Effect of toluene on Pseudomonas stutzeri ST-9 morphology: Plasmolysis, cell size and formation of outer membrane vesicles

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Effect of toluene on *Pseudomonas stutzeri* ST-9 morphology: Plasmolysis, cell size and formation of outer membrane vesicles

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Abstract

Isolated toluene-degrading *Pseudomonas stutzeri* ST-9 bacteria were grown in a minimal medium containing toluene (100 mg L\(^{-1}\)) (MMT) or glucose (MMG) as the sole carbon source, with specific growth rates of 0.019 h\(^{-1}\) and 0.042 h\(^{-1}\), respectively. Scanning (SEM) as well as transmission (TEM) electron microscope analyses showed that the bacterial cells grown to mid log in the presence of toluene possess a plasmolysis space. TEM analysis revealed that bacterial cells that were grown in MMT were surrounded by an additional "material" with small vesicles in between. Membrane integrity was analyzed by leakage of 260 nm absorbing material and demonstrated only 7% and 8% leakage from cultures grown in MMT compared to MMG. X-ray microanalysis showed a 4.3-fold increase in Mg and a 3-fold increase in P compared to cells grown in MMG. FACS analysis indicated that the permeability of the membrane to propidium iodide was 12.6% and 19.6% when the cultures were grown in MMG and MMT, respectively. The bacterial cell length increased by 8.5%±0.1 and 17% ±2 as measured using SEM images and FACS analysis, respectively. The results obtained in this research show that the presence of toluene led to morphology changes such as plasmolysis, cell size and formation of outer membrane vesicles. However, it does not cause significant damage to membrane integrity.

Key words: *Pseudomonas*, plasmolysis, toluene, outer membrane vesicles, morphology
Introduction

Bacterial cells have evolved various biochemical and physiological adaptation mechanisms, which protect them from the lethal effects of organic solvents such as benzene, ethylbenzene, toluene and xylene. These adaptation mechanisms include: (1) alteration of the properties of the bacterial cell membrane (Tsitko et al. 1999); (2) morphological changes such as bacterial cell size and formation of outer membrane vesicles (OMV) (Kobayashi et al. 2000); (3) changes in the bacterial proteome which include stress proteins and proteins involved in energy metabolism, efflux pumps and porins (Segura et al. 2005).

The bacterial cell envelope is the first target for the organic solvents. The cell wall of Gram-negative bacteria is composed of inner and outer membranes. The inner lipid bilayer membrane contains mainly phosphatidylethanolamine. The outer membrane consists of an asymmetric bilayer, where the inner leaflet is enriched with phosphatidylethanolamine and the outer leaflet is enriched with lipopolysaccharides (LPS). The inner and outer leaflets are separated by a periplasmic space that contains a hydrophilic peptidoglycan which provides additional stability (Schirmer 1998; Van Gelder et al. 2000). The outer membrane of Gram-negative bacteria is a hydrophobic barrier, which facilitates the passage of ions and molecules. Molecules with a molecular mass higher than 600-1,000 Da cannot penetrate through the outer membrane into the periplasm. The low permeability of the outer membrane to hydrophobic molecules is ascribed to the presence of the LPS (Sikkema et al. 1995).

Several Gram-negative bacteria constantly discharge outer membrane vesicles (OMVs) during their growth. OMVs are bilayered spherical membranous structures, with a diameter ranging between 50 and 250 nm. They contain LPS, outer membrane proteins, phospholipids and periplasmic constituents. OMVs are a secretion and delivery structure through which soluble molecules are released in a complex with insoluble material (Kulp and Kuehn 2010). The OMVs
provide a route for delivery of virulence factors such as phospholipase C, proteases, proelastase, and hemolysins (Kadurugamuwa and Beveridge 1995).

The phenomenon of a plasmolysis space pertains to spaces along the length of the bacterial cell where the cytoplasmic membrane shrinks and retracts from the wall (Woldringh 1994). Bacterial plasmolysis is known to occur in response to osmotic stress (20-30% sucrose) and commonly occurs in Gram-negative species. However, they have also been documented in Gram-positive bacteria (Korber et al. 1996; Schall et al. 1981; Woldringh 1994). A NaCl osmolality of 0.03 caused plasmolysis of *E. coli* which led to a reduction in cytoplasmic and cell water to 20% and 50% of their original values, respectively, and increased periplasmic water by 300% (Cayley et al. 2000).

