
- h) using of said selected features to build the model.

The inventors have shown that this represents a particularly efficient way of determining a model.

In a further implementation of the method according to the first aspect, exposing the test cell sample to the stressor and exposing the one or more reference cell samples is performed in parallel on the same day with the same batch of cells, preferably in the same multiwell plate. In parallel here may refer to that at the exposure is performed in approximately the same time, wherein small deviations (e.g. seconds or minutes) are still considered as parallel.

In a further implementation, the test cell sample, the one or more reference cell samples, and the non-exposed cell sample are from the same batch of cells. This has the advantage that differences between different batches are excluded.

In a further implementation, the method further comprises selecting the features by sequential feature selection and feature importance evaluation using decision trees. These have been shown to be particularly useful for selecting informative features that thus lead to accurate classification of the stressors.

In a further implementation, the sample mass spectrometry profiles is acquired using matrix-assisted laser desorption/ionization time-of-flight, MALDI-TOF.

In a further implementation, the cell sample comprises bacteria, the stressor is an antibiotic and a concentration of the antibiotic is equal or lower than a minimal inhibitory concentration of the antibiotic for the cell sample, in particular lower than 50%, preferably lower than 25%, of the minimal inhibitory concentration of the antibiotic for the cell sample.

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In a further implementation, the classification is with respect to

- (i) the presence of the stressor,
- (ii) the type of the stressor, in particular a type of antibiotic,
- (iii) the mechanism of the stressor, and/or
- 10 (iv) the potency of the stressor.

Experiments of the inventors have shown that the method of the first aspect can be used not only to indicate a presence of a stressor, but also to classify the stressor into different stressor classes. Furthermore, by training a model with reference stressors of the different mechanism of action or different potency, it is possible to classify unknown stressors also with regard to mechanism of action or potency, e.g. relative potency compared to the known reference stressors.

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In a further implementation,

- 20 - if the type of the cell sample is *Escherichia coli*, the features comprise one or more of the following m/z peaks: 5098; 5411; 6504; 9066,
 - if the type of the cell sample is *Staphylococcus aureus*, the features comprise one or more of the following m/z values: 5698; 5873; 5932; 6978; 7007,
 - if the type of the cell sample is human, in particular HeLa, the features comprise at
 - 25 least one of the following m/z peaks 4966 and 6085,
- wherein a accuracy of +/- 2 of the m/z is considered as belonging to the same peak

Experiments of the inventors have shown that these represent particularly informative peaks for classification of unknown stressors.

30 In a further implementation,

- if the type of the cell sample is *Escherichia coli*, the features comprise at least three, in particular at least five, of the following m/z peaks: 5098; 5326; 5869; 6277; 7117; 7159; 8601; 8874; 9631; 9726;
- if the type of the cell sample is *Staphylococcus aureus*, the features comprise at least
- 35 three, in particular at least five, of the following m/z peaks: 4074; 4668; 5331; 5932; 6889; 7006; 8151; and/or

- if the type of the cell sample is human, in particular HeLa, the features comprise at least three, in particular at least five, of the following m/z peaks: 4210; 4593; 4939; 4965; 6012; 6048; 6056; 6085; 6111; 6650; 7262; 8409, wherein a deviation of +/- 2 is considered as belonging to the same peak.

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A second aspect of the invention refers to a device for analyzing an action of a stressor on a cell sample, the device comprising:

- an input unit for obtaining a sample mass spectrometry profile of the cell sample,
- a determining unit for determining one or more values of one or more features of the sample mass spectrometry profile, wherein a choice of one or more features is based on a type of the cell sample, and
- a classification unit for classifying the stressor using a model-based comparison of the values of the features of the mass spectrometry profile with one or more reference stressors.

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The device of the second aspect may be configured to carry out the method of the first aspect or of one of the implementations of the first aspect.

In a first implementation, the device of the second aspect comprises a database for storing identifiers for a plurality of cell types and for each of the plurality of cell types one or more features. For example, the database might comprise an entry that indicates which features (e.g. which peaks) should be used for cells of the type *Escherichia coli*. Thus, the device may automatically use the correct features when analyzing the action of a stressor on cells of the type *Escherichia coli*.

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A third aspect of the invention refers to a computer-readable storage medium storing program code, the program code comprising instructions for carrying out the method of the first aspect or one of the implementations of the first aspect.

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BRIEF DESCRIPTION OF THE DRAWINGS

To illustrate the technical features of embodiments of the present invention more clearly, the accompanying drawings provided for describing the embodiments are introduced briefly in the following. The accompanying drawings in the following description are merely some embodiments of the present invention, modifications on these embodiments are possible without departing from the scope of the present invention as defined in the claims.

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FIG. 1 is a flow chart illustrating a method in accordance with an embodiment of the present invention,

FIG. 2 is a diagram of a typical mass spectrum acquired in accordance with the present invention,

FIG. 3A is a diagram of relative feature importances of different features as determined in accordance with the present invention,

FIG. 3B is a diagram indicating average spectra of antibiotics grouped to mechanism of action at highest tested concentration in accordance with the present invention,

FIG. 4A is a diagram indicating a number of times features have been selected by forward feature selection in accordance with the present invention,

FIG. 4B is a diagram indicating a number of times features have been selected by sequential backward feature selection in accordance with the present invention, and

FIG. 5 is a diagram illustrating details of MALDI-TOF spectra near important peaks as identified by the random forest algorithm and sequential feature selection in accordance with the present invention.

DETAILED DESCRIPTION OF EMBODIMENTS

The foregoing descriptions are only implementation manners of the present invention, the scope of the present invention is not limited to this. Any variations or replacements can be made by a person skilled in the art. Therefore, the protection scope of the present invention should be subject to the protection scope of the attached claims.

Mass spectrometry, in particular matrix assisted laser desorption ionization time of flight (MALDI-TOF), is an economic method to obtain a fine-grained picture of the molecular composition of an organism, resembling a fingerprint of its metabolic and proteomic state. Until now, analysis of mass spectral data of biological samples remained limited to subjective approaches such as visual, non-automated identification of individual peaks and simplistic un-

supervised learning algorithms. We applied state-of-the-art machine learning and computational data analysis methods to leverage the detailed information content of mass spectra. We investigated this approach, termed “Phenotypic profiling by Mass Spectrometry and Machine Learning” (PhenoMS-ML), to study and model the phenotypic response of bacteria to a variety of antibiotic drugs. The mass-spectral data was investigated by a variety of machine-learning methods. This resulted in predictive models that are capable of identifying an antibiotic effect at concentrations well below the minimal inhibitory concentration and to determine the mechanism of action of unknown antibiotic compounds. We also show that the method is applicable to study the influence of, such as steroidal drugs, tubulin ligands and antifungal compounds on the mass-spectral profile of eukaryotic cells such as HeLa and fungi. PhenoMS-ML requires minor sample preparation and is amenable to high-throughput screening. Furthermore, it employs wild-type cells without the need for reporter genes or (radio)labels, and provides a fine-grained phenotypic readout. It allows the pharmacological characterization of weakly active compounds that will be missed by traditional antibacterial or other phenotypic screening methods. PhenoMS-ML has considerable potential in drug discovery, toxicology, plant protection and related settings where phenotypic responses of organisms to an external influence, such as a drug or pesticide, are studied.

A single MALDI-TOF mass spectrum may comprise signal intensity data for thousands of m/z data points. For non-complex samples, such as a purified protein, the mass spectrum shows a number of peaks, of which one is the protein of interest. The analysis then comprises the identification of the desired peak and of possible contaminant peaks. Interpretation of such spectra can be done without the involvement of computational data analysis tools. In contrast, if a cellular lysates or other complex samples are studied by mass spectrometry, whether or not coupled to liquid chromatography, analysis has to be aided by designated software packages, as mass spectra can contain thousands of peaks. Therefore, it is almost impossible to interactively investigate and interpret the full breadth of data.

Feature selection, dimensionality reduction and other machine learning methods can be applied to interpret and analyse mass-spectral data obtained by MALDI-TOF of cellular samples. These methods allow to:

1. Identify and select the most relevant features, i.e., those parts of the signal which carry most of the information content and are expected to discriminate the spectra that were obtained from cell cultures treated with various stressors. This step is known as feature selection.
2. Use the data, which is now reduced to its most meaningful parts, to build models that allow the classification of spectral data. This step, which may involve a variety of computational methods, is denoted as model building.

3. In a third step, the classification models obtained in step 2 may be applied to novel spectra, e.g. spectra that were obtained by treating the biological system with as-yet uncharacterized compounds, in order to determine the biological effects of these compounds.

The first proof-of-concept for the presented method ("PhenoMS-ML") was pursued in the area of antibiotic drug discovery. Reasons for this were the high and continuing medical need for novel antibiotics, the availability of biological material and the availability of antibacterial compounds with known and diverse mechanisms of action.

The target-directed drug discovery paradigm, which was successful in several other fields, provided only limited results in the area of antibacterial compounds. During the initial years of the post-genomic era, considerable effort was put into the pursuit of target proteins (such as penicillin-binding proteins, bacterial gyrases and bacterial ribosomal machinery) that are unique to bacteria. Their relative easy expression and production through biotechnological methods, made them widely available for target-oriented high-throughput screening campaigns and structure-based drug design. Notably, some of the most important targets (the lipopolysaccharide membrane) for existing antibiotics are notoriously difficult to study with biochemical systems and were therefore not pursued to a large extent with high-throughput screening or structure-based design methods.

Pharmacokinetic effects, such as insufficient permeation into cells, or export processes, are major factors that cannot be studied in target-oriented biochemical assays. It should be noted that some of the most important targets of existing antibacterial drugs are located in the cell wall or within the periplasm, and inhibitors of these targets (such as β -lactams that bind penicillin-binding proteins and polymyxins, which bind to the lipopolysaccharide in the outer membrane) do not need to enter the bacterial cytoplasm. In contrast, many of the antibacterial target proteins pursued in the post-genomic are localized in the cytoplasm and are only accessible by cell-penetrating drugs (such as quinolones inhibiting DNA gyrase, macrolides binding to the ribosomal 50S subunit and tetracycline antibiotics binding to the ribosomal 30S subunit). Furthermore, the biophysical microenvironment of many targets (such as membrane association) is ignored in most biochemical assays, although the environment can have an effect on the folding and accessibility of the target.

Most of the currently used antibiotics were developed on the basis of phenotypic observations, usually a lethal or growth-inhibiting effect on bacteria. Pharmacokinetic factors are naturally integrated in such phenotypic systems. Whole-cell approaches also capture the effect of compounds that interact with targets which are difficult to characterize in a biochemical assay, such as penicillin-binding proteins. Therefore, it appears that phenotypic approaches have a particular value in antibacterial drug discovery.

However, phenotypic screening in antibacterial drug discovery is hampered by the relatively simplistic readout: "growth" versus "no growth" at the tested concentration. If the tested concentration is too low to inhibit growth, then a molecule which may still have a significant effect on a relevant target, and which may be developed further into a highly potent drug, is most likely missed in the screen.

During the first stages of drug discovery, it is of importance to capture even small effects, since the screening library of compounds may not contain a meaningful hit with sufficient activity and further optimization potential. However, phenotypic approaches, particularly those that have a binary readout like bacterial growth, are prone to miss weak activities of drugs. While the binary, growth-based, approaches in antibacterial screening have produced good results in the past, we now face a situation where most highly active compounds from natural product libraries have already been identified. With the low-hanging fruit already picked, it appears worthwhile to consider hits with lower potency but a potential for further optimization.

In target-based drug discovery, the value of low-affinity ligands has been realized since some time and led to a highly successful concept termed fragment-based drug discovery (FBDD). The paradigm of FBDD is that weakly potent chemical fragments can be combined into a single molecule and thereby yield a high-affinity drug. In its ideal form, FBDD also integrates structural information about the binding mode of the fragments to the target, so that the choice of fragments and their connection can be designed in a rational manner. It is therefore not be possible to use FBDD to its full potential without structural information, but the initial knowledge of a phenotypic activity of a weakly active molecule (i.e., a fragment) can trigger additional target-based approaches.

The inventors have shown that it is feasible to combine a multivariate analytical method and machine learning methods to study the response of phenotypic biological systems to external influences (e.g. well characterized and target-specific drugs) and to subsequently classify the response of unknown stressors (e.g. other drugs/compounds, uncharacterized drugs) based on the phenotypic response of the biological system. A focus is laid on pharmacological effects (by antibiotics or other stressors) on cultured cells (bacteria, fungi, eukaryotic cells), studied with MALDI-TOF MS as the analytical method.

A stressor may refer to a chemical substance or other external influence that induces changes in the proteomic composition of the organism.

"Reference stressor" may refer to a chemical substance or other external influence, defined and characterized with respect to its target and mode of action, which induces changes in the proteomic composition of the organism.

“Mass spectrometry profile” may refer to a mass spectrum obtained from a biological sample under the influence of a stressor or reference stressor, or obtained from an untreated sample.

A “feature” may refer to a property of a mass spectrometry profile.

A feature can be, in particular:

- 5 - the presence, absence or relative intensity of a signal (peak) in a mass spectrum at a certain mass-over-charge (m/z) value;
- the relative intensities of two or more signals.

“Reference mass spectrometry profile” may refer to a mass spectrometry profile obtained from a cell culture in the absence of a reference stressor or from a cell culture that has been
10 treated with reference stressors at defined concentrations.

“Sample mass spectrometry profile” may refer to a mass spectrometry profile obtained from cell cultures that have been treated with stressors of unknown activity.

“Spectral processing” may include any method for treating mass spectrometry profiles or reference mass spectrometry profiles with the intention to obtain mass spectrometry profiles
15 or reference mass spectrometry profiles suitable for further analysis. In particular, this may involve one or more of the following procedures:

- signal (peak) alignment;
- background subtraction;
- noise reduction;
- 20 - spectral intensity normalization.

Feature selection encompasses methods to select a subset of features present in a reference mass spectrometry profile that are most relevant in model construction. For the present invention, this is in particular:

- estimating feature importance using a random forest of decision trees;
- 25 - sequential forward and backward feature selection.

Model building may denote the use of supervised machine learning techniques which train a classification model. Supervised machine learning techniques include, in particular, Support Vector Machines (SVM) and random forests of decision trees.

30 The presented method may as input the selected features (see Feature Selection) of the reference mass spectrometry profiles (see above) and trains the model.

