INTRODUCTION

*Burkholderia cenocepacia* is one of the species constituting the *Burkholderia cepacia* complex (BCC), a group of phenotypically very similar micro-organisms capable of infecting cystic fibrosis (CF) patients, sometimes with lethal outcome. CF is the most common inherited disease in the Caucasian population; the genetic defect involves the CF conductance transmembrane regulator protein and results in altered secretions across epithelia (Govan & Deretic, 1996). In particular, the bronchopulmonary epithelium is covered with a hyperviscous mucus, which impairs mucociliary clearance and favours bacterial colonization and infection. Of the 20 species that currently make up the BCC (Mahenthiralingam et al., 2000; Coenye et al., 2003; Peeters et al., 2013; De Smet et al., 2015), all can cause CF infection, with *B. cenocepacia* being an important CF pathogen, generally more aggressive than other BCC species. When BCC infects CF patients, the clinical outcome is uncertain, with some patients being colonized for years before showing adverse symptoms and others quickly experiencing diminished respiratory capacity and dying of 'cepacia syndrome' (Govan & Deretic, 1996). Mucoid as well as non-mucoid strains are isolated from CF patients, but the relationship between mucoid phenotypes and virulence still needs to be clarified (Silva et al., 2013; Zlosnik et al., 2011). The presence of biofilm in the lungs has been undoubtedly established for *Pseudomonas aeruginosa* (Singh et al., 2000; Høiby et al., 2011), but not yet for BCC species. Recent literature reports on BCC identification in CF lungs as single cells or small clusters (Schwab et al., 2014) within phagocytes and mucus, but not in biofilm-like structures. Although biofilms produced by BCC members have not yet been found in the CF lungs, BCC species do form biofilms in vitro on conventional supports (Conway et al., 2002) and on epithelial cell lines (Caraher et al., 2007), and they possess the genes required for biofilm formation (Huber et al., 2002). Since in biofilms bacteria exhibit increased persistency and resistance to antimicrobials, the investigation of biofilm structure in...
terms of macromolecular composition is relevant to understanding their biological functions. In fact, it is generally recognized that exopolysaccharides (EPOLS) are important components of the biofilm matrix, but little is known about the type of EPOLS produced in BCC biofilms because most of the literature data derive either from genetic studies or from polysaccharides produced in non-biofilm mode.

In this context, investigations carried out mainly in our laboratory on the type of EPOLS produced by BCC species have revealed a variety of structures (Cuzzi et al., 2014), with the EPOL cepacian produced by almost all the isolates examined when growth was developed on yeast extract–mannitol (YEM) medium (Sage et al., 1990) which promotes the mucoid phenotype. Cepacian is characterized by a highly branched (Cescutti et al., 2010) and heavily acetylated repeating unit structure (Cescutti et al., 2010); its polysaccharide chain shows a rather rigid macromolecular conformation and the ability to form two- and four-stranded aggregates, as detected by light scattering and atomic force microscopy (AFM) (Sist et al., 2003; Nogueira et al., 2005; Herasimenka et al., 2008), thus suggesting that it might be a good candidate in the formation and/or maintenance of BCC biofilm architecture. Cepacian was also proved to be a reactive oxygen species scavenger (Cuzzi et al., 2012) and to quench the bactericidal action of antimicrobial peptides, even if to a lesser extent than alginate (Fosciatti et al., 2009).

The majority of the EPOLS of the BCC have been purified from bacteria grown on agar plates, a condition that does not strictly respond to the definition of biofilm, as reported by Donlan & Costerton (2002). Only very recently we developed biofilms on cellulose membranes deposited on agar media (Merritt et al., 2011) and isolated and characterized the EPOLS produced in the matrices, showing that they can differ from those biosynthesized in a non-biofilm mode of growth (Cuzzi et al., 2014). The present investigation was carried out in order to determine the influence of different media and solid supports on the macromolecular composition of the biofilm matrix produced by B. cenocepacia strain BTS2, a CF clinical isolate that was chosen because it is an abundant biofilm producer. Visualization of the biofilms and determination of their thickness were obtained with AFM, and confocal laser scanning microscopy (CLSM) and phase-contrast microscopy (PCM) were used for general biofilm qualitative description.