*Pseudomonas stutzeri* bacterium belongs to the Gamma proteobacteria class, which is widely distributed in a variety of environments, such as soil, plants, water, air and clinical specimens. This is a ubiquitous Gram-negative bacterium with a high degree of physiological and genetic adaptability (Rius et al. 2001; Sikorski et al. 2002). Taxonomically, *P. stutzeri* strains have been grouped into 22 genomovars (Sikorski et al. 2005; Zhang et al. 2014). The isolated toluene-degrading bacterium *P. stutzeri* STF9 belongs to genomovar 3 and its draft genome was recently published (Gomila et al. 2015).

In the present study, *P. stutzeri* ST-9 was grown in a minimal medium with toluene as the sole carbon source. SEM analysis showed that when *P. stutzeri* ST-9 was grown in the presence of toluene, the cells possess "hole like" structures. Further investigation using TEM analysis showed these "hole like" structures are plasmolysis spaces where the cytoplasmic membrane shrinks and retracts from the wall. In order to determine whether this phenomenon causes damage to the bacterial cell envelope, the membrane integrity was analyzed by leakage of 260 nm absorbing material, X-ray microanalysis and FACS. A change in cell size and OMV formation were also observed.
Materials and methods

Minimal Medium (MM): 1 liter MM was composed of 2.44 g Na$_2$HPO$_4$, 1.52 g KH$_2$PO$_4$, 0.5 g (NH$_4$)$_2$SO$_4$, 0.2 g MgSO$_4$·7H$_2$O, 0.05 g CaCl$_2$·2H$_2$O and 10 mL Trace element solution SL-4.

Trace element solution SL-4 was composed of 0.2 g FeSO$_4$·7H$_2$O, 0.5 g EDTA and 100 mL Trace element solution SL-6. Trace element solution SL-6 was composed of 0.1 g ZnSO$_4$·7H$_2$O, 0.03 g MnCl$_2$·4H$_2$O, 0.3 g H$_3$BO$_3$, 0.01 g CuCl$_2$·2H$_2$O 0.03 g Na$_2$MoO$_4$·2H$_2$O, 0.02 g NiCl$_2$·6H$_2$O, 0.2 g CoCl$_2$·6H$_2$O (457 mineral medium DSMZ). MMG- MM with (1%) glucose as the sole carbon source. MMT- MM with toluene (100 mg L$^{-1}$) as the sole carbon source.

Bacterial strains: P. stutzeri STF9, was previously isolated from toluene-contaminated soil (Gomila et al. 2015). Pseudomonas putida F1 (DSM 6899) was purchased from DSMZ, Germany.

Growth conditions. P. stutzeri STF9 as well as P. putida F1 were inoculated to 0.15 OD$_{590}$ nm in MMG or MMT at 30ºC with shaking at 170 rpm. For growth curve examination, samples were measured using a spectrophotometer (Thermo Electron Genesys 10UV, USA) at 590 nm.

Scanning Electron Microscopy (SEM). P. stutzeri ST-9 was washed and fixed with Karnovsky solution (2.5% glutaraldehyde and 2% paraformaldehyde) for 1 h or was fixed with a diluted fixative (containing a smaller concentration of glutaraldehyde and paraformaldehyde) solution containing 0.5% formaldehyde and 0.1% glutaraldehyde, followed by 1% osmium tetroxide. The cells were then dehydrated by incubation in increasing ethanol concentrations. The specimens were gold-coated using a Polaron device. Scanning was performed with a JEOL 840 scanning electron microscope at an accelerating voltage of 20 kV.

Transmission electron microscopy (TEM). P. stutzeri ST-9 was washed and fixed with Karnovsky solution (2.5% glutaraldehyde and 2% paraformaldehyde) for 1 h or was fixed with a diluted fixative of 0.5% formaldehyde and 0.1% glutaraldehyde, at room temperature, and washed
three times in 0.1 M sodium cacodylate buffer. The samples were post-fixed with 1% OsO₄ for 1 h followed by dehydration with solutions of increasing concentrations of ethanol (50%, 70%, 90% and 100%) and propylene oxide. Afterwards, the samples were embedded in Agar 100 resin, Agar Scientific, UK. Thin sections (70 nm) were cut, stained with uranyl acetate and lead citrate, then observed under transmission electron microscope FEI Tecnai 120 kV.