“The model” may be a set of rules, defined by logical and mathematical quantities and operations, in particular for SVM a set of hyperplanes, obtained from the model building process. The model can be used to classify the sample mass spectrometry profiles with respect to one or more of the following:

- 5 - determine the presence or absence of any stressor;
- determine the type or mechanism of action of the stressor;
- determine the relative potency of the stressor.

10 The systems used with the presented method are laid out in Table 1. In all cases, the biological system is a cell culture, the analytical method is mass spectrometry, and the stressor (external influence) is a chemical substance. Because of its practical advantages, particularly in sample preparation, we focused on MALDI-TOF-MS as mass spectrometric method of analysis. It is expected that other soft ionization methods (such as electrospray ionization; ESI) could produce similar results to MALDI-TOF MS, albeit possibly with a higher effort for sample preparation. Since the biological systems (bacteria vs. fungi vs. human cells) have different receptors and response mechanisms for chemical agents, the chemical agents used to influence the organisms are varied accordingly.

20 Wet-lab techniques have been optimized with a focus on miniaturization of both Gram-positive (*S. aureus*) and Gram-negative (*E. coli*) bacterial assay conditions in 384-well plates, and towards high-throughput processing in a MALDI-TOF mass spectrometer. Similar efforts were performed on human (HeLa) and fungal cells (*C. albicans*). FIG. 1 is a schematic overview of the different steps in the PhenoMS-ML procedure.

Table 1: Biological, analytical and data-analysis methods discussed.

Biological kingdom	Organism	Stressor	Analytical method	Data analysis
Bacteria	<i>Escherichia coli</i> ; <i>Staphylococcus aureus</i>	Antibiotic drugs	Mass spectrometry	Variety of methods to be explored, among which Random forests and support vector machines
Fungi	<i>Candida albicans</i>	Antifungal drugs		
Animalia	<i>Homo sapiens</i> (HeLa)	Various pharmacological agents		

Instead of the traditional optical density readout from a cell proliferation assay, we apply mass spectrometry to determine subtle proteomic changes after exposure to stressors. This goes beyond using mass spectrometry as readout for growth, as described by others who introduce an internal standard and use the overall relative intensity of the mass spectral signal as readout. Rather, we search for specific alterations in the mass spectra. Thereby the aim is to make better use of the information content within the mass spectrum, including the relative peak intensities, and not just the total ion count. By investigating the relative quantity of certain peaks, the use of expensive (radio)labels and internal standards is avoided.

It is important to use identical, standard operating procedures such as defined growth conditions when comparing mass spectra. The analysis of multiple mass spectra can be computationally aided with a variety of open source software packages such as MicrobeMS (Lasch, Nattermann et al. 2008) (<https://www.microbe-ms.com/>), the R based package MALDIquant (Gibb and Strimmer 2012), Mass-Up (Lopez-Fernandez, Santos et al. 2015) and Bruker's commercial Biotyper software and ClinProTools.

Mass spectra should be properly processed before any kind of analysis can be performed. These processing steps involve, but are not limited to, alignment of spectra, baseline correction, normalization, denoising (also referred to as smoothing) and peak detection. For identification purposes, comparison of acquired spectra to a reference database of spectra is performed, where the composite correlation index (CCI) for each spectrum is calculated. Based on the similarity score which results from the CCI, a spectrum can be assigned as a match to a reference spectrum in the database, or assigned as unknown. As this method yields only a simplistic correlation number for spectral similarity in a typing setting, we extended our evaluation towards more advanced methods of data analysis and model building. This will allow us to make predictions based on novel data, which can elucidate, for example, the mechanism of action of an unknown stressor.

Data handling and analysis can be performed in a fully automated manner using the software MATLAB, which implements several specialized functions for handling mass spectrometry data in its BioInformatics Toolbox. Among these steps performed are pre-processing of the raw data (including re-sampling, baseline subtraction, noise reduction, normalization, outlier detection, peak alignment and peak picking).

After data processing, mass-over-charge (m/z) peaks are extracted that represent differentially expressed proteins. Proteins differentially expressed between the experimental conditions (e.g. treatment with different classes of antibiotics) will be designated as features. These features are extracted from the dataset and are subsequently used to construct a classification model. The model requires the input of mass spectral peaks of bacterial (or cell type) cultures that were exposed to non-lethal concentrations of antibiotics or other stressors. This provides

information about different stress-induced phenotypes, which can be subsequently used to screen compounds, in search for antibiotics or compounds with other pharmacological activities. After training of the model, the model will return the class label (presence/absence of stressor, mechanism of action of stressor, identity or relative potency of stressor) that is in agreement with the phenotypic response represented by those mass spectral peaks.

The experimental work can be conceptually split in two phases (see FIG. 1): the model construction using mass spectra from cells treated with known stressors. These mass spectra are processed and features (peaks in the mass spectrum) are selected to be used for model training and internal validation. In a parallel phase (the classification phase) cells are subjected to unknown stressors, after which their mass spectra are recorded and processed in the same way as during the model building phase. Values of selected peaks in spectra originating from cells treated with unknown stressors are extracted and fed into the model to make predictions of their antibiotic effect. The PhenoMS-ML procedure can be operated as a self-contained system, which adapts to the mass spectral data fed into the algorithm in the model building phase. We describe the validation of the models generated in the model building phase. In order to do so, the obtained method was evaluated on a novel, blind set of compounds, including inactive compounds and compounds that were not used in the model training phase. The results confirm the applicability of the presented method as a black-box, operator-independent, procedure for a drug-screening application.

Chemicals and culture media were obtained from commercial suppliers, as further outlined below. Chemicals used as stressors (antibiotics, antifungals, other pharmacological agents) came from commercial suppliers (Sigma-Aldrich, Fagron, TCI, etc.) or from the substance library of the Institute of Pharmacy and Molecular Biotechnology (IPMB) Medicinal Chemistry department.

Consumable research materials, such as plastic ware, were obtained from various commercial suppliers as further indicated below.

Mass spectra were obtained using a Bruker MALDI-TOF MicroFlex LT instrument (Bruker Daltonics, Bremen, Germany). Other procedures were performed using standard laboratory equipment.

FlexControl software (Version 3.3, Build 108.2, Bruker Daltonics, Bremen, Germany) was used to acquire mass spectra using AutoXecute runs.

FlexAnalysis software (Version 3.3, Build 80, Bruker Daltonics, Bremen, Germany) was used to export raw mass spectrometry data into *.txt files.

Data analysis was performed with MATLAB (version R2018a) on a Windows 64-bit computer (16 GB random access memory, i5-4690 CPU at 3.5 GHz).

The organisms with the following identification numbers were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ; German collection of microorganisms and cell cultures):

Escherichia coli: ATCC® 25922™ (DSMZ 1103)

5 *Staphylococcus aureus*: ATCC® 29213™ (DSMZ 2569)

Homo sapiens HeLa: DSMZ ACC 57

Candida albicans: ATCC® 90028™ (DSMZ 11225)

10 The MICs of selected antibiotics were determined as described previously, which is in accordance with the CLSI and EUCAST guidelines (CLSI 2013) (EUCAST 2016) for antimicrobial susceptibility testing. The MIC was determined for the gram-negative EUCAST reference *Escherichia coli* strain (ATCC 25922) and the Gram-positive *Staphylococcus aureus* (ATCC 29213). Antibiotics were selected to cover a diverse range of mechanism of action, see Table A. 1 and Table A. 2 in the appendices. The following antibiotics were dissolved in water: benzylpenicillin, cefotaxime, cefuroxime, moxifloxacin and vancomycin. The following antibiotics were dissolved in DMSO/water (50%/50% v/v): amoxicillin, ciprofloxacin, erythromycin, gentamicin, neomycin, tetracycline, trimethoprim, nitrofurantoin, and rifampicin. The following antibiotics were dissolved in DMSO: chloramphenicol, clarithromycin, and doxycycline. Unknown antibiotics and other compounds for the blind validation screen were dissolved in DMSO to a final stock solution of 10 mM. Antibiotics were dissolved to a final concentration of 1280 mg/L and filtered using a cellulose acetate membrane (0.2 µm pore size, GE Healthcare Life Science, Freiburg, Germany) to ensure sterility. Stock solutions were stored at 4° Celsius. Prior to use, antibiotic stock solutions were diluted in sterile cation-adjusted Mueller-Hinton (MH) medium.

25 The MIC determination to antifungal drugs (listed in Table A. 3) was performed as described by the European Committee on Antimicrobial Susceptibility Testing-Subcommittee on Antifungal Susceptibility Testing (EUCAST-AFST) guidelines with minor alterations. In brief: *Candida albicans* (ATCC 90028) was grown on yeast-peptone-dextrose (YPD) agar plates overnight. Five representative colonies were suspended in DPBS and diluted to yield a density of colony forming units/mL (CFU/mL), as determined by colony plate count. This solution was subsequently diluted in RPMI 1640 medium (supplemented with L-glutamate, 2% glucose, with phenol red, buffered with sodium bicarbonate) to yield approximately 3×10^5 CFU/mL. Drug stock solutions (in DMSO, sterile filtered) were diluted in RPMI 1640 medium and mixed 1:1 with 100 µL of yeast cells in a 96-well plate (polystyrene U-bottom; Greiner Bio-One GmbH, Frickenhausen, Germany) to yield a final inoculum of 1.5×10^5 CFU/mL.

Plates were incubated overnight at 35° C at saturated humidity, after which growth was assessed visually.

HeLa cells (HeLa-ACC 57; obtained from DSMZ; Braunschweig, Germany) were grown in DMEM medium (Dulbecco's Modified Eagle's Medium, Sigma-Aldrich, Munich, Germany) supplemented with 10% fetal bovine serum (Gibco FBS, ThermoFisher Scientific, Waltham, MA, USA), penicillin and streptomycin (100 units/mL and 100 mg/L respectively; Gibco, ThermoFisher Scientific, Waltham, MA, USA) to approximately 90% confluence at 37° C in the presence of 5% CO₂ under humidified atmosphere. Cells were washed with DPBS (Dulbecco's phosphate buffered saline, Sigma-Aldrich, Munich, Germany) and harvested using the commercial available Accutase® cell detachment solution (Innovative cell technologies, Inc., San Diego, USA) according to the manufacturer's protocol. Cell seeding density was determined using a hemocytometer (Neubauer improved, Marienfeld Superior, Lauda-Koenigshofen, Germany) and adjusted to 20.000 cells/90 µL/well (96 well plate; Cellstar® polystyrene flat-bottom plates, Greiner Bio-One, Frickenhausen, Germany).

Drugs (see Table A. 4 in the appendix, 10 µL/well) were mixed in the plate upon seeding cells and incubated for 24 hours at 37° Celsius under a humidified atmosphere in the presence of 5% CO₂ to determine CC₅₀ values. Cell viability was assessed using the resazurin-based CellTiter-Blue® reagent (Promega Corporation, Madison, USA) by adding 20 µL of CellTiter-Blue® assay solution to each well. The CC₅₀ is here defined as the drug concentration where half of the expected cell viability is observed, compared to untreated cells (which have a viability of 100%). After incubation for 1-2 hours at 37° C, fluorescence was measured at 570 and 600 nm using a FLUOstar Omega plate reader (BMG Labtech GmbH, Ortenberg, Germany). Data was exported in Microsoft Excel format. Using OriginPro software (version 2015; OriginLab Corporation, Northampton, USA) the data was fitted to a sigmoidal dose-response curve and the CC₅₀ was determined.

Acquisition of reference and sample MS profiles

The following sections describe in more detail the general workflow as depicted in FIG. 1 for each of the assayed organisms (the bacteria *E. coli* and *S. aureus*, the yeast *C. albicans* and human HeLa cells)

All experiments were performed using cation adjusted Mueller-Hinton broth (MH medium; Sigma-Aldrich, Munich, Germany) prepared according to the manufacturers' guidelines.

After initial experiments, it was observed that although inoculum density (measured in McFarland units) and viable cell count (colony forming units/mL, measured by means of agar plating) were kept constant, cell cultures of *E. coli* showed considerable differences in their mass spectra among different days of experimentation. It is known that a culture of *E. coli* cells within a constant environment (i.e. exponential growth phase) show a variability in

growth rate, generation time and cell size. To circumvent dealing with such a heterogeneous cell culture, and thereby avoiding considerable inter-day differences between mass spectra of cells, the replication and division cycles within the culture were synchronized. To achieve this, cells were starved of essential nutrients, after which they were supplemented with fresh MH medium. This method is similar to work performed by Cutler & Evans and Lomnitzer & Ron, where cultures of *E. coli* are being grown in a nutrient-limiting environment (by either induced heat shock or late stationary-phase growth) which exhibit synchronous division after supplementation of said cultures with a complete medium. The heat shock is thought to make the cells temporarily methionine-deficient due to heat inactivation of essential metabolic enzymes.

E. coli cells were grown in 50 mL tubes for approximately 8 hours in MH medium in a Minitron incubator (Infors AG, Bottmingen, Switzerland) at 120 rotations per minute (25 mm shaking throw) at 37° C, after which cells were centrifuged at 2000×g for 10 minutes. Residual medium was decanted to waste and the cell pellet was resuspended in sterile DPBS (Dulbecco's phosphate buffered saline). Cell cultures were put back in the incubator and starved in this environment overnight for approximately 16 hours. After starvation, cells were centrifuged again for 10 minutes at 2000×g. Supernatant was decanted and cells were resupplied with fresh MH medium and diluted to McFarland standard of 1.

The starvation period arrests all cells at the same point in their cell division cycle. Supplying fresh MH medium after the starvation period causes the cell culture to initiate synchronized division. It appeared that after starvation overnight in DPBS, a McFarland value of 1 still corresponds to roughly 1×10^8 CFU/mL in the case of *E. coli* (ATCC 25922). However, for *S. aureus* cultures after starvation it was found that a McFarland standard of 1.0 corresponded to roughly 1×10^7 CFU/mL. Cells were allowed to adapt to the nutrient rich medium for at least one division cycle (approximately 70 minutes in the case of *E. coli*; approximately 90 minutes in the case of *S. aureus*) to a McFarland of 2 (*E. coli*) and 2.6 (*S. aureus*) before addition to the antibiotics in the 384-well plate.