METHODS

Bacterial strain and growth media. B. cenocepacia BTS2 was isolated from a CF patient attending the Regional Centre for Cystic Fibrosis in Trieste, Italy. The following solid and liquid media were used: King A (KA), Luria–Bertani supplemented with 20 % glucose (LBG), Mueller–Hinton (MH), synthetic CF medium (SCFM) and yeast extract–mannitol (YEM); media components are reported in Table S1, available in the online Supplementary Material. To prepare solid media, agar (1.5 %) was added to all media except SCFM, which was supplemented with agarose (1.2 %).

Biofilm production on cellulose membranes. Cellulose membranes (cut-off 14 000 Da; Sigma) were cut into circles the size of the Petri dishes to be used (90 mm diameter), washed first in 5 % Na2CO3 and then in water, autoclaved and placed over Petri dishes containing the selected medium. An overnight liquid culture of B. cenocepacia BTS2 was diluted to OD600 0.013 (about 106 c.f.u. ml⁻¹) and 10 μl was placed on each membrane, after drying the excess water. The liquid medium used was the same as in the seeded Petri dish. After 5 and 7 days of incubation at 30 °C, the material on the membranes was recovered by scraping in 5–10 ml 0.9 % NaCl, centrifuged at 3200 g at 4 °C for 20 min, and finally filter-sterilized (Millipore membranes, 0.22 μm). As control, 10 μl portions of the liquid cultures were seeded directly on the agar media and recovered as described above, but using 2 ml NaCl solution. When YEM medium was used, separation of the cells was achieved by centrifuging at 49 200 g at 4 °C for 30 min, owing to the viscosity of the extracellular polymeric substance. The recovered material was kept at −20 °C to avoid enzymic degradation.

Biofilm production on glass slides. Glass microscope slides were placed in 50 ml Falcon test tubes containing 25 ml of the desired liquid medium and autoclaved. Ten tubes were used for each medium. For SCFM medium, glass slides were autoclaved separately and then placed in Falcon tubes containing sterile SCFM. An overnight liquid culture of B. cenocepacia BTS2 was diluted to OD0.013 (about 10⁶ c.f.u. ml⁻¹) and 10 μl was used to inoculate each Falcon tube, which was then incubated at 30 °C at an angle of 22°. After 7 days, the glass slides were gently rinsed in 0.9 % NaCl to remove planktonic bacteria, and the biomass was recovered by scraping in the same solution. When biofilm also formed on the test tube walls, the liquid medium was discarded and 0.9 % NaCl was added with thorough mixing. In both cases, bacteria were removed by centrifuging at 3200 g for 20 min at 4 °C followed by filtration (Millipore membranes, 0.22 μm) and stored at −20 °C.

Growth curves of B. cenocepacia BTS2. B. cenocepacia BTS2 was grown at 30 °C for 16 h in liquid MH and YEM media, and diluted to a concentration of 10⁶ c.f.u. ml⁻¹; 10 μl portions were used to produce a biofilm on cellulose membranes (seven for each growth medium), as explained above. The same volume of bacterial culture was used to seed Petri dishes containing MH or YEM medium. The biomass was collected every day for 7 days and serially diluted in 10 mM phosphate buffer pH 7.70, 0.9 % NaCl, and 100 μl of each dilution was used for bacterial counting. Bacterial colonies were counted after incubating for 48 h at 30 °C. The results are the mean of two independent growths and two replicates.

Swimming motility. An overnight culture of B. cenocepacia BTS2 was point-inoculated into LB medium containing 0.3 % agar and incubated at 30 °C for 1 day and at room temperature for 3 days in order to check for swimming motility (Huber et al., 2001).

Colorimetric assays and water content. Protein content was determined using the Bradford assay (Bradford, 1976) with BSA as reference, while saccharides were quantified according to Dubois et al. (1956), using glucose as standard. At least three different samples from membranes and two from agar were used. The colorimetric tests were always performed in duplicate. Water content was determined as the difference between the biomass weight before and after lyophilization; nine membranes deposited on YEM and six on MH were used.