Determination of membrane integrity by leakage of 260 nm absorbing material, X-ray microanalysis and FACS:

Leakage of 260 nm absorbing material analysis. Nucleic acids absorb UV light at a wavelength of 260 nm. The presence of nucleic acids in a suspension can be used as an indicator of damage to the cell membrane caused by leakage of materials into the surrounding. *P. stutzeri* ST-9 was grown in MMG or MMT to the early and mid log phase and the release of nucleic acid components into the medium during growth was measured. At indicated times, the cultures were adjusted with MM to achieve a bacterial suspensions of 0.15 OD 590 nm. The suspensions were harvested (8,000 g 10 min at 4°C) and the absorbance of the cell-free supernatant was recorded at 260 nm (Gaur and Khare 2009; Je and Kim 2006; Oliva et al., 2004; Vanhausteghem et al. 2013). Since toluene absorbs at 260 nm, similarly to nucleic acids, pretreatment of the toluene remaining in the supernatant (of the culture growing in MMT) was carried out by extracting the toluene twice using hexane. A culture of bacterial cells that was grown in MMG as in the experiment, but which was treated with ethanol before harvesting, served as a positive control (calculated as 100% of nucleic acids released). Absorbance at 260 nm was measured using a Thermo Electron Genesys 10UV Spectrophotometer (USA).

X-ray microanalysis (XRMA). *P. stutzeri* ST-9 grown in MMG or MMT to mid log phase were harvested, washed twice with 0.1 M ammonium acetate and resuspended in 30 µL ammonium acetate. Each suspension (20 µL) was attached to an aluminum grid, air-dried at room temperature for at least 24 h and then coated with a layer of carbon (Cahan et al. 2008; Daniel-Hoffmann et al. 2008; Daniel-Hoffmann et al. 2009).
XRMA was performed using an eXL Link X-ray system attached to a JEOL 840 scanning electron microscope. Each spectrum was determined with approximately $10^6$ cells. The background level was the same during all measurements.

**FACS analysis.** The propidium iodide (PI) dye is excluded by intact membranes of viable cells. The presence of the dye within the cell therefore indicates disruption of the cell membrane. Flow cytometry (FACS) analysis provides an opportunity to make rapid and quantitative measurements within a few minutes of PI uptake by individual bacterial cells (thousands of cells) (Gottfredsson et al. 1998; Jiang et al. 2014; Lv et al. 2014; Vanhauteghem et al. 2013). To determine membrane integrity, *P. stutzeri* ST-9 was grown in MMT as well as MMG to the mid log phase. The cells were harvested (8,000 g, 10 min, 4°C) and the pellet was resuspended in 1 mL PBS (cell concentration of $3\times10^8$ cells mL$^{-1}$). Followed by staining with propidium iodide (PI) (1 mg/mL stock solution; Sigma-Aldrich, USA) for 5 min. PI fluorescence intensity was measured by FACS Calibur (Becton Dickinson, USA) using excitation with an argon laser at 488 nm and emission was collected in the FL2 cytometric channel.

**Statistics.** Data were expressed as means ± SD (standard deviation) of three replicates and were statistically analyzed by ANOVA single factor analysis. The difference between the results was considered significant if the $P$-value was less than 0.05.

**Results and discussion**

Toluene-degrading bacteria were isolated from toluene-contaminated soil and were identified using biochemical and molecular biology methods, as *P. stutzeri* genomovar 3, and designated as *P. stutzeri* ST-9 (Gomila et al. 2015). The bacterial cells were grown in a minimal medium containing toluene (100 mg L$^{-1}$) (MMT) or glucose (MMG) as the sole carbon source (Fig 1). The culture that was grown in MMG reached a maximum OD of about 1.1 at 590 nm after
84 h and a specific growth rate of 0.042 h\(^{-1}\), whereas the culture that was grown in MMT reached a maximum OD of about 0.6 at 590 nm after 96 h and a specific growth rate of 0.019 h\(^{-1}\).