Exposure of cells to antibiotics was performed in clear polystyrene 384-well plates (flat-bottom; Greiner Bio-One GmbH, Frickenhausen, Germany). Concentrations of each antibiotic (2-fold dilution series in MH) were made to ensure that the highest final assay concentration was 1×MIC of that antibiotic. 50 µL of antibiotic stock (2×MIC) solution were added to each well. Subsequently an inoculum with a McFarland standard of 2.0 (*E. coli*) or 2.6 (*S. aureus*) was added (also 50 µL) to the plates using a multichannel pipette. This ensured a final cell concentration which corresponds to an approximate cell density of approximately 1×10^8 colony forming units per mL (CFU/mL). Plates were sealed using sealing film (Seal-Plate® film, Excel Scientific Inc, Victorville, CA, USA) and placed in a preheated microplate

incubator (Thermo Scientific iEMS Incubator/Shaker, ThermoFisher Scientific, Waltham, MA, USA) at 37° C and shaken at 1150 rotations per minute for 2 hours.

After incubation, plates were centrifuged (Rotina 420R, Hettich Lab Technology, Tuttlingen, Germany) equipped with a swinging bucket rotor at 2000×g for 10 minutes at 4 °C . Supernatant was discarded and cell pellets were washed with 100 µL 35% ethanol (v/v) and incubated in the microplate incubator for 5 minutes at 1150 rotations per minute. Cell debris was centrifuged again and washed a second time with 100 µL of 35% ethanol. After removal of 90 µL the supernatant, remaining cells were resuspended in the remaining 10 µL 35.0% ethanol, sealed and stored at 4 °C. Prior to MALDI-TOF MS analysis, bacterial cell pellets were resuspended in the plate. Cell suspension was mixed 1:1 with freshly prepared α -cyano-4-hydroxycinnamic acid (CHCA; 10 mg/mL in 50.0% acetonitrile, 47.5% H₂O, and 2.5% trifluoroacetic acid) and approximately 1 µL was spotted on a MALDI target plate (MSP 96 polished steel BC microScout target, Bruker Daltonics, Bremen, Germany). Samples were air-dried at room temperature.

All experiments were performed using RPMI 1640 medium supplemented with L-glutamate, 2% glucose, phenol red and buffered with sodium bicarbonate (Sigma-Aldrich, Munich, Germany).

Exposure of cells to antifungals was performed in clear polystyrene 384-well plates (Flat-bottom; Greiner Bio-One GmbH, Frickenhausen, Germany). Concentrations of each antifungal (2-fold dilution series in RPMI 1640 supplemented as described below) were made to ensure that the highest final assay concentration was 1×MIC. To each well 10 µL of antifungal stock solution was added. Subsequently an inoculum of 90 µL with a McFarland standard of 4.4 was added to the plates using a multichannel pipette. This ensured a final cell concentration which corresponds to an approximate cell density of 2.5×10^7 colony forming units per mL (CFU/mL). Plates were sealed using sealing film (SealPlate® film, Excel Scientific Inc, Victorville, CA, USA) and placed in a preheated microplate incubator (Thermo Scientific iEMS Incubator/Shaker, ThermoFisher Scientific, Waltham, MA, USA) at 35° C and shaken at 1150 rotations per minute for 2 hours. Fungal cells were exposed to antifungal compounds at the following concentrations: 1×MIC, $\frac{1}{2}$ ×MIC and $\frac{1}{4}$ ×MIC. At lower concentrations (i.e. $\frac{1}{8}$ ×MIC and lower) the cell's spectra could not be distinguished from untreated cells.

After incubation, plates were centrifuged (Rotina 420R, Hettich Lab Technology, Tuttlingen, Germany) equipped with a swinging bucket rotor at 2000×g for 10 minutes at 4 °C . Supernatant was discarded and cell pellets were washed with 100 µL 35% ethanol (v/v) and incubated in the microplate incubator for 5 minutes at 1150 rotations per minute. Cell debris was centrifuged again and washed a second time with 100 µL of 35% ethanol. After removal of 90 µL the supernatant, remaining cells were resuspended in the remaining 10 µL 35.0% ethanol,

sealed and stored at 4 °C. Prior to MALDI-TOF MS analysis, fungal cell pellets were resuspended in the plate. Cell suspension was mixed 1:1 with freshly prepared α -cyano-4-hydroxycinnamic acid (CHCA; 10 mg/mL in 50.0% acetonitrile, 47.5% H₂O, and 2.5% trifluoroacetic acid) and approximately 1 μ L was spotted on a MALDI target plate (MSP 96 polished steel BC microScout target, Bruker Daltonics, Bremen, Germany). Samples were air-dried at room temperature.

HeLa cells were grown in DMEM medium (Dulbecco's Modified Eagle's Medium Sigma-Aldrich, Munich, Germany) supplemented with 10% fetal bovine serum (Gibco FBS, ThermoFisher Scientific, Waltham, MA, USA), penicillin and streptomycin, as described previously.

HeLa cell cultures exposed to the CC₅₀ concentrations of the selected stressors were prepared as described in previously.

After approximately 24 hours of incubation in the presence of different stressors (see Table A. 4), HeLa cells were harvested. Cells were washed with 100 μ L DPBS and resuspended in 50 μ L ice-cold 35% ethanol. Using a disposable micropipette tip the well surface was scratched to promote cells detachment. Cell suspension (1 μ L) was mixed with 1 μ L CHCA matrix (prepared as described above) and spotted on a steel MALDI target plate. It appeared that upon treatment of cells with paclitaxel at the determined CC₅₀, there was an insufficient amount of cells to yield a proper signal using the MALDI. The experiment was repeated at the IC₈₀ (0.030 mM).

The following procedure was used for all samples, irrespective of cell type. Target plates were positioned in the mass spectrometer (MALDI-TOF microflex LT, Bruker Daltonics, Bremen, Germany) fitted with a nitrogen laser (337 nm, 60 Hz). Spectra were acquired in linear mode with a mass range of m/z 2,000-15,000 using AutoXecute runs of the FlexControl software (Version 3.3, Build 108.2, Bruker Daltonics). The laser was set to fire 100 shots at 80% power per location (attenuator set to 20-30%), while moving in a small spiral raster over 7 locations per sample spot to assure appropriate signal intensity. The sum of 700 shots yields spectra with ion intensities in the order 10⁴-10⁵ ion counts for the most abundant ions. Sample rate was set to 1.00 GS/s; detector gain was set to 3.7 $\times$; electronic gain was set to 200 mV and Realtime Smooth was disabled; Default delayed ion extraction was fixed at 140 ns. Calibration of the instrument was regularly evaluated using Brukers 'Protein Calibration Mix I' and if necessary, adjusted accordingly.

Using Bruker's FlexAnalysis software the collected raw spectra were exported to a *.txt file in ASCII format. Subsequently the spectra were imported in MATLAB (R2018a; The MathWorks Inc., Natick, USA) and preprocessed as follows. Spectra were resampled in order to obtain a homogenous mass/charge (m/z) vector for each sample. The baseline of each indi-

vidual spectrum was estimated and subtracted using a sliding window filter. Noise was reduced using locally weighted scatter plot smoothing regression method (LOWESS filter). Spectra were normalized to their total ion current (TIC; in this case identical the total area under the curve, the AUC). Subsequently each mass spectrum was aligned towards multiple inter-spectra conserved high intensity peaks. Spectral processing was identical for spectra originating from the same organism, to allow for comparison. For example, the majority of the highly abundant proteins that can be observed in a typical *E. coli* mass spectrum are large and small ribosomal (RL and RS) associated proteins. By aligning spectra during the initial processing step towards several of these highly intense and consistently observed peaks, errors in peak location are reduced. In the case of mass spectra of *E. coli*, the peaks used for alignment were observed at the following m/z values (protein name; UniProt accession number in parenthesis, post translational modification if applicable): 4365.333 (RL36; PoA7Q6), 5381.396 (RL34; PoA7P5), 6255.416 (RL33; PoA7N9 initiator methionine removed, methylated), 6316.197 (RL32; PoA7N4, initiator methionine removed), 7158.746 (RL35; PoA7Q1, initiator methionine removed), 7274.456 (RL29; PoA7M6) and m/z 10300.100 (RS19; PoA7U3, initiator methionine removed). Peaks were putatively identified by searching the UniProt database (release 2018_07) of reference proteome up000000625 of *Escherichia coli* strain K12 (Taxonomy identifier 83333) using the TagIdent tool (Gasteiger, Hoogland et al. 2005). Subsequently, average masses of proteins were calculated using the primary sequence data and the Fragment Ion Calculator (Proteomics Toolkit, Institute for Systems Biology).

For *S. aureus*, the peaks used for alignment were observed at the following m/z values (protein name; UniProt accession number in parenthesis, post translational modification if applicable): m/z 4306.36380 (RL36; Q2FW29), 5303.35368 (RL34; Q2FUQ0, initiator methionine removed), 5873.73892 (RL33; Q2FY22), 6354.35216 (RL32; Q2FZF1, initiator methionine removed), 6554.68044 (RL30; PoAoG2), 8091.25832 (RL29; Q2FW14), 9627.02076 (DNA-binding protein HU; Q5HFV0) and m/z 11537.45936 (RL24; Q2FW17). Peak identities were found in the UniProt database using the reference proteome up000008816 of *Staphylococcus aureus* strain NCTC 8325. Theoretical average masses were calculated as described for *E. coli*.

In the case of HeLa mass spectra, the peaks used for alignment were observed at the following m/z values (protein name; UniProt accession number in parenthesis, post translational modification if applicable): 4965.52320 (Minor histocompatibility protein HB-1; O97980) 6085.18252 (Metallothionein-2; P02795, acetylated), 6648.86572 (40S ribosomal protein S30; P62861), 8565.85228 (Ubiquitin; PoCG47, position 1-76). Theoretical average masses were calculated as described for *E. coli*.

In the case of *Candida albicans* mass spectra, alignment peaks were observed at m/z 4163.6168 (alpha mating pheromone; AoMETHODD8PI68), 6059.2549 (R L40; C4YHX3),

6198.3745 (RL3; Q96W55, initiator methionine removed), 6468.3638 (RS29A; C4YMQ1, initiator met removed), 6981.1323 (RL29; C4YCU6, initiator methionine removed).

A peak detection algorithm was applied to identify centroid peak locations (Coombes, Tsavachidis et al. 2005) (Morris, Coombes et al. 2005). Subsequently, peak binning was performed to obtain a common m/z vector to describe the peaks observed in the spectra. This yields a common m/z vector containing approximately 170 peaks in the m/z 2000-15000 Da region in the case of *E. coli*. Similar amount of peaks are observed for mass spectra of *S. aureus* (127 peaks) and HeLa cells (170 peaks) and *C. albicans* (82 peaks).

Based on the TIC values, training data were grouped into quartiles and the interquartile range (IQR) was calculated. To determine outliers from the bulk TIC data, the upper fence (UF) and the lower fence (LF) were computed using Equation 1 and Equation 2:

$$UF = Q3 + 1.5 \times IQR \quad \text{Equation 1}$$

$$LF = Q1 + 1.5 \times IQR \quad \text{Equation 2}$$

Where Q3 depicts the third quartile (75th percentile) and Q1 the first quartile (25th percentile) of the TIC values. Spectra with TIC values above the upper fence or below the lower fence were considered outliers and removed from the dataset. This eliminates the requirement to visually inspect each spectrum and gives an objective verdict about the relative quality of the mass spectrum. Removing these samples reduces the chance of overfitting a model and reduces model complexity.

Additionally, an outlier filter was added that removes any spectrum whose intensity was higher than the upper fence based on the intensity of the mass spectrum at m/z 12500. In none of the mass spectra of the assayed organisms, a peak was observed at this m/z . Therefore the relative intensity at this m/z provides an easy way of removing bad quality spectra. As a threshold, spectra with relative intensity above the third quartile plus 3 times the interquartile range at m/z 12500 (where no peak is expected) were removed. In practice, this threshold meant that all spectra with intensity roughly above 1% at m/z 12500 were removed.

The datasets of both *E. coli* and *S. aureus* included spectra from cultures treated with over 15 different antibiotics assayed at several concentrations of each antibiotic (2-fold dilution series). Eight biological replicate spectra were measured per antibiotic concentration. The concentrations at which experiments are performed are denoted as a fraction of the MIC in the following manner throughout the remainder of the document: i.e. $\frac{1}{8} \times \text{MIC}$ for an experiment performed at one-eighth of the MIC value. Unless indicated otherwise, a distinction between untreated cell cultures and cultures treated with an antibiotic was not possible below $\frac{1}{8} \times \text{MIC}$.

Feature Selection

The following abbreviations are used for the respective feature selection methods:

Random forest: RF; sequential backward feature selection: SBS; sequential forward feature selection: SFS.

5 a) Feature evaluation using correlation

Not all the peaks in the spectrum contain sufficient discriminatory and relevant information for classification model construction. Peak values were evaluated using their Pearson correlation coefficient to identify highly correlating, redundant features. Ideal features are strongly correlated with their associated class labels (e.g. antibiotic mode of action or identity), but
10 are uncorrelated to each other and describe unique variance in the dataset. The Pearson correlation coefficients of all features from the dataset can be graphically represented in a heat map of the feature correlation values. A strong correlation (close to +1 or -1) is indicative that the peaks have a direct linear dependency on each other, being either positive (+1) or negative (-1) respectively. A correlation of 0 refers to uncorrelated features. Features with a low
15 correlation coefficient are describing unique variance in the data. The correlation of the features to each other gives an indication about the fitness of each feature to describe unique variance in the dataset.