1H-NMR spectroscopy. Biofilm matrices obtained after 5 days of incubation on cellulose membranes were purified by dialysis against 0.1 M NaCl and then water, taken to pH 6.8, and freeze-dried. Apart from SCFM, the liquid medium was used.
from the sample produced on YEM, the others were treated with protease (Streptomyces griseus; Sigma) in 50 mM Tris/HCl, pH 7.5 at 37 °C for 16 h, centrifuged at 3200 g for 10 min at 4 °C, dialysed as reported above, taken to neutral pH and freeze-dried. When EPOL solutions were too viscous, molecular masses were decreased by treatment with a Branson sonifier equipped with a 2.8 Å microtip. Samples (1 g l−1) were cooled in an ice bath and sonicated using five bursts lasting 1 min each, separated by 1 min intervals. Biofilm matrices developed on glass slides and on test tubes were dialysed as above, taken to neutral pH and lyophilized. For recording NMR spectra, all samples were prepared by dissolution in 99.9 % D2O followed by lyophilization three times, and finally dissolved in 0.7 ml 99.96 % D2O. Spectra were recorded on a 500 MHz VARIAN spectrometer operating at 50 °C. Two-dimensional experiments were performed using standard VARIAN pulse sequences and pulsed-field gradients for coherence selection when appropriate. Heteronuclear single-quantum coherence (HSQC) spectra were recorded using 140 Hz one-bond J/Hz constant. Total correlation spectroscopy (TOCSY) experiments were carried out using 100 ms spin-lock time.

Electrospray ionization MS (ESI-MS). ESI-MS were recorded on a Bruker Esquire 4000 ion-trap mass spectrometer connected to a syringe pump for the injection of the samples. The instrument was calibrated using a standard mixture provided by Bruker. After reduction with NaBD4 and permethylation (Dell, 1990), samples were dissolved at an appropriate concentration in 1 : 1 methanol : chloroform containing 11 mM ammonium acetate, and injected at 180 μl h−1. Detection was performed in positive-ion mode.

Polysaccharide linkage analysis. The determination of glycosidic linkages was achieved by methylation analysis. The lyophilized sample was permethylated (Harris et al., 1984), and then hydrolysed with 2 M trifluoroacetic acid; the products were reduced to alditol with NaBH4 and subsequently peracetylated (Albersheim et al., 1967) to obtain partially methylated alditol acetate derivatives. The mixture was analysed by GLC on a Perkin-Elmer Autosystem XL gas chromatograph equipped with a flame-ionization detector and SP2330 capillary column (30 m; Supelco), using He as carrier gas. In order to quantify each sugar derivative, peak areas were corrected by the effective carbon response factor (Sweet et al., 1975). The mixture of partially methylated alditol acetates was also analysed by GLC-MS using an Agilent Technologies 7890A gas chromatograph coupled to an Agilent Technologies 5975 C VL MSD, equipped with the same capillary column and using He as carrier gas. The temperature programme used was 150–250 °C at 4 °C min−1.

CLSM. Sterile glass coverslips were placed in six-well plates containing 4 ml of each culture medium inoculated with a BTS2 overnight culture to give a final concentration of 106 c.f.u. ml−1. After excitation with an argon ion laser operating at λ = 514 nm. The images were acquired with a ×20 objective. Digital images were collected using EZ-C1 software and analysed using ImageJ software; the brightness and contrast of images were adjusted before export to Adobe Photoshop CS. Each coverslip was examined on both its surfaces to check the biofilm distribution and select representative views. Three to five views were considered for each coverslip.

AFM. Biofilms on glass coverslips were prepared as described in the previous paragraph, except that incubation was performed for 5 days at 30 °C with gentle orbital shaking to maximize surface film homogeneity. Glass coverslips were washed once in 0.9 % NaCl and twice in water and treated for 2 min with 1 ml methanol to fix the biomass. After drying, they were kept at 22 °C under an N2 atmosphere until AFM characterization.

