Supporting Information

Raman spectroscopic characterization of packaged *L. pneumophila* strains expelled by *T. thermophila*

Dragana Kusić,[1] Anuradha Ramoji,[2,3] Ute Neugebauer,[2,3,4] Petra Rösch,[1*]

and Jürgen Popp[1,2,3,4]

[1]Institut für Physikalische Chemie and Abbe Center of Photonics, Friedrich-Schiller-Universität Jena, Helmholtzweg 4, D-07743 Jena, Germany
[2]Leibniz Institute of Photonic Technology (IPHT), Albert-Einstein-Straße 9, D-07745 Jena, Germany
[3]Center for Sepsis Control and Care (CSCC), Jena University Hospital, Erlanger Allee 101, D-07747 Jena, Germany
[4]InfectoGnostics Forschungscampus Jena e.V., Zentrum für Angewandte Forschung, Philosophenweg 7, D-07743 Jena, Germany

E-mail: petra.roesch@uni-jena.de

EXPERIMENTAL SECTION

Tetrahymena thermophila and *Legionella pneumophila*

The *Legionella pneumophila* strains Philadelphia-1 DSM 7513, Los Angeles-1 DSM 7514 and U8W DSM 7515 were purchased from the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig), while *Tetrahymena thermophila* CCAP 1630/1M was obtained from the Culture Collection of Algae and Protozoa (CCAP), Scottish Marine Institute, Oban.

Sample preparation

The *Legionella* strains were maintained on Buffered Charcoal Yeast Extract (BCYE) agar (Sigma-Aldrich, 86558-500G-F) containing *Legionella* Growth Supplement (Sigma-Aldrich 42981-1KT) for three days at 37 ºC in a humid atmosphere (≈85% humidity) with 5% CO_2. A few grown colonies were transferred into 25 ml of Yeast extract (10 g/L) with *Legionella* Growth Supplement (Sigma-Aldrich 42981-1KT) and incubated at 37 °C for 24 h. Afterwards, bacteria were washed by centrifugation for 3 minutes at 5000 rpm and finally suspended in 1 ml of sterile-filtered tap water (Pall Life Sciences, Acrodisc PF syringe filters with 0.2 µm Supor membrane), before being used to feed *T. thermophila*. The final bacterial concentration was ~10^8 CFU/ml.

1 ml of a stock culture of *T. thermophila* was transferred into 20 ml of Proteose pepton Yeast Extract Medium (PPY) and incubated at 26 °C for four days. Afterward, the cells were concentrated by two times washing via centrifugation for 5 minutes at 10,000 rpm and suspended in 1 ml of filter-sterilized tap water so that the concentration of *T. thermophila* was ~10^5/ml. Twenty-four h after adding bacteria to the protozoa and incubating them at 30 °C, expelled pellets containing *L. pneumophila* strains were investigated via both confocal laser scanning microscopy and Raman microspectroscopy. For the control sample, washed and concentrated *T. thermophila* were incubated in tap water for 24 h at 30 °C in the absence of bacterial cells. To account for the effects of biological variability, all the experiments were performed in triplicate.

Experiments with immuno-labeled bacteria

L. pneumophila Philadelphia-1 cells were immuno-labeled with fluorescent polyclonal antibodies (Anti-*Legionella pneumophila* LPS-FITC Antibody; GenWay, 18-251-404448, concentration: 4.0 mg/ml). The bacteria were prepared as previously described and incubated with 50 µl of anti-*L. pneumophila* LPS-FITC antibodies for 30 minutes at room temperature in the dark. The immuno-labeled bacteria were then washed, suspended in 1 ml of filter sterile tap water and used to feed *T. thermophila* cells at 30 °C protecting the sample from light. Accumulations of *T. thermophila* food vacuoles filled with immuno-labeled Philadelphia-1cells were investigated after 3 h of incubation, while pellets of the same strain expelled by *T. thermophila* were examined 24 h after incubation via both confocal laser scanning microscopy (CLSM) and the fluorescence mode of the Raman microscope.

Staining with Propidium Iodide and Live/Dead staining

Pellets containing Philadelphia-1 cells labeled with FITC conjugated antibodies obtained after 24 h of incubation in sterile-filtered tap water were stained with Propidium Iodide (PI) (LIVE/DEAD BacLight bacterial viability kit, Invitrogen, L13152) at room temperature in the dark for 15 minutes. Afterwards, the sample was filtered through a filter with 5 µm pore size (Supor membrane, Pall) suitable to retain a sufficient number of pellets for the CLSM analysis. Pellets were then washed three times by filtering 2 ml of sterile distilled water through the filter. An inverse filtration step with 0.5 ml of sterile distilled water was used to aspirate the retained pellets on the filter. PI is a red fluorescence dye that binds to double stranded DNA of a membrane damaged bacterium and is used here to stain nonviable Philadelphia-1 cells within pellets.