Features which are highly correlated to each other might be redundant, as they describe similar variance in the data. These features may be removed from the dataset, as they might cause
20 overcomplicating or overfitting of the models. It can be expected that the MALDI-TOF data contains both single and double charged molecules of the same ion, which can be easily identified using this method.

b) Feature selection using random forest

A way to evaluate features is using decision trees. In a classification decision tree, the so-called leaves represent class labels and the nodes connecting the branches represent conjunctions of features that lead to the respective branch (path) in subsequent nodes to the leaf.
25 Advantages of decision trees are that they are relatively easy to interpret, provide direct information about the fitness (or redundancy) of features, and are computationally inexpensive. A bootstrap aggregated ('bagged') random forest (RF) of 1000 decision trees was grown
30 to evaluate the feature importance. The amount of 1000 trees gives a good estimation of the feature importance considering the data size and complexity. By evaluating the so called 'out-of-bag error', each feature's importance with regard to impact on classification performance can be evaluated. The relative feature importance can be used as filter for features (peaks in the dataset) which do not hold any discriminatory value and cause unnecessary complication
35 of the model. This type of feature selection allows for redundant features to be removed,

which decreases overfitting of the data and reduces model complexity. As a threshold, features with a relative feature importance higher than the mean importance plus one and a half standard deviation were incorporated in the models.

c) Feature selection using sequential feature selection

5 Another way to filter out redundant features is sequential feature selection. During sequential feature selection, a subset of features is selected that best predict the data until there is no improvement in prediction accuracy. This can be done by creating an initial feature subset and subsequently adding more features (so called sequential forward feature selection; SFS). Another option is taking all features in consideration at the start, and then features are re-
10 moved until accuracy no longer improves (so called sequential backward feature selection; SBS). As sequential feature selection is a computationally intensive operation, only features were considered for sequential feature selection that had a relative feature importance higher than the mean feature importance minus one standard deviation as determined by the random forest.

15 For each new candidate feature subset (after adding or removal of a feature), a 10-fold cross validation was performed using randomly chosen training and test subset populations. Sequential forward feature selection was performed 100 times to identify peaks with a large positive influence on model accuracy. As a threshold, features were selected based on the mean amount of times they were selected (out of the 100 times) plus one standard deviation
20 of the amount of times they were selected.

Sequential backward feature selection was also performed 100 times using 10-fold cross validation. As a threshold, features that were selected more than mean amount of times they were selected (out of the 100 times) plus one and a half standard deviation of the amount of times they were selected, were included for modeling. If either the threshold for SFS or SBS
25 was selected more than 100 times, which would result in no features selected, a threshold of >99 was taken to select features.

Features selected by two or more of the applied feature selection methods (RF, SFS and SBS) were considered for model building.

Model building and internal validation

30 Using the selected features and corresponding class labels (either treated/untreated, mode of action, stressor identity and mode of action with relative potency as fraction of MIC), models were constructed under MATLAB's default settings in the *classificationLearner* application. The models were internally validated using a 10-fold cross-validation and 34% hold-out validation. During hold-out validation, a part of the data (in these cases approximately $\frac{2}{3}$ of the
35 data, 66%) was used to construct the model. The model is then evaluated by making classifi-

cation predictions on the remaining 34% of the data. It was found that Support Vector Machine-based classifiers (SVM) performed among the best on our data sets.

Confusion matrix

Model performance is in all cases evaluated with the overall correct rate, a number between 0% and 100%, indicating the percentage of spectra classified correctly (see Equation 3). Additionally, for the majority of the models described the false negative rate (FNR, commonly also referred to as ‘miss rate’), the false positive rate (FPR, commonly also referred to as fall-out), the true positive rate (TPR, commonly also referred to as ‘recall’ or ‘sensitivity’ value) and the true negative rate (TNR, commonly also referred to as ‘selectivity’ or ‘specificity’ value), defined as in Equation 4, Equation 5, Equation 6 and Equation 7 (yielding a number between 0 and 1).

$$\text{Overall accuracy} = \frac{(\text{amount of positive samples})}{(\text{amount of samples})} \times 100\% \quad \text{Equation 3}$$

$$\text{FNR} = \frac{(\text{false negatives})}{(\text{all output negatives})} \quad \text{Equation 4}$$

$$\text{FPR} = \frac{(\text{false positives})}{(\text{all output positives})} \quad \text{Equation 5}$$

$$\text{TPR} = \frac{(\text{true positives})}{(\text{all output positives})} \quad \text{Equation 6}$$

$$\text{TNR} = \frac{(\text{true negatives})}{(\text{all output negatives})} \quad \text{Equation 7}$$

External validation of models with blind datasets

For the bacterial models, an external validation with a blind set of compounds was performed. These compounds were provided to the PhenoMS-ML operator without any further information, except that the set contained a number of active compounds which were not included in the training dataset, along with a number of compounds from the training set and (presumably) inactive compounds. These compounds were subjected to the PhenoMS-ML method at a fixed concentration of 10 μM , which represents a typical concentration in HTS campaigns. The trained model was externally validated by making predictions on novel data that was explicitly not included in the training phase. The set of compounds used for the antibacterial screens on *E. coli* and *S. aureus* and details of their predictions are provided in Table 6 and Table 10. For the validation, four models were built for each bacterial strain; one using a binary classifier, returning only whether the spectra belonged to cells treated with an antibiotic or is untreated. The second model that was built used the mode of action of the antibiotics as class labels (as given in Table A. 1 and Table A. 2). The third model used the

antibiotic identity as class labels. As the model cannot predict for antibiotics that are not contained in the training data set, a prediction was considered correct if at least the mode of action of the predicted antibiotic was correct. The fourth model used the mode of action and the relative potency of the antibiotic as fraction of the MIC as class label.

5 RESULTS

MIC determination for bacteria

All MIC values were within the acceptable range ($\pm$ a 2-fold dilution step) of the reference values, as depicted in Table A. 1 (*E. coli*) and Table A. 2 (*S. aureus*) in the appendices. The bacterial cultures were treated with antibiotics in subsequent PhenoMS-ML experiments at sub-lethal concentrations. This highest concentration by which *E. coli* cultures were treated was at the MIC, as determined by the CLSI and EUCAST guidelines (Wiegand, Hilpert et al. 2008). Subsequently, a 2-fold serial dilution series was prepared and bacterial cultures were treated with a fraction of the MIC (i.e. $\frac{1}{2}$ times the MIC, $\frac{1}{4}$ times the MIC, $\frac{1}{8}$ times the MIC, etc).

15 MIC determination for fungi

The MIC values found for compounds were within acceptable range of EUCAST-AFST and CLSI reference values, as provided in (EUCAST 2018) and (NCCLS 2002). The MIC values of tested antifungals are listed in Table A. 3.

HeLa toxicity: CC₅₀

20 The CC₅₀ of several stressors on HeLa cells was determined using the conventional CellTiter-Blue® assay. The stressors and their respective CC₅₀ values are reported in Table A. 4. These stressors were selected to cover a diverse range of pharmacological classes, from anticancer drugs (i.a. tubulin polymerization inhibitors such as vinblastine) to steroid hormones (corticosteroids, i.a. prednisolone). For some anticancer drugs (i.a. paclitaxel, vinblastine), the CC₅₀ was in the nM range, while CC₅₀ values for some other drugs (i.a. hormones, opioids) was in the mM range. This is expected, as the anticancer drugs are intended to kill the cells, while hormones are not.

PhenoMS-ML for Gram-negative bacteria exposed to antibiotic stressors

30 The following procedure describes the exposure of bacterial culture to antibiotics at their MIC concentrations or below. However, the inoculation cell density in the present procedure is higher and the incubation time is shorter than in the classical MIC determination methods, as provided by the CLSI. Both factors are known to cause deviations of the actual MIC. The CLSI guidelines refer to this as the inoculation effect, where a higher (or lower) inoculum density may cause under- or over-estimation of the actual MIC. Although inoculum density is

higher and incubation times shorter in the here presented MALDI-TOF MS assay compared to CLSI, the MIC value was used as benchmark here.

Multiple high-abundance proteins in mass spectra of *E. coli* have been identified previously, with the majority being ribosomal proteins. This allows us to reduce errors in peak location by aligning spectra during the initial processing step towards several of these known reference peaks (see FIG. 2 for a typical mass spectrum of *E. coli* and corresponding errors observed for reference peaks). After alignment to the known reference peaks, the errors are well within the mass tolerance limit of ± 300 ppm recommended by the manufacturer of the MS instrument (Bruker Daltonics). Theoretical masses and pI values were calculated using the MS/MS Fragment Ion Calculator (Proteomics Toolkit, Institute for Systems Biology, available through <http://db.systemsbiology.net:8080/proteomicsToolkit/FragIonServlet.html>).

FIG. 2 shows a mass spectrum of *E. coli* (average spectrum of 161 spectra; originating from untreated cell cultures). Indicated are reference peaks used for spectra alignment during spectral processing, with their respective protein name and mass. RL corresponds to Ribosomal Large subunit (50S) and RS to Ribosomal Small subunit (30S) followed by the respective protein unit number. Table inset shows mass accuracy in ppm of the experimentally determined m/z ('Exp.') and theoretical calculated average mass (Th.). Note that the theoretical pI of most ribosomal proteins is relatively high (above 9.5). This is in agreement with previous observations.

The recorded mass spectra represent the proteomic composition of the bacterial cells. The (dis)appearance and shifts in peaks are caused by changes of cellular composition induced upon antibiotic treatment. After normalization and baseline subtraction, the resulting peak intensity reflects changes in relative abundance of a compound. This means each peak (a feature) in a mass spectrum holds a conceivable amount of information about the phenotype of the *E. coli*. This information is two-fold: presence or absence of peaks (m/z); and if a peak is present, it holds information about its relative abundance (relative intensity of the peak, depicted in %, normalized to the most abundant peak).

Feature selection

The peaks in the mass spectrum were investigated in terms of their Pearson correlation towards one another. As a rule of thumb, features with a correlation coefficient ≤ 0.30 are considered weakly correlated and describe unique variance in the data. Features with a correlation between 0.3 and 0.85 can be considered medium correlated, and features with a correlation ≥ 0.85 are considered to be highly correlated and could be considered for removal, as they don't describe unique variance. Especially in the lower m/z region (peaks between m/z 3800 and 4100), the correlation of some the features is over 0.85. On average in this region the Pearson correlation is over 0.62, indicating a considerable degree of redundancy among

these features. Therefore it is sensible to remove redundant features from the dataset before proceeding to modelling.

Using the random forest (RF), the relative feature importance of the features was evaluated. The relative feature importance also depends on the class labeling level used (either antibiotic mode of action, antibiotic identity and antibiotic identity in combination with its relative strength as fraction of the MIC). Results when using the antibiotic mode of action are depicted in FIG. 3. This dataset uses for each antibiotic concentrations ranging from $1\times$ down to $\frac{1}{8}\times$ MIC, as in general distinction below $\frac{1}{8}\times$ MIC could not be made (unless indicated otherwise). Details of features that are considered most important and least important by the random forest are depicted in FIG. 3 as well.

FIG. 3 on the left side (A) shows all 174 peaks (features) from the dataset as evaluated on their importance by growing a forest of 1000 decision trees, with the mode of action as class label. From this graph, one of the most important features is feature 59 (indicated by the arrow), corresponding to m/z 5098. Threshold for feature selection (mean importance plus one standard deviation) is indicated by horizontal dashed line. Details of this peak are depicted on the right side (B). Average spectra are shown of antibiotics grouped to mechanism of action at highest tested concentration (see legend). Note the difference in relative intensity of the peak observed at m/z 5098 (arrow), especially comparing the untreated cells and the cells treated with a protein synthesis inhibitor (PRT) and DNA synthesis inhibitors (DNA). Intensity of CW and OTH seems identical to untreated cells (UNT).

Previously, it was found that the majority of proteins detected using whole cell MALDI-TOF on *E. coli* are ribosomal proteins. The protein with m/z 5098 can be identified as being stationary-phase-induced ribosome-associated protein (previously referred to as 30S ribosomal protein S22 (also known as RS22; UniProtKB P68191, actual mass of single charged peptide is 5096.8287 Da). It is known that this protein associates with the 30S subunit of the ribosome and that its copy-number increases from 0.1 per ribosome particle in the exponential growth phase to 0.4 per ribosome particle once the culture reaches stationary growth phase. It was found the peak at m/z 5098 to be altered in abundance dependent on the growth stage of bacterial cultures. Possibly, the influence of antibiotics on the growth process of bacteria has an influence on the abundance of this protein. In our case, see FIG. 3B, this protein seems especially decreased in abundance upon treatment with protein synthesis inhibitors, DNA interfering antibiotics and other antibiotics. This effect is not seen when cells are treated with cell wall synthesis inhibitors. In total, 9 peaks were selected by the random forest algorithm, if mode of action was used as class label.

FIG. 4 shows features selected by forward feature selection (A) and backward feature selection (B). Only features selected more than the threshold value (horizontal dotted line) were

considered for model building. In the case of SFS, a total of 10 features were considered. In the case of SBF, a total of 18 peaks were selected for consideration in the model.

Forward feature selection was performed with all peaks having a relative feature importance larger than the mean feature importance as previously determined by the random forest.

5 Forward feature selection was performed 100 times using 10-fold cross validation with the mode of action as class label. The feature selection process is terminated when the cross validation misclassification error does not decrease anymore by adding more features. As the sequential feature selection algorithm evaluates the features in a random order, the features that are finally selected by the sequential feature selection differ each round. However, some
10 features will get included in every round of feature selection, as they are good predictors (example in FIG. 4A, where feature 117, 147 and 161 are included in all 100 rounds of feature selection. As a threshold for considering peaks to be included in the model, peaks selected more than the mean amount times selected (out of the 100 times) plus one standard deviation of the amount of times peaks were selected (out of the 100 times) was set (see FIG. 4A,
15 horizontal dotted line). This yielded 10 peaks being selected, some of which were also previously selected by the random forest.

To avoid missing combinations of weak features that display synergistic predictive power, backward feature selection was performed as well. Backward feature selection was performed 100 times, using 10-fold cross validation. As a threshold, peaks were selected based on the
20 amount (out of the 100 times) they were included by SBFS. As a threshold, only peaks selected >99 was used to consider them for model building. This procedure yielded 18 peaks, of which several were also selected by the random forest and forward feature selection algorithm (see FIG. 4).

To create a set with strong predictive peaks, the peaks that were selected in 2 out of the 3 performed feature selection methods (random forest, backward and forward feature selection)
25 were combined. This yielded in total 8 peaks to be used for model building, when using the mode of action as class label. The peaks selected for the four different models are provided in Table 2.

Table 2 below shows features selected for the four different models based on different class
30 labels using the complete *E. coli* data set. Bin: binary classification (treated versus untreated); MoA: classification based on mode of action; ID: classification based on antibiotic identity; MoAP: classification based on Mode of action and relative potency as fraction of the MIC.