Morphological analysis of slides was performed on a customized Agilent 5100 AFM with RHK SPM–1000 electronics. The instrument, optimized for cell analysis, was mounted on an epi-fluorescence microscope (Nikon Eclipse Ti-U). All measurements were performed in air in dynamic mode (no-contact) using Ultrasharp silicon cantilevers (NSC11/CrAu; MikroMasch), characterized by a nominal force constant and a resonance frequency in air of 48 nN nm−1 and 330 kHz, respectively. Biofilm thickness was determined as the difference between the mean height of the organic film covering the surface and the height of a portion of the sample where the film was removed using a scalpel to expose the glass underneath. Height differences were determined as the distance between Gaussian-fitted height distributions of the portion of the sample covered by the polymeric organic matrix and the portion where the film was removed. Each biofilm height is the mean value of three measurements performed on three different regions of the scratch and averaged over three different samples (n=3). Errors were computed as SD.

PCM. PCM images were acquired on samples prepared as for AFM analysis using a Nikon Eclipse Ti-U microscope.

RESULTS AND DISCUSSION

Biofilm formed on cellulose membranes

Comparison of bacterial colonies grown on agar with biofilms formed on cellulose membranes. Biofilms were produced on cellulose membranes deposited on five different growth media: KA; LBG; MH; SCFM (Palmer et al., 2007), which mimics the nutritional conditions in the CF sputum; and YEM (Sage et al., 1990), which promotes the mucoid phenotype. Biofilm biomass was compared with that of the equivalent sample grown on solid agar medium. The growth morphology was completely different, and such diversity increased with the incubation time: round shape on agar and more spread out on cellulose membranes (Fig. S1). The different morphology can be attributed to motility on cellulose membranes. In fact, with the culture conditions used to check for swimming ability (Huber et al., 2001), bacteria showed the formation of chemotactic rings, indicative of swimming motility (see Fig. S2). As expected, YEM was the only medium to induce the mucoid phenotype on agar as well as on membranes; on the latter the biomass produced was the most abundant, it was very viscous but poorly adherent to the support,
and it covered the whole plate after 7 days. In contrast, on the other four media the biomass did not develop as much, it adhered strongly to the cellulose support, and it never looked mucoid. In general, the amount of biomass produced was visibly higher on cellulose membranes than on agar.

Protein and carbohydrate contents were determined by means of colorimetric assays after 5 and 7 days of growth. The results (Table 1) showed that growth on cellulose support always resulted in higher production of both biomolecules. Comparison of the amount of proteins and saccharides after 5 and 7 days of growth on cellulose support showed that: (1) proteins were more abundant than carbohydrates in all media except YEM, where polysaccharides predominated; (2) the absolute amount of proteins and carbohydrates increased from day 5 to day 7; and (3) the relative percentages of the two compounds did not change from day 5 to day 7. These experiments not only showed a medium-dependent biosynthetic behaviour for *B. cenocepacia* BTS2, as similarly reported for other bacterial species (Kives et al., 2006; Chen et al., 2013; Jung et al., 2013; Moryl et al., 2014), but also evidenced differences related to the support used. Since the biomass produced on YEM was different from those developed on all other media, some experiments were conducted using other media, some experiments were conducted using only YEM and MH media. In order to determine if the differences seen in protein and carbohydrate production were correlated with differences in the absolute number of bacteria, cell number was determined from growth on membranes deposited on MH and YEM media as well as from growth directly on MH and YEM agar. The results showed that after 7 days bacteria were still in the stationary phase (Fig. S3) and that their absolute number was the same within the same type of medium. The speed of growth was different: bacteria reached the stationary phase after 1 day on MH medium, but after 3 days on YEM. However, at day 7 the number of cells was 10 times higher on YEM than on MH medium. These data indicated that cellulose membranes promoted biosynthesis of higher amounts of matrix polymers with respect to growth on agar. The water content was determined to be 84 and 95 % (w/w) for the biomass produced on MH and on YEM media, respectively, showing similar values after 5 and 7 days of growth. These figures are likely due to the different composition of the matrices in terms of proteins and saccharides, the latter usually possessing a higher hydrophilic character than the former.