In addition, pellets containing Los Angeles-1 cells were stained with Live/Dead BacLight bacterial viability kit (Invitrogen, L13152) 24 h after incubation in sterile-filtered tap water at room temperature in the dark for 15 minutes, washed through a filter with 5 µm pore size as explained, and then visualized via confocal laser scanning microscopy.

Raman spectroscopic analysis

The Raman spectra of the pellets expelled by *T. thermophila* as well as the Raman spectra of single cells of three *L. pneumophila* strains were obtained via a Raman microscope (Bio Particle Explorer (BPE); Rap.ID Particle Systems GmbH, Berlin, Germany) equipped with an Olympus MPLFLN-BD 100 X microscope objective. In the fluorescence mode of the BPE, the fluorescence emission of fluorescein isothiocyanate (FITC) labeled antibodies used to stain Philadelphia-1 cells was obtained with an excitation wavelength of 470 nm from a LED, and visualized using a single bandpass filter (Semrock, FF01-530/43-25). A solid-state frequency-doubled Nd:YAG laser source with 532 nm excitation wavelength was used to obtain the Raman scattering. The laser light was focused onto the sample with a spot size <1 µm laterally. The laser power used was ~7 mW at the sample. The scattered light was diffracted via a single-stage monochromator (HE 532; Horiba JobinYvon) with a grating of 920 lines mm^{-1} and collected with a thermoelectrically cooled CCD camera (DV401 BV; Andor Technology). The integration time per Raman spectrum was 20 s. To bleach the cytochrome, 20 s preburning

was performed before the Raman spectra of pellets were obtained.[1] The spectral resolution was approximately ~8 cm^{-1}. Depending on the size of the pellets, 3 to 5 Raman spectra were obtained for each pellet from different positions, while a new bacterial cell was selected for each single-cell Raman spectrum.

Confocal laser scanning microscopic analysis

Confocal microscope (LSM 780, Carl Zeiss) equipped with a 63x oil objective (Plan-Apochromat 63x/1.40 Oil DIC M27, Carl Zeiss) as well as Diode 405-30 (405 nm), Argon (458, 488 and 514 nm), DPSS 561-10 (561 nm) and HeNe633 (633 nm) lasers was used. A 488 nm argon laser line was used for the excitation of fluorescein isothiocyanate (FITC) labeled antibodies used to stain Philadelphia-1 cells. The emission was detected using a 500-600 nm band pass filter. Furthermore, the excitation of Propidium Iodide used to stain membrane damaged cells was delivered by a 561 nm laser line (DPSS 561 10), while the emission was detected using a 599-718 nm band pass filter.

For the visualization of Live/Dead stained pellets containing the Los Angeles-1 cells, both lasers DPSS 561-10 (561 nm) and Argon (488 nm) were used. The excitation of Propidium Iodide as well as the emission detection was performed as explained, while the excitation of SYTO 9 was provided by a 488 nm argon laser line and the emission selected using a 500-600 nm band pass filter.

Statistical analysis

All collected Raman spectra were preprocessed as previously described.[2] Briefly, the background of the Raman spectra and cosmic spikes were eliminated. Then, the Raman spectra were wavenumber calibrated via 4-acetamidophenol. Afterwards, the Raman spectra (70–3319 cm^{-1}) were cut from 460 to 1800 cm^{-1} and from 2650 to 3150 cm^{-1}, and vector normalized. Preprocessed Raman spectra were used to calculate the mean Raman spectra of pellets of each *L. pneumophila* strain using the GnuR software. To compare Raman spectra of Philadelphia-1, Los Angeles-1 and U8W cells packaged into pellets with Raman spectra of single cells of corresponding *L. pneumophila* strains, three independent batches of collected Raman spectra of single cells of *L. pneumophila* strains were collected and the mean Raman spectra were calculated. Afterwards, the spectral differences among the normalized mean Raman spectra of single cells of Philadelphia-1, Los Angeles-1 and U8W strains and pellets containing corresponding *L. pneumophila* strains were calculated for each strain. Likewise, the spectral differences between the normalized mean Raman spectra of both spherical pellets with smooth membrane and spherical pellets showing wrinkled membrane were obtained, and the spectral variations between morphologically distinct pellets for each *L. pneumophila* strain were discussed.