Table 2:

Model (Nr. of classes)	Nr. of fea- tures	m/z									
Bin (2)	7	4214	4859	7216	7661	8898	9293	12654			
MoA (5)	8	5098	5411	6256	6280	6504	8120	9066	9720		
ID (18)	8	5098	5411	5814	6256	6504	7929	9066	9275		
MoAP (25)	10	4532	5098	5411	6256	6316	6413	7216	8120	9275	9720

Using the selected features and corresponding class labels (either treated/untreated, mode of action, stressor identity and relative potency as fraction of MIC), models were constructed under MATLAB's default settings in the *classificationLearner* application. The models were internally validated using a 10-fold cross-validation and 34% hold-out validation. During hold-out validation, a part of the data (in these cases approximately $\frac{2}{3}$ of the data, 66%) was used to construct the model. The model is then evaluated by making classification predictions on the remaining 34% of the data. Using MATLAB's *classificationLearner* application, it was found that under default settings the quadratic Support Vector Machine-based classifiers (Q-SVM) performed among the best on our data sets. Therefore only Q-SVM models have been evaluated and will be discussed here.

Under MATLAB's default settings, a Q-SVM model was generated using the subset of selected features and the antibiotics identity as class label. Model performance using 10-fold cross validation and hold-out validation was similar, with 61% of the spectra being identified to the correct class (59% using the hold-out validation). The results of the 10-fold cross-validated model are depicted in table 3.

Table 3 shows a confusion matrix of Q-SVM model using 10-fold cross validation and antibiotic identity as class label using 8 selected features. Overall accuracy is 61%. Numbers on the diagonal indicate number of correctly classified spectra. The numbers of incorrectly classified spectra are off the diagonal. Overall model contains 908 mass spectra, each antibiotic at the range of $1 \times \text{MIC}$ to $\frac{1}{8} \times \text{MIC}$. Boxes with bold borders indicate data that is discussed in detail in the text.

Note especially the inability of the model to distinguish between spectra from cell cultures treated with penicillin-derivatives amoxicillin (AMX), cefotaxime (CFT), cefuroxime (CFX) and penicillin (PEN), as indicated with the black box. This can be explained by the fact that both antibiotics are cell wall synthesis inhibitors.

Comparing these results to the Raman spectral analysis of Athamneh and coworkers, the approach which comes closest to our method with respect to general data analysis, application purposes and amount of different antibiotic identities and mechanism of action classes used, the presented SVM-based model here is roughly equally accurate (Athamneh, Alajlouni et al.

VAN				1	1	3		1	13		6			1				21
Predicted class																		

In a similar fashion as described previously, data was evaluated using only the mechanism of action as class label for feature evaluation and model construction. The results of that Q-SVM model are depicted in Table 4. Table 5 depicts the confusion matrix of the binary classifier model.

Table 4 shows a confusion matrix of Q-SVM model (10-fold cross validation) performance using 8 features and the mode of action of the antibiotics as class labels. Data encompasses over 900 mass spectra from the selected antibiotics ranging between $1 \times \text{MIC}$ and $\frac{1}{8} \times \text{MIC}$. Class labels are as follows: CW: cell wall synthesis inhibitors; DNA – DNA synthesis/replication inhibitors; PRT: protein synthesis inhibitors; UNT: untreated; OTH; other mode of action. Overall accuracy is 75% using 10-fold cross validation and 76% using hold-out validation. For each predicted class is the following indicated: FNR: false negative rate; FPR; false positive rate; TPR: true positive rate; TNR: true negative rate.

Table 4:

True class	n = 908	CW	DNA	OTH	PRT	UNT	FNR	FPR	TPR	TNR
	CW	141	20	7	30	12	0.10	0.31	0.69	0.90
	DNA	28	87	1	16	2	0.06	0.26	0.74	0.94
	OTH	7	1	44	15	11	0.04	0.29	0.71	0.96
	PRT	15	7	9	276	18	0.09	0.20	0.80	0.91
	UNT	12	3	1	10	135	0.04	0.24	0.76	0.96
Predicted class										

Table 5 shows a Confusion matrix of Q-SVM model (10-fold cross validation) performance using 7 features and the presence or absence of antibiotics (binary classification) as class labels. Data encompasses over 900 mass spectra from the selected antibiotics ranging between $1 \times \text{MIC}$ and $\frac{1}{8} \times \text{MIC}$. Class labels are as follows: ANT: spectra from cell cultures treated with mentioned antibiotics; UNT: spectra from untreated cell cultures. Overall accuracy is 93% using 10-fold cross validation and 94% using hold-out validation. For each predicted class is the following indicated: FNR: false negative rate; FPR; false positive rate; TPR: true positive rate; TNR: true negative rate.

Table 5:

True class	n = 908	ANT	UNT		FNR	FPR	TPR	TNR
	ANT	719	28		0.18	0.05	0.95	0.82
	UNT	34	127		0.05	0.18	0.82	0.95
	Predicted class							

Using the mode of action and the relative potency of the stressors, model accuracy was 42% using 10-fold cross validation and 43% using hold-out validation. Confusion matrix of this model is not shown.

For the external and blind validation of models, MS profiles for a set of substances were measured and evaluated using the respective models. The identity, activity and relative potency of these substances remained unknown to the operator during the PhenoMS-ML workflow. The set of unknown compounds (including an undefined number of antibiotics) was tested with 8 technical replicates for each compound. The average mass spectrum of the technical replicates was calculated and peak intensities of the selected peaks were fed into the model. The results of the external validation are depicted in Table 6.

Using the binary classifier, which simply indicates if a mass spectrum is originating from a culture treated with an antibiotic (ANT) or not (-), an accuracy of 95% was achieved. Notably, there were no false positive predictions made by the model, as all the drug compounds without known antibiotic activity were all classified correctly by the model as being 'untreated'. However, one false negative was observed. Using the binary classifier, the spectra from cells treated with tiamulin were not classified correctly as being treated with an antibiotic. This might be due to its low activity against Gram-negative bacteria.

Using the mode of action (MoA) as class label, the model performed equally well, with 95% of the predictions being correct. Similar to the binary classifier, no false positives were observed, but the mass spectra from cells treated with nalidixic acid (a quinolone antibiotic) were incorrectly assigned to belong to the class of protein synthesis inhibitors. Contrary to the binary model, tiamulin is recognized by this model and assigned to the correct mode of action class.

As expected from the internally validated models, the model accuracy drops considerably during external validation when classifying using the antibiotic identity. Although there are no false positives, the number of falsely identified antibiotics rises to 4 and tiamulin is missed. Notably, the two antibiotics (cefuroxime and trimethoprim) that were present in the training set are both identified incorrectly. Spectra from cells treated with cefuroxime, however, are assigned as being treated with penicillin. This is arguably not far off, as cefuroxime is, like penicillin, a β -lactam antibiotic. However cells' spectra treated with trimethoprim are

assigned as being spectra belonging to the class of doxycycline treated cells, which is incorrect.

Model accuracy for model based on mode of action and relative potency as fraction of the MIC (MoAP) is only performing slightly better than the antibiotic identity model with an overall accuracy of 0.80. Again there are no false positives seen among the inactive compounds, but paramomycin is not picked up by the model. All the spectra from cells treated with an antibiotic get assigned a mode of action and a relative potency by the model, but three of them (trimethoprim, nalidixic acid and novobiocin) are incorrect. The antibiotics that are assigned the correct mode of action and a relative potency (azithromycin, ampicillin, cefuroxime, chlortetracycline, tiamulin) are arguably correct comparing the screening dose of 10 μ M to literature MIC values. The EUCAST reports MIC for ampicillin between 2-8 μ g/mL, and in our experiment the screening dose of 3.5 μ g/mL predicted as being 0.250 $\times$ the MIC. This can be assumed correct, as 3.5 μ g/mL is between 0.250-0.500 $\times$ MIC, assuming the 8 μ g/mL is the MIC. The same is true for Azithromycin (EUCAST: MIC 1-8 μ g/mL), where the screening dose of 7.5 μ g/mL was predicted as being equivalent to 1 $\times$ MIC. Cefuroxime (MIC determined at 8 μ g/mL) , which included in the training of the model, was predicted as 0.250 $\times$ CW, although the applied dose at 10 μ M was just above 0.500 $\times$ MIC, at 4.2 μ g/mL. Chlortetracycline and tiamulin are mainly used in veterinary medicine, and MIC data on wild-type *E. coli* is scarce. However, tiamulin has a reported MIC of 12.5 μ g/mL (Ziv, 1980), and the actual dose at 10 μ M (equivalent to 4.9 μ g/mL) is considered correctly predicted at 0.500 $\times$ MIC. In the case of chlortetracycline, the MIC has to be experimentally determined by ourselves before it can be said definitively a correct prediction.

External validation of the models, although with a relatively small set of compounds, show that the models are performing well with real-life data. The fact that we observe similar, and in some cases even better model accuracy using external validation, shows that the models are capable of handling real-life data without losing too much model accuracy.

Table 6 shows Classification results of the Q-SVM model using the set of unknown compounds on *E. coli*. First column displays identity of the compounds (unknown to the operator prior to the assay), with notes an expected prediction result in the second column. Third, fourth, fifth and sixth column show classification result of the binary (Bin), mode of action (MoA), antibiotic identity (ID) and mode of action and relative potency as fraction of the MIC (MoAP) models respectively. The corresponding number of features in the corresponding model is depicted at the top.

Table 6:

Drug name	no. of features Notes; expected prediction for Bin/MoA	7	8	8	10
		Bin	MoA	ID	MoAP
Azithro- mycin	macrolide antibiotic; ANT/PRT	ANT	PRT	CHL	1.000×P RT
Loperamid e	No activity expected; -	-	-	-	-
Paroxetine	No activity expected; -	-	-	-	-
Cefurox- ime	β-lactam antibiotic Antibiotic in training data; ANT/CW	ANT	CW	PEN	0.250×C W
Chlortet- racycline	tetracycline antibiotic; ANT/PRT	ANT	PRT	DOX	0.500×P RT
Thalido- mide	No activity expected; -	-	-	-	-
Fusidic acid	steroid antibiotic protein synthesis inhibitor Only active on Gram positives; -	-	-	-	-
Trime- thoprim	folate metabolism antibiotic Antibiotic in training data; ANT/DNA	ANT	DNA	DOX (wrong)	1.000×P RT (wrong)
Nalidixic acid	quinolone antibiotic Only active on Gram negative; ANT/DNA	ANT	PRT (wrong)	CHL (wrong)	0.031×P RT (wrong)
Metopro- lol	No activity expected; -	-	-	-	
Ampicillin	β-lactam antibiotic; ANT/CW	ANT	CW	PEN	0.250×C W
Sumatrip- tan	No activity expected; -	-	-	-	-
Fen- bendazole	No activity expected; -	-	-	-	-
Paromo- mycin	aminoglycoside antibiotic strong effect on Gram negatives, weak effect on Gram positives; ANT/PRT	ANT	PRT	CFT (wrong)	miss
Novobi-	quinolone antibiotic; ANT/DNA	ANT	DNA	CHL	0.031×P

ocin				(wrong)	RT (wrong)
Brucine	No activity expected; -	-	-	-	-
Umifeno- vir	No activity expected; -	-	-	-	-
Tiamulin	pleuromutilin antibiotic (protein synthesis inhibitor) strong effect on Gram positives, weak effect on Gram negatives; ANT/PRT	miss	PRT	miss	0.500×P RT
Ergota- mine	No activity expected; -	-	-	-	-
Ephedrine	No activity expected; -	-	-	-	-
Correct rate (overall accuracy)		0.95	0.95	0.75	0.80

PhenoMS-ML for Gram-positive bacteria exposed to antibiotic stressors. Exposure to antibiotics and subsequent sample preparation was performed similar to *E. coli*, as described previously.

- 5 For feature evaluation of *S. aureus* spectra, only data of antibiotics in the range from 1×MIC down to 1/8×MIC were considered. Similar to data analysis on *E. coli* mass spectra, a random forest of decision trees was constructed as described previously with the antibiotics' mechanism of action as class labels (DNA, CW, PRT, OTH; untreated cells indicated by UNT). Subsequently, SFS and SBS were performed. A total of seven peaks were selected, observed at
- 10 m/z 5698, 5873, 5932, 6172, 6354, 6978 and 7007. Out of the 7 selected peaks, two peaks (m/z 5873 5932) were putatively identified using TagIdent as being 50S ribosomal protein L32.2 and L32.3. For the other observed masses no hits were found. Similarly, peaks were selected for the binary classification (treated versus untreated, resulting in a total of 10 peaks selected) and antibiotic identity classification (resulting in a total of 5 peaks selected). The
- 15 peaks selected for the four different models are provided in Table 7.

Table 7 shows Features selected for the four different models based on different class labels using the *S. aureus* data set. Bin: binary classification (treated versus untreated); MoA: classification based on mode of action; ID: classification based on antibiotic identity; MoAP: classification based on mode of action and relative potency of the stressors.

Table 9 below shows a confusion matrix of Q-SVM model (10-fold cross validation) performance using 10 features and the binary class labels (spectra from antibiotic treated cells: ANT; spectra from untreated cells: UNT). Data encompasses over 800 mass spectra from the selected antibiotics treated cells, ranging between $1\times\text{MIC}$ and $\frac{1}{8}\times\text{MIC}$. Overall accuracy is 98% using 10-fold cross validation and 98% using hold-out validation. For each predicted class is the following indicated: FNR: false negative rate; FPR; false positive rate; TPR: true positive rate; TNR: true negative rate.

True class	N = 860	ANT	UNT		FNR	FPR	TPR	TNR
	ANT	681	13		0.08	0.01	0.99	0.92
	UNT	8	158		0.01	0.08	0.92	0.99
	Predicted class							

External validation of the model was performed as described for *E. coli*. The results are depicted in Table 10. If all 8 spectra were assigned as being outliers during the spectral processing (based on either their TIC-value or their relative intensity at m/z 12500), it was assumed that the compound in question had killed all the cells, causing the absence of a proper mass spectrum, and therefore would be an antibiotic. In these cases the unknown compound was given the assignment of being an antibiotic, although the model could not make any further predictions as the quality of spectra is deemed insufficient. These results were not included for calculations of the model classification correct rate. For the intended drug-screen application, sample mass spectra that would be rejected based on the TIC or relative intensity at m/z 12500 would be subjected to additional experiments at a lower concentration to assign the mode of action or antibiotic identity. Notably, for the *S. aureus* data, 10 μM of all antibiotics except ampicillin and trimethoprim proved to yield poor quality spectra that could not be considered for further evaluation. The false positive rate of the model was 0, as all of the non-antibiotic drugs were assigned to the class of untreated. Considering the mass spectra that were eligible for classification, the classification performance is good, with 95% classified correctly using both the binary and the MoA-based classifiers. Both these classifiers misclassify paromomycin as being not an antibiotic, but this could be due to its weak activity for Gram positive bacteria. Notably, using the antibiotic identity for classification, trimethoprim is misclassified despite being present in the training dataset. The other antibiotic in the set, ampicillin (a β -lactam) is assigned as being most similar to cefotaxime, another β -lactam, and therefore regarded as a correct classification.