**Table 1.** Extracellular carbohydrates (Carb.) and proteins (Prot.) produced by *B. cenocepacia* BTS2 grown on cellulose membranes and agar plates

<table>
<thead>
<tr>
<th>Medium</th>
<th>Carbohydrates</th>
<th>Proteins</th>
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<tr>
<td></td>
<td>Carb.</td>
<td>Prot.</td>
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<tr>
<td>KA</td>
<td>0.961 ± 0.147 (38)</td>
<td>1.552 ± 0.252 (62)</td>
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<tr>
<td>LBG</td>
<td>1.055 ± 0.420 (19)</td>
<td>4.359 ± 0.672 (81)</td>
</tr>
<tr>
<td>MH</td>
<td>0.361 ± 0.006 (13)</td>
<td>2.507 ± 0.348 (87)</td>
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<tr>
<td>SCFM</td>
<td>0.175 ± 0.085 (37)</td>
<td>0.303 ± 0.097 (63)</td>
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<tr>
<td>YEM</td>
<td>5.367 ± 0.968 (97)</td>
<td>0.171 ± 0.116 (3)</td>
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<tr>
<th>Medium</th>
<th>Cellulose membranes</th>
<th>Agar</th>
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<tr>
<td>KA</td>
<td>1.889 ± 0.516 (33)</td>
<td>3.827 ± 0.927 (67)</td>
</tr>
<tr>
<td>LBG</td>
<td>1.761 ± 0.543 (19)</td>
<td>7.327 ± 0.644 (81)</td>
</tr>
<tr>
<td>MH</td>
<td>0.421 ± 0.174 (8)</td>
<td>4.806 ± 0.773 (92)</td>
</tr>
<tr>
<td>SCFM</td>
<td>0.235 ± 0.104 (35)</td>
<td>0.432 ± 0.049 (65)</td>
</tr>
<tr>
<td>YEM</td>
<td>9.001 ± 1.848 (95)</td>
<td>0.517 ± 0.262 (5)</td>
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**1H-NMR spectroscopy of EPOLS produced in biofilms developed on cellulose membranes.** 1H-NMR spectroscopy of EPOL solutions purified from biofilm biomass recorded after protease treatment revealed the type of polysaccharides present in the matrices. In Fig. 1 the expansion of the proton anemic region for each sample is shown; comparison with known spectra revealed that the EPOL galactan-Kdo (Cescutti et al., 2003) was present in the biomass produced on all media used, while cepacian (Cescutti et al., 2000) was very
abundant only in the matrix from YEM, present in traces in MH medium and not detected in the other media. Galactan-Kdo is a linear polysaccharide containing three galactose residues, one 3-deoxy-D-manno-oct-2-ulosonic acid (Kdo) and one acetyl group per repeating unit, a structure very different from that of cepacian (Fig. S4). The resonances at 4.64 ($J_{H1,H2}=7.94$) and 5.16 p.p.m. (labelled ‘New-S’ in Fig. 1) indicated two signals that did not belong to any known EPOL of the BCC; since the intensity of both these resonances increased concurrently, most likely they both belong to a single, yet-unknown EPOL. In general, biomass rich in proteins was more adherent to the membrane and less spread out, while the biomass richest in cepacian occupied the whole membrane, but was less adherent.

**Fig. 1.** $^1$H-NMR spectra anomeric regions of the EPOLs isolated from the biomass produced on membranes on the solid media KA (a), LBG (b), MH (c), SCFM (d) and YEM (e). CEP, Resonances due to cepacian; G-K, resonances due to galactan-Kdo; New-S, new signals due to yet-unknown EPOL.
The constant presence of galactan-Kdo in the biomass from all media strongly suggested a role in the biofilm architecture.

**Biofilm formed on glass slides and test tube walls**

When *B. cenocepacia* BTS2 was grown in test tubes each containing a glass slide, biofilms were formed on the glass at the liquid/air interface in all media used and, with the exception of YEM, also on the portion of the slide completely immersed in the liquid medium and on the test tube walls, even on the parts not in contact with the liquid, thus indicating relevant microbial adhesive and motility characteristics (see Fig. S5). The biomass was always less abundant than that produced on cellulose membranes and showed a different degree of adhesiveness, which again was medium-dependent. In fact, biofilms formed in YEM medium were easily removed, while the ones produced in the other media stuck more strongly to the glass; remarkably, biofilms formed in SCFM were the most adherent to the glass.