Support vector machines (SVMs) [3] were used to discriminate between the Raman spectra of both pellets without intact bacteria and spherical pellets containing Philadelphia-1, Los Angeles-1 and U8W cells, while a tenfold cross-validation was used to establish the accuracy of the classification model. The cost factor of the SVM calculation was 4, while the sigma parameter was optimized via the kernlab function from the R package.[4] The Raman spectra of pellets without intact bacteria (pellets* in Table 1) comprise the Raman spectra of both pellets from the control sample as well as pellets without fluorescence obtained from the sample where *T. thermophila* was fed with fluorescently immuno-labeled *L. pneumophila*. The Raman spectra of spherical pellets containing Philadelphia-1, Los Angeles-1 and U8W cells comprise the Raman spectra of pellets with smoothed membrane as well as pellets with wrinkled membrane. Three independent batches of collected Raman spectra were included in the Raman dataset of pellets expelled by *T. thermophila*.

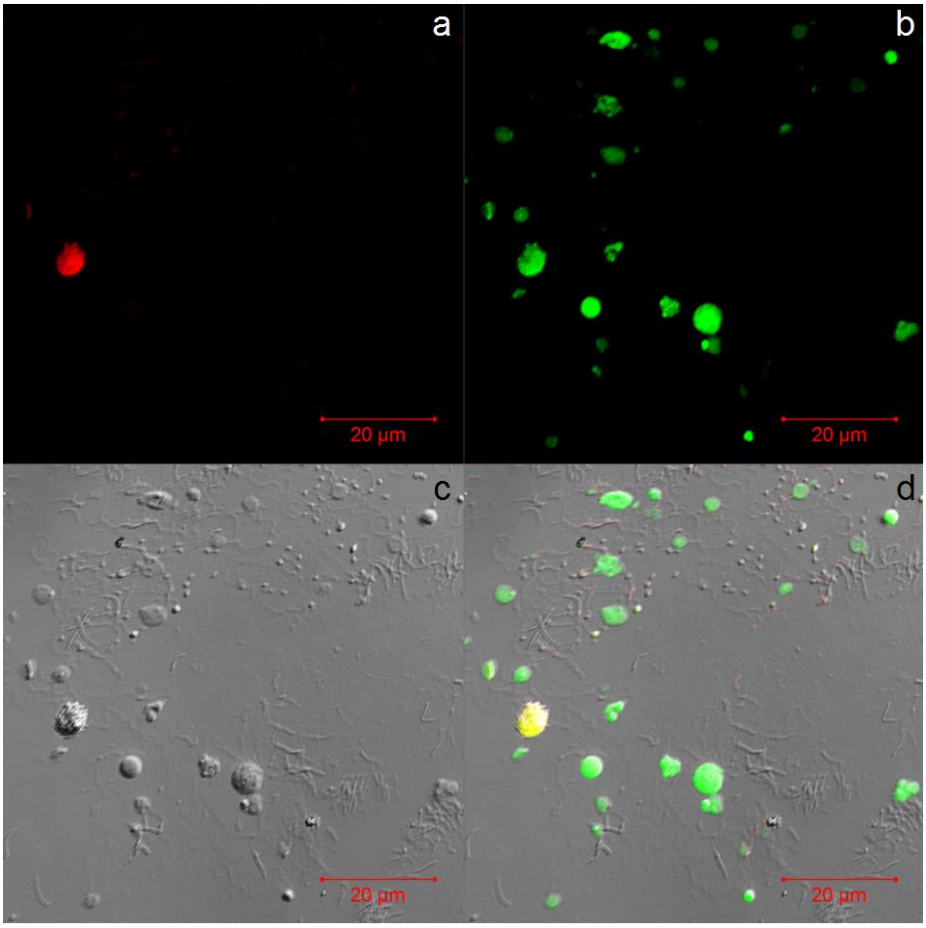

Figure S1. Pellets containing the cells of *L. pneumophila* Los Angeles-1 strain expelled by *T. thermophila* 24 h after incubation. The pellets were stained with the LIVE/DEAD BacLight bacterial viability kit (Invitrogen, L13152). The SYTO 9 stain labels all bacteria within pellets green, whereas Propidium Iodide (red) binds to a double stranded DNA of nonviable bacteria. The individual emission channels of pellets (a, b), bright field image (c) as well as the composite image of pellets containing Los Angeles-1 cells are shown (d).