Using the model to predict the mode of action and relative potency as fraction of the MIC, both trimethoprim ($1.000\times\text{DNA}$) and ampicillin ($0.031\times\text{CW}$) are assigned the correct mode

of action and arguably the correct relative potency as fraction of the MIC. At the screened concentration of 10 μM is equivalent to 2.9 $\mu\text{g/mL}$, being approximately $1\times\text{MIC}$ (as literature MIC of trimethoprim is 0.25-2.0 $\mu\text{g/mL}$). *S. aureus* is in general resistant to ampicillin, and the EUCAST does not provide MIC reference breakpoint values, some other publications estimate the MIC for ampicillin to range between 2 $\mu\text{g/mL}$ and 32 $\mu\text{g/mL}$. The screening concentration of 10 μM ampicillin is equivalent to 3.5 $\mu\text{g/mL}$, roughly one-tenth of the MIC (assuming 32 $\mu\text{g/mL}$ as the MIC). The model predicts slight lower at $0.031\times\text{MIC}$, not far off. Paramomycin is missed by this model as well.

It would be most interesting in follow-up work to assay the drugs for which improper quality spectra were obtained at a concentration lower than 10 μM , and attempt to assign the mode of action when spectra of sufficient quality are available. Overall, the quality control of the mass spectral pre-processing and the final external validation of the models show that our developed workflow is more than capable of accurately detecting antibiotics based on the mass spectra of the cells.

Table 10 shows classification results of the Q-SVM model using the set of unknown compounds on *S. aureus*. First column displays identity of the compounds (unknown prior to the assay), with notes an expected prediction result in the second column. Third, fourth, fifth and sixth column show classification result of the binary (Bin), mode of action (MoA), antibiotic identity (ID) and mode of action and relative potency as fraction of the MIC (MoAP) models respectively. The corresponding number of features in the corresponding model is depicted at the top.

Table 10:

Drug name	No. of features	10	7	5	8
	Notes; expected prediction for Bin/MoA	Bin	MoA	ID	MoAP
Azithromycin	macrolide antibiotic; ANT/PRT	*	*	*	*
Loperamide	No activity expected; -	-	-	-	-
Paroxetine	No activity expected; -	-	-	-	-
Cefuroxime	β -lactam antibiotic. Antibiotic in training data; ANT/CW	*	*	*	*
Chlortetracycline	tetracycline antibiotic; ANT/PRT	*	*	*	*
Thalidomide	No activity expected; -	-	-	-	-
Fusidic acid	steroid antibiotic, protein synthesis inhibitor. Only active on Gram positives; ANT/PRT	*	*	*	*
Trimethoprim	folate metabolism antibiotic. Antibiotic in training data; ANT/DNA	ANT	DNA	(miss)	1.000× DNA
Nalidixic acid	quinolone antibiotic. Only active on Gram negatives; -	-	-	-	-
Metoprolol	No activity expected; -	-	-	-	-
Ampicillin	β -lactam antibiotic; ANT/CW	ANT	CW	CFT	0.031× CW
Sumatriptan	No activity expected; -	-	-	-	-
Fenbendazole	No activity expected; -	-	-	-	-
Paromomycin	aminoglycoside antibiotic strong effect on Gram negatives weak effect on Gram positives; ANT/PRT	(miss)	(miss)	(miss)	(miss)
Novobiocin	quinolone antibiotic; ANT/DNA	*	*	*	*
Brucine	No activity expected; -	-	-	-	-
Umifenovir	No activity expected; -	-	-	-	-
Tiamulin	pleuromutilin antibiotic (protein synthesis inhibitor). Strong effect on Gram positives, weak effect on Gram negatives; ANT/PRT	*	*	*	*

Ergotamine	No activity expected; -	-	-	-	-
Ephedrine	No activity expected; -	-	-	-	-
Correct rate (overall accuracy)		0.95	0.95	0.90	0.95

PhenoMS-ML for fungi exposed to antifungal stressors

Cell cultures of *Candida albicans* were exposed to antifungal compounds (listed in Table A. 3), as described below.

Feature selection was performed as described for *E. coli* and *S. aureus*. Data included spectra of cells subjected to 1×MIC, ½×MIC and ¼×MIC. Of each condition there were 8 replicate spectra. As class labels the mode of action of the antifungal was taken (see Table A. 3). A total of five peaks were selected for model building. Using the antifungal identity, a total of 6 peaks were selected. For the binary classifier, a total of 4 peaks were included for modeling, for details see Table 11.

Table 11 below shows features selected for the four different models based on different class labels using the *C. albicans* data set. Bin: binary classification (treated versus untreated); MoA: classification based on mode of action; ID: classification based on antibiotic identity; MoAP: classification based on Mode of action and relative potency as fraction of the MIC.

Model (Nr. Of classes)	Nr. Of features	m/z					
Bin (2)	4	5753	7152	7216	7567		
MoA (5)	5	4552	5784	6399	6907	7127	
ID (6)	6	4290	4484	4552	6399	7127	10388
MoAP							

When classifying with the antibiotic mode of action as class labels, model accuracy was 78% for a quadratic-SVM based model using 10-fold cross validation (76% using hold-out validation) as depicted in Table 12. Using the binary classification model, only the class labels 'treated' and 'untreated', an accuracy of 97% was achieved using 10-fold cross validation (98% using hold-out validation), as depicted in Table 13. Using the classification model based on the antifungal identity an accuracy of 72% was achieved using 10-fold cross validation (74% using hold-out validation), as depicted in Table 13. All classifications were performed using mass spectra of cells subjected to 1×MIC, ½×MIC and ¼×MIC with eight technical replicates per condition.

Table 12. Confusion matrix of Q-SVM model (10-fold cross validation) using 5 features and mode of action of antifungals as class labels. Overall accuracy is 78%. Data encompasses 129 spectra from selected antifungals at 1×, ½× and ¼×MIC. Class labels are as follows: CW: the cell wall synthesis inhibitor caspofungin); DNA: fungal DNA synthesis inhibitor flucytosine;

MEM: membrane integrity disruptor amphotericin B; STB: ergosterol synthesis disruptors (amorolfen and miconazole); UNT: spectra from untreated cells. For each predicted class is the following indicated: FNR: false negative rate; FPR; false positive rate; TPR: true positive rate; TNR: true negative rate

	n = 129	CW	DNA	MEM	STB	UNT		FNR	FPR	TPR
True class	CW	13	1	1	3	2		0.05	0.07	0.93
	DNA	1	15		6	1		0.07	0.12	0.88
	MEM			22	2			0.00	0.04	0.96
	STB	1			43	2		0.06	0.27	0.73
	UNT				7	9		0.04	0.21	0.79
		Predicted class								

5

Table 13. Confusion matrix of Q-SVM model (10-fold cross validation) using 4 features and the presence or absence of antifungal drugs (ANT and UNT respectively). Data encompasses 129 spectra from selected antifungals at $1\times$, $1/2\times$ and $1/4\times$ MIC. Overall accuracy is 94%. For each predicted class is the following indicated: FNR: false negative rate; FPR; false positive rate; TPR: true positive rate; TNR: true negative rate

10

	n = 129	ANT	UNT		FNR	FPR	TPR	TNR
True class	ANT	112	1		0.10	0.06	0.94	0.90
	UNT	7	9		0.06	0.10	0.90	0.94
		Predicted class						

Table 14. Confusion matrix of Q-SVM model (10-fold cross validation) using 6 features and the presence or absence of antifungal drugs at $1\times$, $1/2\times$ and $1/4\times$ MIC. Overall accuracy is 72%. Antifungal identity class labels are given in Table A. 3.

	N = 129	5FC	AMB	AMO	CAS	MZO	UNT		FNR	FPR	TPR	TNR
True class	5FC	13		5		3	2		0.09	0.24	0.76	0.91
	AMB		22			2			0.02	0.00	1.00	0.98
	AMO	3		13		2	4		0.09	0.54	0.46	0.91
	CAS			3	17				0.03	0.00	1.00	0.97
	MZO			3		21			0.03	0.34	0.66	0.97
	UNT	1		4		4	7		0.08	0.46	0.54	0.92
		Predicted class										

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Mass spectra of HeLa cell cultures were recorded between 4 and 15 kDa. Computational analysis of the HeLa MALDI-TOF mass spectra were performed in a similar fashion as described previously for *E. coli* and *S. aureus*. Spectra obtained from HeLa cell cultures treated with stressors at their CC₅₀ concentration (stressors listed in Table A. 4).

5 To evaluate the predictive power of the peaks, a random forest of 1000 decision trees was constructed as described previously for *E. coli* and *S. aureus*. Some of the peaks with the most predictive powers were found in the range of m/z 6000-6100 (see FIG. 5). Using the TagIdent software, we identified the peaks observed at m/z 6057 and 6086 as metallothionein proteins (acetylated metallothionein-1E; P04732 and acetylated metallothionein-2; P02795 respectively). Metallothioneins are cysteine-rich, low-molecular weight proteins
10 known to interact with heavy metals and are transcriptionally regulated by heavy metals and corticoids. This is in agreement with a 4-fold increase in abundance for peak observed at m/z 6081 upon treatment with prednisolone and dexamethasone 21-phosphate. The increase in metallothioneins in HeLa cells upon treatment with dexamethasone had already been described in 1979 by Karin and Herschman (Karin and Herschman 1979). Later it was established that this effect is a more general response towards corticosteroids (Frings, Kind et al. 1989).
15

The combined efforts of the RF, SBF and SFF yielded a total of six peaks for model construction (mode of action as class label). These peaks were observed at the following m/z values:
20 4592, 4966, 6048, 6057, 6085 and 10795.

FIG. 5 shows details of MALDI-TOF spectra near the important peaks at m/z 6048, 6056 and 6085 as identified by the random forest algorithm and sequential feature selection. Average spectra depicted are from at least 18 replicate spectra; see Table A. 4 for stressor abbreviations and CC₅₀ values. Most prominently, there is an increase of the peak intensity at m/z 6085 upon treatment with the corticosteroids (CORT) compared to other classes of stressors and untreated cells' spectra.
25

In a similar manner as for *E. coli* and *S. aureus*, using the mass spectra obtained from the HeLa cells a SVM model was constructed and evaluated using 10-fold cross-validation, which showed 64.2% overall prediction accuracy. The confusion matrix of this classifier is given in
30 Table 15. Spectra were classified according to their mechanism of action (e.g. vinblastine, combretastatin, paclitaxel and colchicine are all classified as tubulin ligands 'TUB', see Table A. 4 in the appendix).

For some classes the prediction accuracy is close to 100%, e.g. for the corticosteroids (CORT; dexamethasone and prednisolone), tretinoin and the group of tubulin ligands (TUB; paclitaxel, combretastatin, colchicine and vinblastine), all of which act on targets that are omnipresent in mammalian cells. These types of stressors appear to have a specific and profound effect
35

on the mass spectra of the cell cultures (see FIG. 5 as an example for the case of the corticosteroids). On the other hand the drugs L-thyroxine (thyroid hormone; TH), loperamide (opioid receptor agonist; OPID), pravastatin (statin; STAT), tamoxifen (selective estrogen receptor modulator; SERM) and ergotamine (neurotransmitter agonist, NTA) were mainly confused with each other (see Table 15).

The response signatures of the HeLa cells to these non-specific stressors is still distinct to untreated HeLa cells which can be explained by the fact that they are stressed at their CC_{50} concentrations. The results also indicate that the specific HeLa stressors, especially corticosteroids and tubulin polymerization inhibitors, cause a distinct, specific type of stress if applied at CC_{50} concentrations. Other assayed drugs, particularly L-thyroxine, loperamide, tamoxifen, pravastatin and ergotamine show fewer distinct alterations in their respective mass spectra, and therefore a correct classification is limited for those non-specific stressors. They do however still yield spectra that can be distinguished from untreated HeLa cells, thus providing an entry point for subsequent, alternative methods to further elucidate their mechanism of action.

The reason that, especially in this case, the drugs loperamide and pravastatin do not yield a single distinct reaction might be that the expression levels of their target or receptor proteins and mechanisms in HeLa is lower than in other tissues. For example, loperamide is a ligand of opioid receptors which are predominantly expressed in intestine and brain tissue. Therefore a specific effect of loperamide or other opioids on cervix HeLa cells appears *a priori* less likely.

Table 15. Confusion matrix of the 10-fold cross validation of quadratic SVM model. For construction of this model, 6 peaks per mass spectra were used, based on the evaluation of the feature importance as determined by forest of random decision trees. In total, the dataset contains 241 mass spectra, with at least 18 biological replicates per class. Abbreviations: TUB - tubulin (de)polymerization inhibitor; CORT - corticosteroid; TH - thyroid hormone; SERM - selective estrogen receptor modulator; IMM - immunosuppressant; STAT - statins; TRE - tretinoin (retinoic acid receptor ligand); OPID - opioid receptor agonist; NTA - neurotransmitter receptor ligand. For each predicted class is the following indicated: FNR: false negative rate; FPR; false positive rate; TPR: true positive rate; TNR: true negative rate.

