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RESULTS ON TAXONOMY AND PHYSIOLOGICAL STATE OF BACTERIA DERIVED FROM LASER-INDUCED SINGLE CELL ANALYSIS

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Resume - Dans cette publication on décrit l'application de la microanalyse par sonde laser et spectrographie de masse à l'étude de cellules bactériennes isolées. Cette méthode donne des informations précieuses aussi bien sur leur physiologie que sur leur classement taxonomique.

Abstract - In this paper the application of laser microprobe mass analysis to single bacterial cells is described. It is shown that this methodology yields information on the physiological state of a bacterium as well as on its taxonomical position.

Introduction: The laser microprobe mass analysis as supplied with the LAMMA-500 instrument makes possible the mass spectrometric analysis of single bacterial cells because of its high lateral resolution together with its low detection limits for atomic as well as molecular ions. The intracellular contents of certain cations correlates with the physiological state of the individual cell /1/. The measurement of these quantities from a larger number of single cells allows the calculation of their distributions within a population. From the character of these histograms more detailed information on the physiological state and its alterations by environmental influences (e.g. chemotherapeutics) may be obtained than from the inevitably averaged data from the established microbiological gross methods.

Informations from the molecular (fragment) ions can at present be drawn only via mass fingerprints because - due to yet unknown fragmentation processes involved in the laser ionization of a complex biological matrix - it is not possible to reliably identify single molecular peaks. The fingerprints comprise a large number of mass peaks with often minute differences between different strains - even of the same species - making visible inspection of the spectra or calculation 'by hand' - although possible /2/ - rather inadequate. However, extensive statistical procedures allow the differentiation between e.g. drug sensitive and the respective resistant strain from a limited number of single cell mass fingerprints.

Materials and Methods: Briefly, in the LAMMA instrument a high-energy UV-laser pulse (pulse duration 15ns) is focused through the objective of a light microscope onto the sample to be analyzed and evaporates and partly ionizes it at a lateral resolution down to approximately 1 μm, dependent on sample conditions and on the laser energy chosen. The produced atomic and molecular (fragment) ions are registered by means of a time-of-flight mass spectrometer at a mass resolution of about 300 (for the lead isotopes).

For single cell analysis the bacteria (E.coli and various mycobacterium strains, see below) were treated as follows: the cells were harvested from the appropriate growth medium and washed twice quickly - to avoid ion dislocation - in destilled water and brought onto Formvar coated Cu-grids and excess fluid was drained off with tissue. This way a wide spread distribution of the bacteria was achieved allowing the laser evaporation of one single cell at a time.

The influence of a drug (nitrofuran derivative HN32) on the Na,K-ratio was investigated with E.coli grown in modified Anton's medium /1/. For this, the drug was added at minimum inhibitory concentration (20 μmol/l) at the beginning of the exponential growth. Untreated cultures were kept as controls. At different times during growth...
bacterial samples were prepared and up to 200 cells were analyzed from each sample and the distribution of the $^{23}\text{Na},^{39}\text{K}$-ratio was determined. (It should be mentioned that this ratio must not necessarily reveal the true intracellular ratio because of different ionization efficiencies for the two cations.) For the differentiation between various species of the same genus or between drug-resistant strains via mass fingerprints an extensive computerized evaluation procedure was established.

The data processing involves the following steps:

1. Measurement and data preparation for a multivariate analysis
   - registration of 90 spectra of each strain
   - conversion to line-spectra and normalization with respect to total intensity to correct for variations in sample size and laser intensity
   - data reduction for each strain to 3 spectra, each of which representing the average over 30 single spectra

2. Checking for statistical significant differences (this analysis follows a procedure described in /3/)
   - calculation of weight factors for each mass, these so-called characteristics are determined for each mass by comparing the deviation in the peak intensities within spectra of the same strain (intra-strain deviation) with that derived from the different strains (inter-strain deviation). The characteristic resembles a signal to noise ratio.

3. Calculation of mathematical expressions for dissimilarities between two spectra
   - each spectrum - reduced to the n masses with the highest characteristics - is interpreted as a point in a n-dim. room
   - the Euclidean interpoint distance, weighted by the characteristics, can be taken as a numerical measure of the dissimilarity between two spectra
   - computation of a dissimilarity matrix comprising the dissimilarities between all spectra (when having K spectra the matrix consists of $K(K-1)/2$ elements)

4. Evaluation of the dissimilarity matrix
   - calculation of the nearest neighbours of each spectrum
   - graphical representation in a 2- or 3-dim. plot to show the information stored in the matrix in a more convenient form. The procedure is based on a nonlinear mapping algorithm given in /4/.

Results and discussion: In fig.1 the growth kinetics of the untreated control and of the HN32-treated sample are given. Fig.2 shows the corresponding distributions of the $^{23}\text{Na},^{39}\text{K}$-ratio of various samples taken at the times marked in the growth kinetics. Clearly, the distributions 1-3 show increasing broadening and a shift of the median to higher values of the Na,K-ratio due to a longer action of the chemotherapeutic. Distribution 4, however, implies - much more pronounced than the growth kinetics - a recovery of the cells. Furthermore, from the changes in these histograms the beginning of the recovery becomes evident earlier. The shapes of the distributions imply also that an averaged value for the Na,K-ratio over all cells as it is obtained from the gross methods can give only incomplete information.

In fig.3 the 2-dim. nonlinear map of the dissimilarity matrix representing the relationship between 21 mycobacteria spectra is shown. Points (spectra) originating from the same strain are interconnected. Strains belonging to different species are well separated except those of M.kansasi and M.gastri, which are known to be closely related in biochemical terms.
Fig. 1: Growth kinetics of E. Coli cells treated with the drug HN32 (a nitrofuran derivative) at minimal inhibitory concentration and of the respective untreated control.

Fig. 2: Distributions of the $^{23}\text{Na}, ^{39}\text{K}$-ratio within bacterial cell populations treated with HN32 at minimal inhibitory concentration. The histograms correspond to the various sampling times marked in fig. 1.
Fig. 3: Nonlinear, 2-dim. map of 21 *Mycobacterium* spectra of various species.

Fig. 4: Nonlinear, 2-dim. map, showing the numerical correlation between *M. tuberculosis* bacteria resistant to different drugs.

The result of the numerical relationship of 4 *M. tuberculosis* strains resistant to different chemotherapeutics and the respective sensitive strain (control) is given in fig. 4. The bacteria made resistant to kanamycin and to viomycin can not be differentiated clearly (possibly due to a cross resistance of the bacteria).

These first results demonstrate that the computerized fingerprint evaluation of the laser desorption mass spectra of single cells may offer useful information for microbiological research.

/4/ J.K. Kruskal, Psychometrika 29, 1 (1964)