The effect of toluene on the bacterial morphology was examined at the log phase and was compared to a control culture, which was grown in MMG. Samples of bacterial cells were washed and fixed in a Karnovsky solution (2.5% glutaraldehyde and 2% paraformaldehyde) and examined using SEM analysis (Fig 2 A, B). As can be seen, \textit{P. stutzeri} ST-9 bacterial cells that were grown in MMG (Fig 2 A) showed a normal structure, exhibiting a typical rod shape with smooth surfaces. However, when the bacterial cells were grown in MMT, plasmolysis spaces were observed (Fig 2 B). In an attempt to verify that the plasmolysis spaces were not due to an effect of the Karnovsky solution which contains relatively high fixative concentrations (2.5% glutaraldehyde and 2% paraformaldehyde), the same experiment was performed, but with a diluted fixative solution which contained a smaller concentration of glutaraldehyde and formaldehyde (0.5% formaldehyde and 0.1% glutaraldehyde) (Fig 2 C, D). As can be seen, the diluted fixative solution led to the same results, where the bacterial cells which were grown in the presence of glucose (Fig 2 C) exhibited a normal structure, whereas the culture that was grown in the presence of toluene (Fig 2 D), demonstrated plasmolysis spaces (indicated by arrows). The effect of toluene on the morphology of \textit{P. putida}, which is known as a toluene degrader (Cho et al. 2000), was performed in order to clarify whether the plasmolysis induced by toluene is a phenomenon unique to \textit{P. stutzeri} ST-9.

\textit{P. putida} was grown in MMG as well as MMT and fixed using Karnovsky solution. Plasmolysis spaces also appeared when \textit{P. putida} cultures were grown in MMT (Fig 2 F). When these bacterial cells were grown in MMG (Fig 2 E), the cells exhibited a normal structure.

Further morphology investigation of the effect of toluene on \textit{P. stutzeri} ST-9 was carried out using TEM analysis. The bacterial cells were harvested at the log phase and fixed with Karnovsky solution (Fig 3 A-E). The control bacterial cells, which were grown in MMG (Fig 3
A), exhibited a normal intracellular structure with diffuse DNA and integrity of the outer and inner membranes. In the presence of toluene (MMT), several phenomena were observed: 1- plasmolysis spaces (indicated by arrows and designated "P", Fig 3 B); 2- bacterial cells surrounded by an additional "material" (indicated by arrows and designated "M") with small vesicles in between (indicated by arrows and designated "V") (Fig 3 C and D); 3- bacterial cells surrounded by a "wide material" (indicated by arrows and designated "WM" Fig 3 E). Larger pictures were added to Supplementary Information (supplementalF1 and supplementalF2). In order to determine whether the relatively high fixatives concentration in the Karnovsky solution led to plasmolysis, the bacterial cells were also prepared for TEM analysis, with the diluted fixative solution (Fig 3 F). Plasmolysis was also observed when the samples were prepared with the diluted fixative.

The phenomenon of bacterial plasmolysis as a consequence of osmotic stress is well known (Alemohammad and Knowles 1974; Korber et al. 1996; Woldringh 1994). However, bacterial plasmolysis due to organic solvents is less known. Using SEM analysis, Park and co-authors showed "destructive openings on the cell envelopes" in Pseudomonas sp. DJ-12 bacterial cells that were exposed to aromatic hydrocarbons such as biphenyl, 4-chlorobiphenyl (4CB) 4-hydroxybenzoate (4HBA) (Park et al. 2001). Duque and co-authors examined a mutant cyoB strain of the solvent-tolerant P. putida DOT-T1E after exposure to solvents. SEM analysis showed plasmolysis in the mutant cells but not in the wild type (Duque et al. 2004).

The vesicles, which are seen in Fig 3 D, were also reported in several studies. OMVs increase bacterial survival by removing a variety of harmful intrinsic or extrinsic agents from the bacterial population (McBroom and Kuehn 2007). The toluene-tolerant microorganism P. putida H-2000 was found to release OMVs in the range of about 80 to 100 nm, in response to toluene exposure. The toluene molecules, which adhere to the bacterial outer membrane, were eliminated by OMVs formation. These OMVs contained a higher concentration of toluene molecules than the
concentration found in the cell membrane. The maximum amount of toluene associated with the
OMVs was 0.625 mol/mol of lipid at 0.5 h after the addition of toluene, which decreased to 0.172
mol/mol at 6.0 h (Kobayashi et al. 2000). The adherence of toluene to OMVs is an ideal model of
organic solvent tolerance. This model supports the hypothesis that the outer membrane of Gram-
negative bacteria is an active eliminator of toxic organic solvents, and not just a simple barrier.