Table 15:

	n = 241	COR T	IM M	NT A	OPI D	SER M	STA T	T H	TR E	TU B	UN T	FN R	FP R	TP R	TN R
Predicted class	CORT	34									1	0.0 0	0.0 0	1.0 0	1.0 0
	IMM		13	3		1				1		0.0 2	0.1 9	0.8 1	0.9 8
	NTA		2	7	1	3	1				3	0.0 4	0.5 3	0.4 7	0.9 6
	OPID			1	2	7	1	2			5	0.0 7	0.8 9	0.1 1	0.9 3
	SERM			1	5	8		2			2	0.0 5	0.7 2	0.2 8	0.9 5
	STAT			1	1	2	8	5				0.0 4	0.5 0	0.5 0	0.9 6
	TH				5	5	1	7				0.0 5	0.6 1	0.3 9	0.9 5
	TRE							1	9		6	0.0 3	0.0 0	1.0 0	0.9 7
	TUB		1				4			57	1	0.0 3	0.0 2	0.9 8	0.9 7
	UNT			2	5	3	1	1			9	0.0 6	0.6 7	0.3 3	0.9 4
	True class														

The PhenoMS-ML method combines mass spectrometry with advanced data processing and analysis, which allows the classification of phenotypic responses of organisms towards stressors, in particular substances with a pharmacological or toxicological effect. In the examples of *E. coli* and *S. aureus* bacteria stressed by antibiotics, classification of the phenotypic response was successful for 18 antibiotics with multiple mechanisms of actions. The effects of the antibiotics were detectable at sub-MIC levels as low as 1/8 of the MIC, which is far beyond the capabilities of other methods.

Additional advantages of PhenoMS-ML are that it is label-free, involves relatively short incubation times and minor sample workup, making it amenable for high-throughput screening. In addition, the method can be performed with wild-type micro-organisms without introduction of reporter genes and does not require special (e.g., radioactive) reagents. The applicability of the method has also been illustrated for the response of mammalian cells and fungi to a variety of stressors at sub-lethal concentrations. The applicability of PhenoMS-ML can be

expected for the study of a multitude of cells that originate from other organisms and tissues, which will consequently show a phenotypic sensitivity for other classes of pharmacological agents, and could be extended for the study of, for example, phytopathogens.

5 The model building procedure can be carried out with minimal intervention of the user, eliminating cognitive bias and allowing an automated, daily training of the system. The latter may be superficially seen as disadvantage in comparison to other mass-spectrometry based classification methods that integrate a hard-coded model or databases, which is the case for established bacterial identification procedures. However, intra-day and site-specific model building has the significant advantage that the state of the biological system and its momentary
10 response to the stressor, which depends on many (partially uncontrollable) factors, is integrated into the model. Therefore, a classification model for a biological system that is derived from intra-day on-site training data has a higher validity and sensitivity than a fixed model which needs to account for a large variety of cellular states or other experimental influences. PhenoMS-ML can be performed in a self-contained, autonomous and operator-independent
15 mode, thereby increasing robustness and transferability between laboratories.

Many important drug targets, particularly in the area of antibacterial drug discovery, are difficult to study in biochemical assays. Examples include the bacterial ribosome, gyrases or penicillin-binding proteins. The PhenoMS-ML method provides swift access to a specific readout for these targets under highly relevant biological conditions, i.e., in a system where
20 these targets are located in their native biophysical and pharmacokinetic (micro)environment.

The PhenoMS-ML method is currently performed in 96 and 384-well format, depending on the type of cell culture. Given the current capabilities of laboratory technology, which also includes automatic processing of microplates and MALDI target plates, it will be straightforward to increase sample throughput and further reduce usage of materials, increase throughput, minimize human workload and interaction, and to adapt the assay to smaller formats.
25 This will further open the way toward employing the method in a high-throughput screening setting.

Table A. 1. Reference MIC (mg/L) values for several *E. coli* strains. NA = not assessed. From MIC tables reported by (Stock and Wiedemann 1999), (Andrews 2001) and (EUCAST 2016) the MIC which was reported the most frequent was taken as reference.

			(Stock and Wiedemann 1999)	(Andrews 2001)	(Andrews 2001)	(EUCAST 2016)	Present method
Strain			Multiple strains	NCTC 10418	ATCC 25922	ATCC 25922	ATCC 25922
Antibiotic	ID Class	MoA Class	MIC (mg/L)	MIC (mg/L)	MIC (mg/L)	MIC (mg/L)	MIC (mg/L)
Amoxicillin	AMX	CW	4	2	4	4	8
Benzylpenicillin	PEN	CW	16	NA	NA	NA	32
Cefotaxime	CFT	CW	0.031	0.031	0.063	0.063	0.031
Cefuroxime	CFX	CW	NA	2	4	4	8
Chloramphenicol	CHL	PROT	4	2	4	8	8
Ciprofloxacin	CIP	DNA	0.031	0.016	0.016	0.016	0.004
Clarithromycin	CLR	PROT	32	NA	NA	NA	16
Doxycycline	DOX	PROT	2	NA	NA	2	2
Erythromycin	ERY	PROT	64	NA	NA	NA	32
Gentamicin	GNT	PROT	0.5	0.25	0.5	0.5	1
Moxifloxacin	MOX	DNA	NA	0.031	0.031	0.063	0.063
Neomycin	NEO	PROT	1	NA	NA	1	2
Nitrofurantoin	NIT	OTH	NA	NA	NA	NA	16
Rifampicin	RIF	OTH	8	16	NA	NA	16
Tetracycline	TET	PROT	2	1	2	2	1
Trimethoprim	TRM	DNA	0.3	0.12	0.25	0.5	2
Vancomycin	VAN	CW	256	NA	NA	NA	128

Table A. 2. Reference MIC values and MIC values found and used in this work for *S. aureus*.

Antibiotic	ID	MoA	(EUCAST 2016)	Presented method
	Class	Class	MIC (mg/L)	MIC (mg/L)
Amoxicillin	AMX	CW	NA	4
Benzylpenicillin	PEN	CW	0.125	0.5
Cefotaxime	CFT	CW	NA	0.5
Cefuroxime	CFX	CW	NA	2
Chloramphenicol	CHL	PROT	8	8
Ciprofloxacin	CIP	DNA	1	0.125
Doxycycline	DOX	PROT	1	0.5
Erythromycin	ERT	PROT	1	0.25
Gentamicin	GNT	PROT	1	0.25
Moxifloxacin	MOX	DNA	NA	0.25
Neomycin	NEO	PROT	NA	0.25
Rifampicin	RIF	OTH	0.0625	0.004
Tetracycline	TET	PROT	1	0.25
Trimethoprim	TRM	DNA	2	0.25
Vancomycin	VAN	CW	2	1
Nitrofurantoin	NIT	OTH	64	16

Table A. 3 List of antifungal compounds used in model building, with MIC values from literature and from this work against *C. albicans* ATCC 90028. (NA = not assigned in either CLSI or EUCAST reference literature).

Name	Abbr.	Target	Class abbreviation	MIC ($\mu\text{g/mL}$)	Reference MIC ($\mu\text{g/mL}$)
Miconazole	MZO	Lanosterol 14α -demethylase Inhibitor	STB (Sterol biosynthesis)	32	NA
Amorolfine	AMO	inhibiting $\Delta 14$ -sterol reductase and cholesterol Δ -isomerase	STB (Sterol biosynthesis)	0.008	NA
Amphotericin B	AMB	Binds ergosterol, thereby disrupting membrane integrity.	MEM (Membrane disruptor)	0.5	0.5-2.0 (CLSI) 1 (EUCAST)
Caspofungin	CAS	Inhibits $\beta(1,3)$ -D-glucan-synthase, involved in cell wall synthesis	CW (Cell wall synthesis)	0.125	NA
Flucytosine	5FC	Inhibits RNA and DNA synthesis	DNA (deoxyribonucleic acid synthesis)	0.5	0.5-2.0 (CLSI)

Table A. 4. List of compounds and their respective CC₅₀ values used to treat HeLa cells prior to MALDI-TOF MS. For compounds where no CC₅₀ could not be determined (N.A = not applicable), cells were screened at a fixed dose of 20 µM of the corresponding stressor. The CC₅₀ readout was determined after 24 h incubation of the compounds with the cells.

Name	Abbr.	Stressor class/mode of action	Class	CC ₅₀ (mM) (present method)
Combretastatin	CBT	Tubulin polymerization inhibitor	TUB	0.234
Paclitaxel	PTX	Tubulin depolymerization inhibitor	TUB	0.062
Vinblastine	VIN	Tubulin polymerization inhibitor	TUB	0.066
Colchicine	CLC	Tubulin polymerization inhibitor	TUB	0.381
Dexamethasone-21-phosphate	DEX	Glucocorticoid	CORT	0.150
Prednisolone	PDS	Glucocorticoid	CORT	0.240
L-Tyroxine	LTX	Thyroid hormone T4 substitute	TH	0.127
Tamoxifen	TMX	Selective estrogen receptor modulator (prodrug)	SERM	0.020
Ciclosporine	CSP	Immunosuppressant, cyclophilin binder	IMM	0.021
Tretinoin	TRE	Retinoic acid receptor and Pin1 ligand	TRE	0.127
Pravastatin	PRV	Statin (HMG-CoA reductase inhibitor)	STAT	0.425
Loperamide	LPD	Opioid-receptor agonist	OPID	0.026
Ergotamine	ERG	neurotransmitter receptor ligand	NTA	0.055

CLAIMS

1. A method for analyzing an action of a stressor on a cell sample, the method comprising the following steps:
 - a) exposing the cell sample to the stressor,
 - 5 b) acquiring a sample mass spectrometry profile of the cell sample,
 - c) determining one or more values of one or more features of the sample mass spectrometry profile, and
 - d) classifying the stressor using a model-based comparison of the one or more values of the features of the sample mass spectrometry profile with one or
10 more reference stressors.
2. The method of claim 1, wherein the model is determined by:
 - e) exposing one or more reference cell samples to one or more reference stressors,
 - 15 f) acquiring one or more reference mass spectrometry profiles of the reference cell samples and a non-exposed cell sample which is not exposed to a reference stressor, and
 - g) selecting features on said reference mass spectrometry profiles, and
 - h) using of said selected features to train the model.
 - 20
3. The method of one of the previous claims, wherein exposing the test cell sample to the stressor and exposing the one or more reference cell samples is performed in parallel using the same batch of cells on the same day, preferably on the same multiwell plate.
- 25 4. The method of one of the previous claims, wherein the test cell sample, the one or more reference cell samples, and the non-exposed cell sample are from the same batch of cells.
5. The method of one of the previous claims, further comprising selecting the features by sequential feature selection and feature importance evaluation using decision trees.
30
6. The method of one of the previous claims, wherein the sample mass spectrometry profiles are acquired using matrix-assisted laser desorption/ionization time-of-flight, MALDI-TOF.
35
7. The method of one of the previous claims, wherein the cell sample comprises bacteria, the stressor is an antibiotic and a concentration of the antibiotic is equal or lower than

the minimal inhibitory concentration of the antibiotic for the cell sample, in particular lower than 50%, preferably lower than 25%, of the minimal inhibitory concentration of the antibiotic for the cell sample.

- 5 8. The method of one of the previous claims, wherein the classification is with respect to
 (i) a presence of the stressor,
 (ii) a type of the stressor, in particular a type of antibiotic,
 (iii) a mechanism of the stressor, and/or
 (iv) a potency of the stressor .
- 10
9. The method of one of the previous claims, wherein
- if the type of the cell sample is *Escherichia coli* , the features comprise one or more of the following m/z peaks: 5098; 5411; 6504; 9066,
 - if the type of the cell sample is *Staphylococcus aureus*, the features comprise
 - 15 one or more of the following m/z values: 5698; 5873; 5932; 6978; 7007,
 - if the type of the cell sample is human, in particular *HeLa*, the features comprise at least one of the following m/z peaks 4966 and 6085,
- wherein a deviation of +/- 2 is considered as belonging to the same peak.
- 20 10. The method of one of the previous claims, wherein
- if the type of the cell sample is *Escherichia coli*, the features comprise at least three, in particular at least five, of the following m/z peaks: 5098; 5326; 5869; 6277; 7117; 7159; 8601; 8874; 9631; 9726;
 - if the type of the cell sample is *Staphylococcus aureus*, the features comprise
 - 25 at least three, in particular at least five, of the following m/z peaks: 4074; 4668; 5331; 5932; 6889; 7006; 8151; and/or
 - if the type of the cell sample is human, in particular *HeLa*, the features comprise at least three, in particular at least five, of the following m/z peaks: 4210; 4593; 4939; 4965; 6012; 6048; 6056; 6085; 6111; 6650; 7262; 8409,
- 30 wherein a deviation of +/- 2 is considered as belonging to the same peak.
11. A device for analyzing an action of a stressor on a cell sample, the device comprising:
- an input unit for obtaining a sample mass spectrometry profile of the cell sample,
 - 35 - a determining unit for determining one or more values of one or more features of the sample mass spectrometry profile, wherein the selection of the one or more features is based on a type of the cell sample, and

- a classification unit for classifying the stressor using a model-based comparison of the values of the features of the mass spectrometry profile with one or more reference stressors.

- 5 12. The device of claim 11, further comprising a database storing a plurality of cell types and for each of the plurality of cell types one or more features.
13. A computer-readable storage medium storing program code, the program code comprising instructions that when executed by a processor carry out the method of one of
- 10 claims 1 to 11.