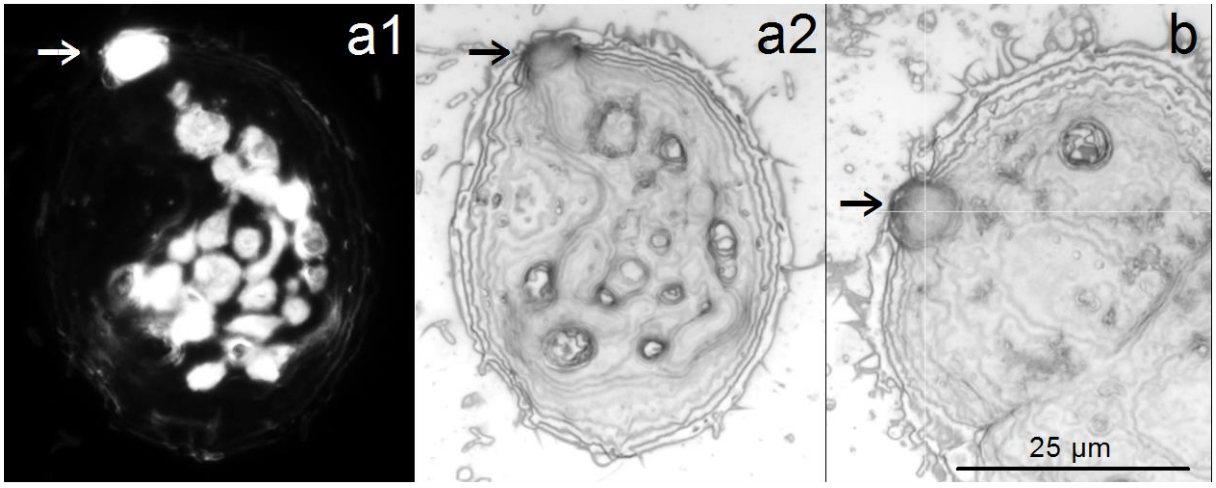

Figure S2. Fluorescence image (a1) of the corresponding *T. thermophila* cell (a2) as well as bright field microscopic images (a2 and b) of a *T. thermophila* cell. The arrows in panels a2 and b point to spherical-shaped pellets with smooth membrane of tightly wrapped Philadephia-1 cells being expelled 3h after incubation in sterile-filtered tap water. Images were obtained by using the Raman microscope.

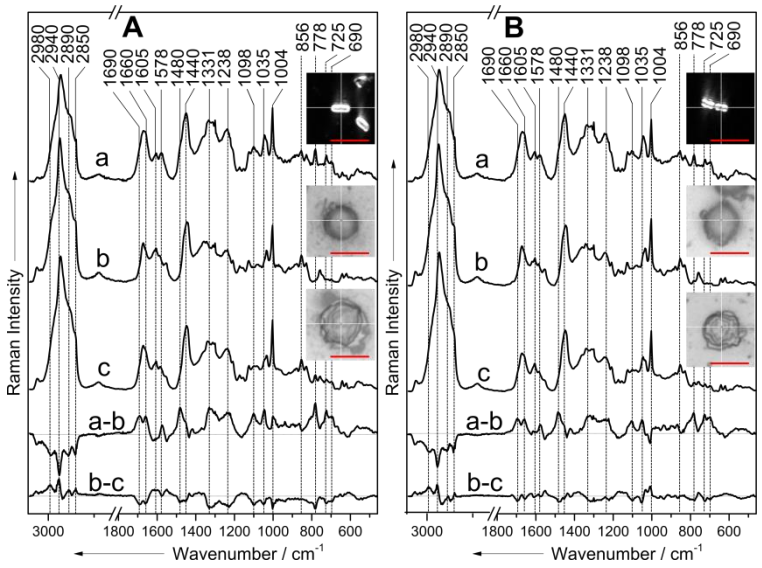

Figure S3. Normalized mean Raman spectra of single cells (a) and pellets with smooth (b) and wrinkled (c) membranes of tightly packaged Los Angeles-1 (A) and U8W (B) cells as well as their spectral differences a – b and b – c for each panel. Scale bars: A-B, 5 μm.