We assume that the additional "material" shown in Fig 3 C-E is exopolysaccharides (EPS) which is known to be produced by many *Pseudomonas* species (Kachlany et al. 2001).
Polysaccharides are believed to protect bacterial cells from desiccation, heavy metals, organic
compounds or other environmental stresses. The strain, the culture conditions, and the type of
carbon source influence the amount and the composition of microbial EPS that is produced by a
certain species (Onbasli and Aslim 2009).

The phenomenon of plasmolysis, which was observed using SEM and TEM analysis, led
to a further investigation of membrane integrity. Determination of the membrane integrity was
performed using analysis of leakage of 260 nm absorbing material (Fig 4), XRAM (Fig 5) and
FACS (Fig 6).

The main materials that absorb UV light at 260 nm are nucleic acids. The presence of
nucleic acids in a medium can serve as an indicator of damage to the cell membrane (Je and Kim
2006). Nucleic acid leakage into the surrounding was determined for cultures that were grown in
the presence of toluene and compared with cultures that were grown in the presence of glucose.
The cultures were harvested at the early and mid-log phase. A positive control of cells with
significant membrane damage was performed by treatment with ethanol prior to harvesting (Fig
4). The absorbance of the positive control was 0.45 and 0.48 OD 260 nm (calculated as 100%
release of nucleic acids) at the early and mid log, respectively. The absorbance of cultures grown
in the presence of toluene at the early and mid-log phase was 0.107 and 0.119 OD, respectively.
This was only 8% and 7% higher than the corresponding cultures grown in the presence of
glucose. The calculated $P$ value of the difference in the percentage of nucleic acids release from
cells that were grown in the presence of toluene compared to those grown in glucose, at the early
and mid-log phase, was 0.07 and 0.08, respectively. These results show that there is no significant
leakage of nucleic acids from cells that were grown in the presence of toluene. This finding may
be correlated to membrane integrity. It was reported that when bacterial cells were grown in a
high solvent concentration (cyclohexane 33% v/v), release of nucleic acid components into the
medium during the growth of a solvent-tolerant strain *Pseudomonas aeruginosa* PseA was a
function of time and showed an absorbance of 0.5 OD at 260 nm (Gaur and Khare 2009).

The integrity of the bacterial membrane was also demonstrated by XRMA (Fig. 5). The
bacterial cells were grown in the presence of glucose or toluene as the sole carbon source (MMG
or MMT, respectively). The cultures were harvested at the mid-log phase and were fixed for
XRMA. The ion composition shown in Fig 5 revealed a 4.3-fold increase in the peak of Mg
(2.45±0.24) as well as a 3-fold increase in P (8.81±0.04) in cells grown in the presence of toluene
compared to those grown in glucose. The increase in Mg and P shows that the integrity of the
membrane was preserved, since damage to the membrane would possibly lead to ion leakage. The
increase in $\text{Mg}^{2+}$ was reported by several studies that showed that divalent cations such as $\text{Mg}^{2+}$
and $\text{Ca}^{2+}$ can help stabilize and maintain the integrity of the outer membrane by binding between
adjacent LPS molecules (Katowsky et al. 1991; Raetz et al. 2007). It was found that in *P. putida*
DOT-T1 grown in the presence of toluene, the addition of $\text{Mg}^{2+}$ led to an increase in membrane
integrity (Ramos et al., 1995). In the case of Pi accumulation, it was reported that many
microorganisms accumulate excess Pi in response to nutrient stress (Alcántara et al. 2014).

The integrity of the membrane was also demonstrated using FACS analysis (Fig 6 A). The
bacterial cells were grown to the mid log phase, in MMT or MMG. The bacterial cells were
stained with PI, which binds to double-stranded DNA, but cannot penetrate the intact cell
membrane of the bacterial cells. The results show that in the presence of toluene, 19.6% of cells
were stained with PI compared to 12.6% when the cells were grown in the presence of glucose. This result again demonstrates that there was no significant damage to the bacterial cell membrane when the cells were grown in the presence of toluene.