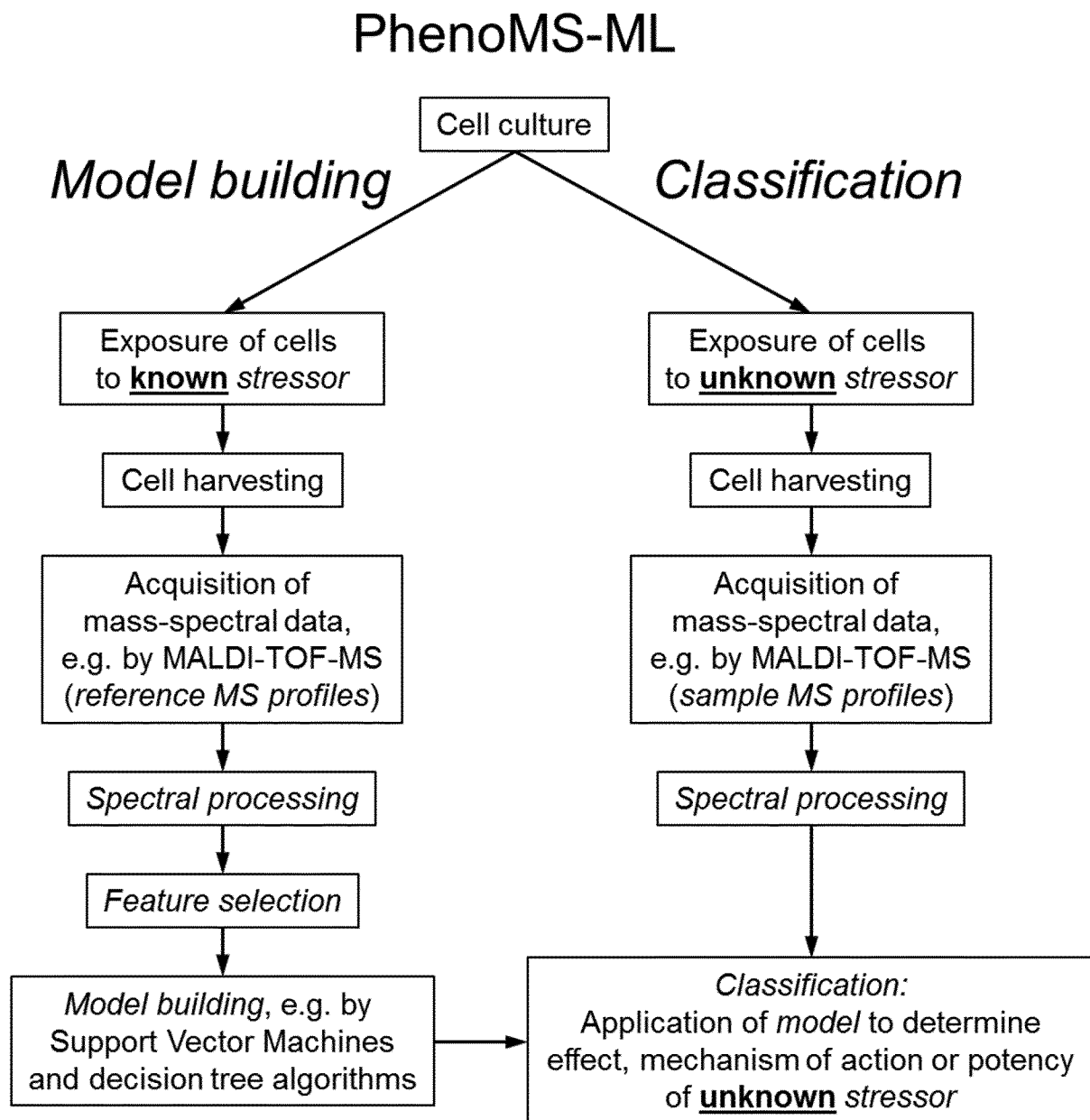


FIG. 1

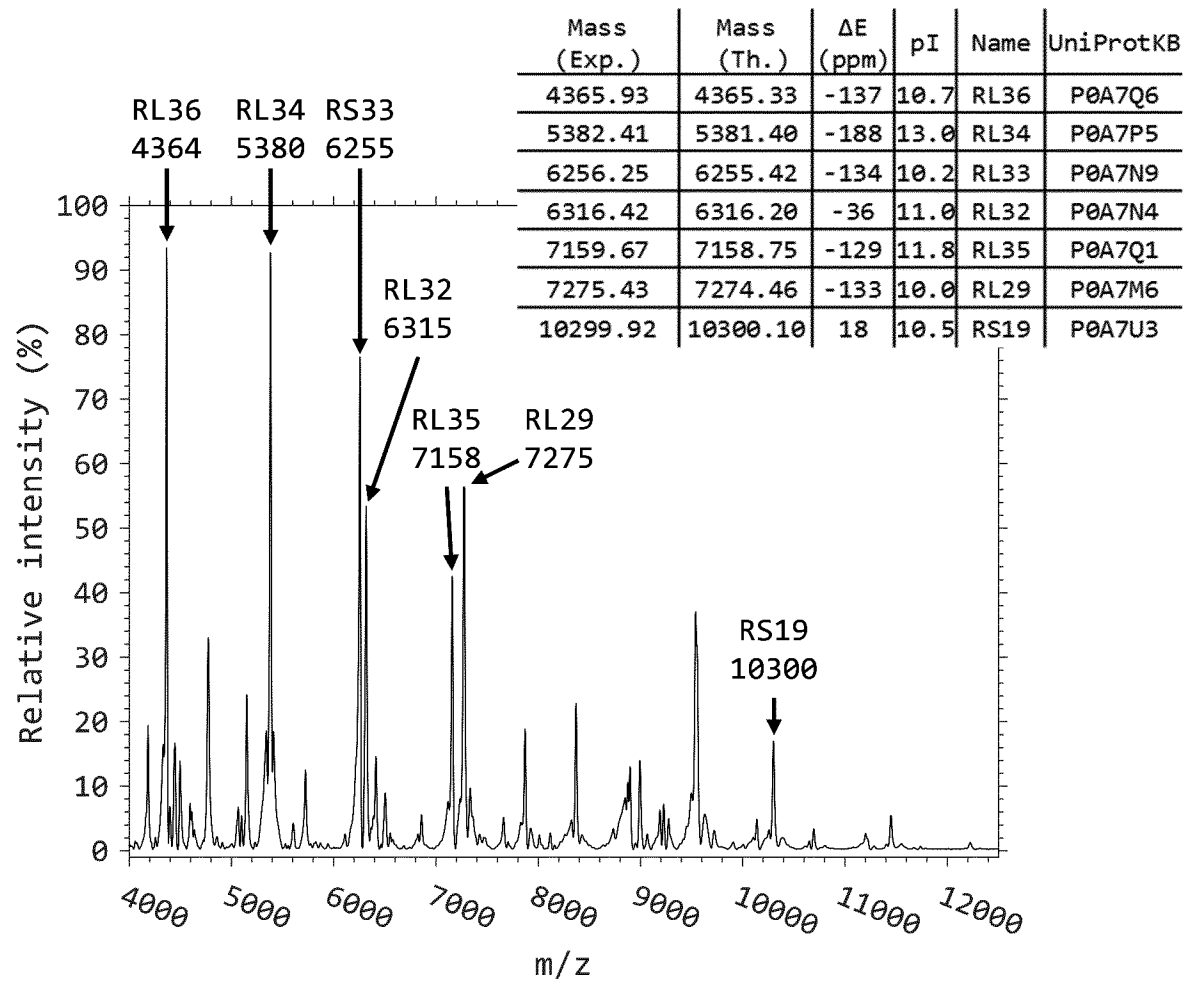


FIG. 2

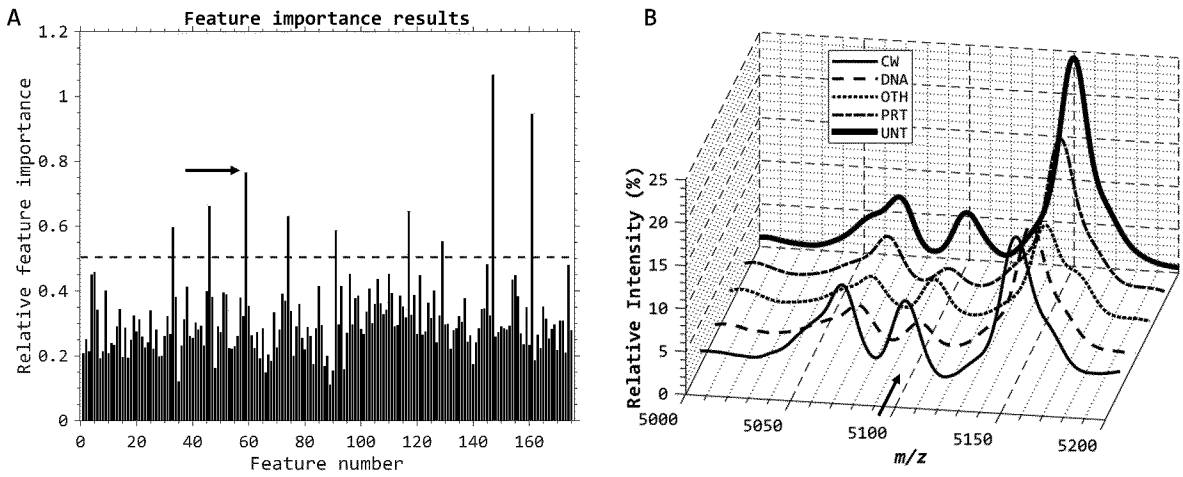


FIG. 3

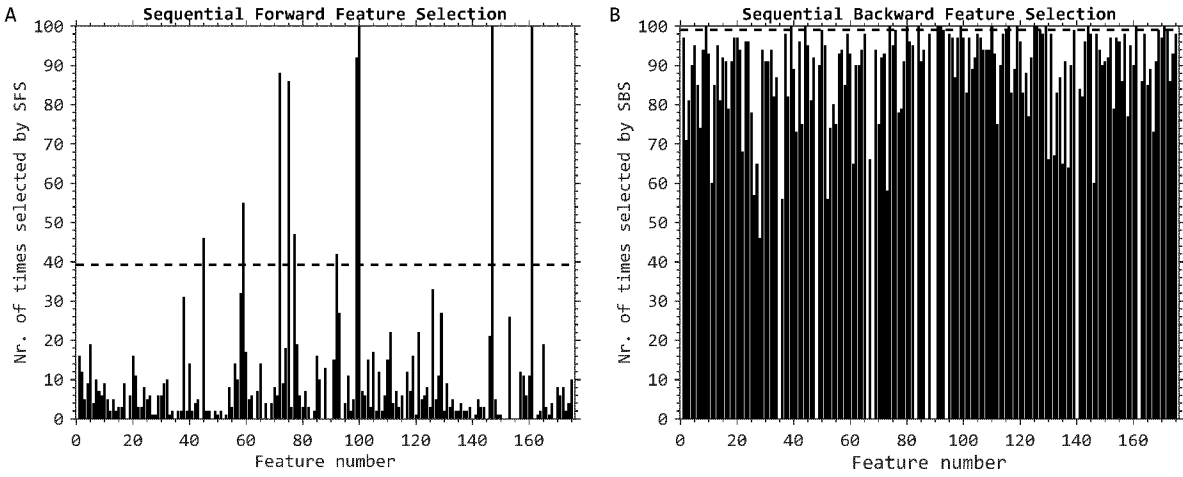


FIG. 4

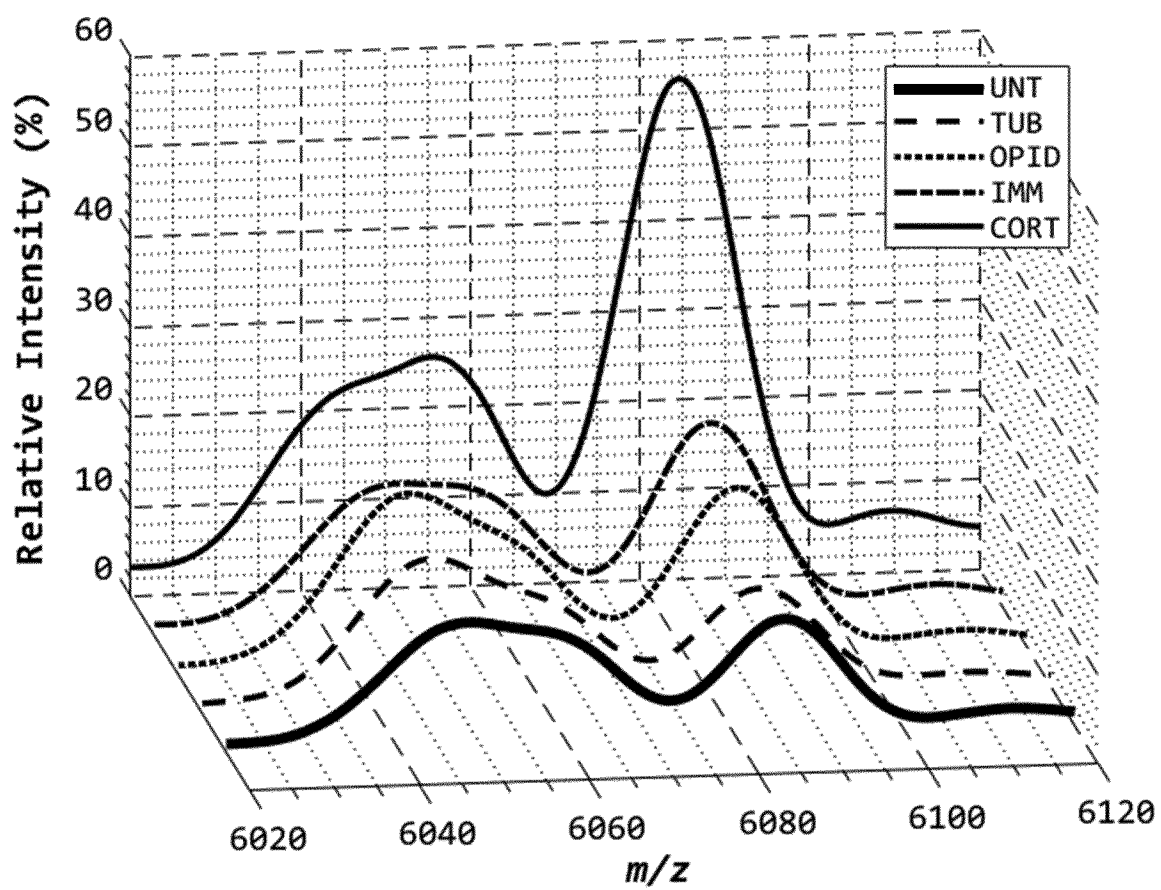


FIG. 5

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2018/079221

A. CLASSIFICATION OF SUBJECT MATTER

INV. C12Q1/18 G01N33/68 G16C20/70 G01N33/50
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C12Q G06F G01N G16C

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, BIOSIS, COMPENDEX, EMBASE, FSTA, INSPEC, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents :

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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

11 July 2019

Date of mailing of the international search report

29/07/2019

Name and mailing address of the ISA/

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INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2018/079221

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 1 650 568 A1 (ESPERION THERAPEUTICS INC [US]) 26 April 2006 (2006-04-26) paragraphs [0001], [0021] - [0026], [0030], [0033], [0037] - [0039], [0043], [0056], [0068] - [0070], [0074], [0075], [0078] paragraphs [0080], [0085], [0095], [0096], [0098], [0108], [0117], [0124] - [0125], [0132] - [0137] paragraphs [0155] - [0156], [0169] - [0171], [0175], [0185] - [0189], [0200], [0206], [0250] - [0256] -----	1,2,8, 11-13
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International application No

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