**Structural analysis of EPOLs in biofilms produced on glass slides and test tube walls.** Biofilms formed on glass slides and on test tubes were collected and analysed separately. Although 10 glass slides were used for each growth medium, the biomass recovered was only sufficient for $^1$H-NMR experiments. The identity of the EPOLs was discovered by comparing the anomeric regions (5.7–4.5 p.p.m.) of their spectra with the same region of known EPOLs (Cuzzi et al., 2014). Despite weak signals due to the low amounts of samples, qualitative compositional data could be collected, and these are reported in Table 2. The spectra also showed non-saccharidic resonance peaks, probably due to proteins and lipids. The $^1$H-NMR spectra of the matrices developed on glass slides were very similar to those developed on the test tube walls, and in all samples signals attributable to galactan-Kdo were present. As an example, the $^1$H-NMR spectra of the matrices developed on glass slides and test tube walls in MH medium are shown in Fig. 2; apart from the signals belonging to galactan-Kdo (labelled with G-K), an intense resonance at 5.37 p.p.m. and a small one at 4.96 p.p.m. (labelled GLY), typical of $\alpha$-anomeric protons, were detected, which were also present in the spectra of matrices produced on KA and YEM. 2D-NMR experiments (correlation spectroscopy, TOCSY and HSQC; spectra not shown) led to the assignment of chemical shifts (Table 3), attributing the signal at 5.37 p.p.m. to H-1 of a (1→4)-linked $\alpha$-glucan, and that at 4.96 p.p.m. to a (1→6)-linked $\alpha$-glucose (Glc), thus identifying the EPOL as glycogen, in good agreement with published data (Barclay et al., 2012). A value of 5 % branching was calculated from integration of the areas of these two signals. An $^1$H-NMR spectrum was recorded again after keeping the sample for 1 month at 4 °C; two signals at 5.22 ($J_{H1,H2}=3.8$ Hz) and 4.64 p.p.m. ($J_{H1,H2}=7.9$ Hz) appeared (spectrum not shown), and they were assigned to the $\alpha$- and $\beta$-reducing ends of glucose, respectively, indicating degradation due to the

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<th>Table 2. Molecular content of biofilms developed on glass slides and on test tubes walls in five different media, as determined by $^1$H-NMR spectroscopy</th>
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<td><strong>Support</strong></td>
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*Present in higher amount than on the other support.
action of a hydrolytic enzyme. The degraded sample was reduced and permethylated before being analysed by ESI-MS (data not shown); the spectra showed disaccharides and trisaccharides, thus confirming extensive depolymerization. In order to confirm the presence of a glycogenase, another portion of the matrix was

Table 3. $^1$H and $^{13}$C chemical shifts of glycogen detected in the biofilm produced in MH broth

<table>
<thead>
<tr>
<th>Glycogenase</th>
<th>$^1$H (p.p.m.)</th>
<th>$^1$C (p.p.m.)</th>
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<tr>
<td>4)-$\alpha$-D-GlcP-(1→4)</td>
<td>5.37 3.62 3.95 3.64 3.83 3.81, 3.86</td>
<td>102.51 74.39 76.12 79.96 74.06 63.37</td>
</tr>
<tr>
<td>4)-$\alpha$-D-GlcP-(1→6)</td>
<td>4.96 3.61</td>
<td>101.33</td>
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dissolved in water and boiled for 15 min to denature the enzyme; $^1$H-NMR spectra recorded 1 month apart did not show any significant change, thus confirming the presence of a specific enzyme. Linkage analysis of the undegraded sample confirmed the NMR data, showing terminal non-reducing glucose, 1,4-linked glucose, and 1,4,6-linked glucose (branched residue); 6% of branching was estimated from integration of the peak areas of the two latter residues, in good agreement with NMR data.