Table S1. Tentative assignments of the Raman bands for Figure 5, 6, 7 and S3[5]

Wavenumber/cm^{-1}	Fig. 5	Fig. 6	Fig. 7	Fig. S3	Tentative assignment	Biochemical component
$2980_{shoulder}$		+		+	$\nu(CH_3)$	Polysaccharide, Protein
$2940_{shoulder}$		+		+	$\nu(CH_3$ and $CH_2)$	Polysaccharide, Protein
2930	+		+		$\nu(CH_3$ and $CH_2)$	Protein, carbohydrate, lipid, cell wall
$2890_{shoulder}$		+		+	$\nu(CH_2)$	Lipid
$2850_{shoulder}$		+		+	$\nu(CH_2)$	Lipid
$1690_{shoulder}$	+			+	$\nu(C=O)$	Protein
$1669_{shoulder}$		+	+		$\nu(C=O)$ Amide I band	Protein
$1660_{shoulder}$	+		+	+	$\nu(C=O)$, $\nu(C=C)_{cis}$	Protein, lipid
1605	+	+		+	$\nu(C=C)_{in\ plane}$, aromatic ring	Protein
1578		+	(+)*	+	guanine and adenine ring stretching and C=C bending mode, aromatic amino acids (Phe)*	DNA/RNA and (protein)*
$1480_{shoulder}$		+		+	guanine, adenine	DNA/RNA
1450	+		+		$\delta(CH_2, CH_3)$ scissoring	Protein, carbohydrate, lipid, cell wall
$1440_{shoulder}$		+		+	$\delta(CH_2, CH_3)$	Protein, lipid
1331	+	+	+	+	$\nu(C\text{-}C)_{skeletal}$, cis conformation and cytosine, uracil (ring stretching)	Polysaccharide and DNA/RNA
1300	+		+		$\tau(CH_2)$	Lipid
1238	+		+	+	Amide III	Protein
1098				+	$\nu(C\text{-}C)_{skeletal}$ and $\nu(C\text{-}O\text{-}C)$ glycosidic link asymmetric stretching	DNA/RNA
1090			+		$\nu(C\text{-}C)_{skeletal}$ and symmetric phosphate stretching vibrations	Protein, polysaccharide
1075	+				$\nu(C\text{-}C)_{skeletal}$	Protein, polysaccharide, cell wall
1050			+		$\nu(C\text{-}C)_{skeletal}$	Protein, polysaccharide, cell wall
1035	+	+		+	$\nu(C\text{-}C)_{skeletal}$	Aromatic amino acids
1004	+	+	+	+	$\nu(C\text{-}C)$ aromatic ring	Protein
856	+	+		+	$\nu(C\text{-}C)_{skeletal}$ or Tyrosine	Protein, polysaccharide
826	+				$\nu(C\text{-}C)_{skeletal}$ or Tyrosine	Protein, polysaccharide
778		+		+	Cytosine, uracil (ring stretching)	DNA/RNA
725	+	+		+	Adenine and C-S	DNA/RNA, protein
690	+	+		+	Guanine	DNA/RNA
560	+				$\delta(C\text{-}O\text{-}C)$ glycosidic ring	Protein, carbohydrate, lipid, cell wall
540	+				$\nu(S\text{-}S)$ disulfide conformation	Protein

ν = stretching, δ = deformation; τ =twisting

REFERENCES

1. P. Rösch, M. Harz, M. Schmitt, K.-D. Peschke, O. Ronneberger, H. Burkhardt, H.-W. Motzkus, M. Lankers, S. Hofer, H. Thiele, *Applied and Environmental Microbiology* 2005, *71*. 1626-1637.
2. (a) D. Kusić, B. Kampe, A. Ramoji, U. Neugebauer, P. Rösch, J. Popp, *Analytical and Bioanalytical Chemistry* 2015, *407*. 6803-6813; (b) D. Kusić, B. Kampe, P. Rösch, J. Popp, *Water Res.* 2014, *48*. 179-189.
3. C. Cortes, V. Vapnik, *Machine Learning* 1995, *20*. 273-297.
4. A. Karatzoglou, A. Smola, K. Hornik. R Foundation for Statistical Computing: Vienna, Austria, 2013.
5. (a) A. C. Williams, H. G. M. Edwards, *Journal of Raman Spectroscopy* 1994, *25*. 673-677; (b) G. J. Puppels, F. F. M. De Mul, C. Otto, J. Greve, M. Robert-Nicoud, D. J. Arndt-Jovin, T. M. Jovin, *Nature* 1990; (c) Z. Movasaghi, S. Rehman, I. U. Rehman, *Applied Spectroscopy Reviews* 2007, *42*. 493-541; (d) S. A. Asher, M. Ludwig, C. R. Johnson, *Journal of the American Chemical Society* 1986, *108*. 3186-3197; (e) J. De Gelder, K. De Gussem, P. Vandenabeele, L. Moens, *Journal of Raman Spectroscopy* 2007, *38*. 1133-1147; (f) A. Rygula, K. Majzner, K. M. Marzec, A. Kaczor, M. Pilarczyk, M. Baranska, *Journal of Raman Spectroscopy* 2013, *44*. 1061-1076.