Examination of bacterial size in response to exposure to toluene was determined by measuring the bacterial size from at least 10 different SEM fields. The total number of bacteria that were counted from those which were grown in the presence of toluene or glucose were 40 and 60, respectively (all cells that were in a clear dividing stage were not taken into account). The results show that the length of bacterial cells grown in the presence of toluene was 8.5%±0.1 higher than the length of bacterial cells grown in the presence of glucose. This result was supported by FACS (Fig 6 B) analysis that revealed that the size of the bacterial cells that were exposed to toluene was 17%±2 higher than the size of cells that were grown in the presence of glucose. In the FACS analysis, all cells in a sample of about 6,000 were taken into account, including those in a dividing stage.

Changes in bacterial cell size were reported as a result of exposure to toxic hydrocarbons. It was found that the cell size of Enterobacter sp. VKGH12 increased with increasing concentrations of n-butanol. This was observed only after the addition of non-lethal concentrations. The decrease in the surface area to cell volume ratio in response to the presence of organic solvents leads to a reduction in the cell surface area, and especially the cytoplasmic membrane, which is the major target for the toxic effects. The reduction of the relative surface area represents an adaptive mechanism to the presence of toxic hydrocarbons (Neumann et al. 2005). On the other hand, a decrease in cell size was observed with Bacillus subtilis which reduced their cell size when treated with aromatic hydrocarbons (Pinkart et al. 1996). Mycobacterium species grown in a two-phase aqueous-organic and organic media tended to shrink and decrease their surface roughness, indicating that they increased their surface area
(Carvalho and Fonseca 2004). In this case, the increase in the cell surface area may enhance their ability for uptake of potential growth substrates.

Conclusions

Isolated toluene-degrading *P. stutzeri* STF9 was grown in a minimal medium containing toluene or glucose as the sole carbon source. Toluene induced plasmolysis was shown using SEM and TEM analysis. In order to verify whether the relative high fixatives concentration in the Karnovsky solution (2.5% glutaraldehyde and 2% paraformaldehyde) led to plasmolysis, the bacterial cells were also prepared for SEM and TEM analysis with a diluted fixative solution (0.5% formaldehyde and 0.1% glutaraldehyde). Plasmolysis was observed also when the samples were prepared with the diluted fixative. The membrane integrity was analyzed by leakage of 260 nm absorbing material, X ray microanalysis and FACS, and revealed no significant damage to the membrane. The bacterial cell length was increased by 8.5%±0.1 or 17% ±2 as measured using SEM images and FACS analysis, respectively. In addition, OMV formation was observed as a function of exposure to toluene.

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Fig. 1  *P. stutzeri* ST-9 growth curve in the presence of toluene (A); glucose (B).

**Fig 2.** SEM images of *P. stutzeri* ST-9 bacterial cells grown in MMG (A and C) and MMT (B and D) and fixed using Karnovsky solution (2% glutaraldehyde and 2% paraformaldehyde) (A and B) or with the diluted fixative (C and D). SEM images of *P. putida* bacterial cells grown in MMG (E) as well as MMT (F) and fixed using Karnovsky solution. Arrows show plasmolysis spaces.

**Fig 3.** TEM images of *P. stutzeri* ST-9 grown in MMT or MMG. A-control bacteria grown in MMG; B-E- bacterial cells that were grown in MMT and fixed using a Karnovsky solution. F-bacterial cells that were grown in MMT and fixed using a diluted fixative. The capital letters: P, V, M, WM designated plasmolysis, vesicles, material and wide material, respectively.

**Fig. 4.** The leakage of 260 nm absorbing material in cultures grown to early and mid log phase, in MM with glucose as the sole carbon source (■) or toluene (○). A positive control that was treated with ethanol prior of harvesting (■). A positive control that was treated with ethanol prior of harvesting (■).

**Fig. 5.** X-ray elemental spectra of *P. stutzeri* ST-9 bacterial cells grown to mid log in the presence of toluene (A) and glucose (b).

**Fig 6:** Flow cytometric analysis. Membrane permeability of *P. stutzeri* ST-9 cells measured using flow cytometry with propidium iodide. The shaded area: MMT; bright area: MMG (A). Histograms
showing an indicative of size and peak height distribution (shown as FSC-H) of *P. stutzeri* ST-9 in toluene (MMT) (solid lines) or glucose (MMG) (dotted lines) (B).
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