**Morphology of biofilms produced on glass slides.** The morphology of biofilms developed on glass coverslips was investigated by means of CLSM, AFM and PCM. CLSM was used to observe 2- and 5-day-old biofilms developed in all five different media after staining with acridine orange. Acridine orange was used as a fluorescent biofilm indicator as it stains cells as well as nucleic acids, which can be a component of the extracellular matrix (Branda et al., 2005). Here, this technique was used to qualitatively illustrate the variety of biofilm structural formations developed in the different culture media. The images obtained after 2 (data not shown) and 5 days of growth (Fig. 3) indicated that the biofilm morphology was indeed medium-dependent. In fact, for both incubation times, the acridine orange stain revealed single cells on the glass incubated in YEM medium, suggesting the absence of extracellular nucleic acids; in LBG and MH media, biofilms appeared with a discontinuous structure, while in KA and SCFM media the coverslips were homogeneously covered by biofilm and the biofilm developed in SCFM was more dense and compact.

In order to distinguish between live and dead bacteria, glass slides were incubated in MH and YEM for 2 and 5 days, followed by staining with the LIVE/DEAD BacLight kit. CLSM revealed that in YEM a significant number of bacteria were already dead after 2 days of growth, while in MH live bacteria were predominant (Fig. 4), even after 5 days of incubation. In order to gain a better idea of the relative amount of dead and live cells, the fluorescence intensity of both dyes was measured. Results are presented as the ratio between the intensity of red fluorescence and the sum of the red and green fluorescence, in order to express the data as the number of dead cells divided by the total cell number. The values obtained (see Table S2) showed that in YEM medium the ratio did not change from 2 to 5 days, while in MH medium the ratio increased, indicating a relative increment of dead cells at day 5. Planktonic growth at day 5 was $3.7 \times 10^8$ c.f.u. ml$^{-1}$ in YEM medium, while no live planktonic cells were detected in MH medium. Inspection of CLSM images in Fig. 4 showed that, although both media support growth, in YEM biofilm production is limited; one possible explanation may be related to the smaller amount of proteins produced in YEM (Table 1) than in the other media, suggesting the lack of important components for adhesion to the support and the lack of an adhesion role exerted by cepacian. For comparison, biofilms were developed in 96-well polystyrene microtitre plates in YEM and MH media, and quantification of the biomass, followed by staining for the conventional crystal violet assay, led to calculation of the biofilm index. This amount is given by the absolute absorbance of crystal violet at 570 nm normalized by the OD$_{590}$, which is related to the number.

![Fig. 3. CLSM images of 5-day-old biofilms of *B. cenocepacia* BTS2 grown in static conditions in the following media: KA (a), LBG (b), MH (c), SCFM (d) and YEM (e). Glass slides were stained with acridine orange. The large panels represent the $x$-$y$ planes while the side panels represent the $y$-$z$ planes referring to the central position of the $x$-$y$ planes. Scale bars correspond to 20 μm.](image-url)
of planktonic cells present. The biofilm index was always higher for the sample in YEM (Table S3), but this was due to less bacterial growth rather than to more abundant production of biofilm.

Biofilms developed on glass coverslips were also investigated by PCM. The images (Fig. S6) show compact and thick biomasses formed in LBG and MH media, while bacteria covered the abiotic surface more evenly in KA and SCFM media. On the contrary, in YEM medium there was scanty biofilm production and the presence of elongated bacterial cells probably indicated stressful conditions (Young, 2006).

AFM was successfully used to determine the thickness of the dehydrated biofilm polymeric matrix in order to avoid contribution by water, which greatly influences the thickness by swelling the matrix. After dehydration,
glass coverslips were partly scratched with a scraper to create a reference surface free of biomass, for the subsequent measurement of the matrix thickness. The AFM images obtained are shown in Fig. 5 and the data, expressed as height values calculated as mean ± SD, revealed that the matrix thickness is medium-dependent, with the lowest value reported for YEM medium, and the highest for LBG and KA media. The matrix thicknesses measured by AFM were: in KA, 21.8 ± 7.6 nm; in LBG, 17.9 ± 3.7 nm; in MH, 7.5 ± 0.8 nm; in SCFM, 7.6 ± 2.0 nm; in YEM, 3.6 ± 1.1 nm. Although the dehydration step used for sample preparation greatly lowered the thickness of the biofilm matrices with respect to that of fully hydrated biofilm (Heydorn et al., 2000), the relative values obtained clearly indicated the different biofilm development. As a matter of fact, dehydration has a dramatic impact on the volume occupied by water–macromolecule systems: for example, alginate gel beads exhibited a 500-fold shrinkage upon dehydration (Skjak-Braek et al., 1989).

**CONCLUSION**

This investigation aimed at determining the influence of different growth media and solid supports on the composition of biofilm matrix formed by *B. cenocepacia* strain BTS2, a CF clinical isolate. Chemical analysis, NMR spectroscopy and three different types of microscopy were used to investigate the biofilms developed on five different media: KA, LBG, MH, SCFM and YEM. Analysis of the biofilms formed on semi-permeable cellulose membranes deposited on agar plates showed that the matrix produced on YEM medium was very different from those developed on the other four media: it contained much more glycan than protein, and the principal EPOL present was cepacian, which is associated with the mucoid phenotype. In the other four media, the matrix contained more proteins than glycans and the main EPOL was galactan-Kdo. Although deposition of planktonic bacteria on cellulose membranes is obviously different from spontaneous adhesion of bacteria to a glass surface, the former proved to be a useful set-up for the production and recovery of...
adequate amounts of biofilm. At the same time, the matrix formed on glass slides was subjected to $^1$H-NMR spectroscopy and it was found to contain the EPOS galactan-Kdo, thus showing a high degree of analogy with the biofilms obtained from cellulose membranes and strongly suggesting a role for this EPOS in biofilm architecture. One main difference between these two supports is the presence of glycogen on glass slides and test tube walls in MH and KA media. Glycogen production by 

*Pseudomonas fluorescens* sessile and planktonic cells has been reported in the recent literature (Quiles et al., 2012; Quiles & Humbert, 2014), but its role in biofilm is not yet clearly established. Moreover, our experimental findings indicated that glycogen was secreted together with a degrading enzyme, and it is well known that in general, enzymes are secreted to depolymerize the matrix during the dispersion stage. CLSM, PCM and AFM images indicated that again the biofilm formed in YEM was very different from those in the other four media. In fact, on the glass slides immersed in YEM medium, the thickness of the matrix was the smallest, no extracellular nucleic acids were detected by acridine orange staining, about half of the cells were dead and they were also elongated, indicating stressful conditions. However, YEM is a peculiar medium, containing 2% mannitol and only 0.2% yeast extract, and may be unsuitable for full biofilm growth. At the same time, mannitol is the carbon source associated with cepacian production (Denman et al., 2014), but how and why it promotes its biosynthesis still needs to be clarified. It is also very interesting that, even when bacteria lacked the mucoid appearance, as in the growth developed on KA, LBG, MH and SCFM media, EPOSs were produced, even if to a lesser extent than in YEM. Therefore, non-mucoid BCC strains may still be capable of polysaccharide biosynthesis and of forming biofilms. A similar finding was described for non-mucoid strains of *P. aeruginosa*, lacking alginate production, but still capable of forming biofilm containing two other structurally different polysaccharides (Mann & Wozniak, 2012). Furthermore, several other articles have shown that the polysaccharides involved in biofilm formation are very diverse in structure and are not only restricted to a few well-known macromolecules such as alginate, cellulose and poly-β-1,6-GlcNAc. An example among the BCC bacteria is the reference strain *Burkholderia multivorans* C1576; when it was grown on cellulose membranes deposited on YEM medium it produced cepacian, while on MH medium it biosynthesized a novel EPOS, containing rhamnose and mannose residues (Cuzzi et al., 2014; Dolfi et al., 2015). The polysaccharides involved in matrix formation probably share some common macromolecular properties, like the capability of interacting with proteins, lipids and other molecules in the biofilm, that render them particularly suitable for forming a polymeric network. Determining these common characteristics will contribute to elucidating biofilm structure and help in finding novel anti-biofilm strategies, avoiding the use of conventional antibiotics against which resistance is increasing (WH0, 2014).

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