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Evaluation of biofilm-forming capacity of *Moraxella bovis*, the primary causative agent of infectious bovine keratoconjunctivitis

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ABSTRACT

The difficulties in preventing and treating infectious bovine keratoconjunctivitis (IBK) and the consequent impact on the cattle industry worldwide emphasize the need to better understand this infectious process along with the biology of *Moraxella bovis*, its primary causative agent. Although there is increasing evidence that bacterial biofilms participate in a variety of ocular infections by direct biofilm formation on the surfaces of the eye, IBK has not been considered as a biofilm-based disease so far, and even more, no information is currently available regarding the ability of *M. bovis* to adopt a biofilm lifestyle. In the present research, we demonstrated the capacity of *M. bovis* clinical isolates and reference strains to form biofilms on different abiotic surfaces and culture conditions, and provided qualitative and quantitative information on the biofilm growth and architecture of mature biofilms. In addition, our data indicated that the type IV pili play a critical role in the biofilm formation *in vitro*. Most significantly, we proved that through exposure to $MgCl_2$ type IV pili are removed from the cell surface, not only preventing *M. bovis* biofilm formation but also disassembling preformed biofilms. These results could constitute a new approach in the understanding of *M. bovis* colonization process in cattle eye and/or nasal cavity, and may aid in the development of future antimicrobial strategies for the control of IBK.

1. Introduction

Moraxella bovis is the primary etiologic agent of infectious bovine keratoconjunctivitis (IBK), the most widespread and highly contagious ocular disease affecting cattle (Brown et al., 1998). The economic impact of IBK in the cattle industry results from the lower level of production because of a reduced weight gain of the animals, the decline in milk production (Webber and Selby, 1981b), and the increase in medical treatment costs. Two virulence factors constitute the major determinants of *M. bovis* pathogenicity: the type IV pili (TFP) – filamentous surface appendages – and a cytotoxin (hemolysin and cytolysin) that damages the cornea (Angelos et al., 2001; Beard and Moore, 1994; Lepper et al., 1995). TFP represent one of the most fundamental surface-associated filaments promoting the attachment to host epithelial cells in several Gram-negative bacteria—*e.g.*, *Neisseria* spp., *Pseudomonas* spp., and *Moraxella catarrhalis* (Helaine et al., 2005; Luke et al., 2007; Semmler et al., 1999). In addition TFP generate

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a form of surface translocation called twitching motility (Semmler et al., 1999). Only piliated *M. bovis* cells were found to be efficient to adhere to corneal epithelial cells and for causing persistent ocular infection and disease (Jayappa and Lehr, 1986).

M. bovis initiates *in vivo* infections by attaching to the corneal epithelial surface (Ruehl et al., 1993). In few days the disease symptoms become evident with deep ulcerations appearing in the centre of the cornea (Weech and Renshaw, 1983). In some animals the ulcers are reduced to scars within 7 days, while in others a complete recovery occurs in three to five weeks. Asymptomatically infected cattle may harbour *M. bovis* in their nasal cavities for long periods of time, thus serving as carriers and allowing the persistence of IBK at particular sites from one year to the next (Pugh et al., 1980). Therefore, since the protection efficiency of the commercial vaccines is low, thus prevention usually fails (Smith et al., 1990; Webber and Selby, 1981a), and the treatment with several antibiotics is not always successful in eradicating the carrier state (McConnel et al., 2007; Sargison et al., 1996), the progression of the infection requires repetitive and costly antibiotic applications (Alexander, 2010). A remarkable aspect of the infection that may be contributing to the persistence and the difficulty to eradicate *M. bovis* from its host – and one that, to our knowledge, has not yet been considered – is that this organism might adopt a biofilm mode of growth on either the cornea and/or on the nasal cavity surfaces. A biofilm is a structured association of bacteria embedded in a self-produced polymeric matrix. This life-style confers upon pathogens several advantageous traits linked to persistence, virulence and resistance to environmental stress, host defences, and antimicrobial compounds (O'Toole and Kolter, 1998b; O'Toole et al., 1999). Although biofilms have been seen to be responsible for a variety of ocular infections (Elder et al., 1995; Murugan et al., 2010; Zegans et al., 2002), *M. bovis* has traditionally been studied *in vitro* as free-floating individual cells. In the present work we demonstrate that *M. bovis* is capable of growing as biofilm on abiotic surfaces under different culture conditions. We found that *M. bovis* TFP are critical for the development and maintenance of a structured biofilm. Within this context we demonstrated an increased antibiotic resistance for *M. bovis* mature biofilms. Finally, we proved that $MgCl_2$ is a strong inhibitor and disassembling agent of the biofilms formed by *M. bovis* on abiotic surfaces.

2. Methods

2.1. Microorganisms and growth conditions

Two clinical isolates – *M. bovis* 1194 03, a "local" (Argentina) isolate, recovered from a cow with IBK, and *M. bovis* 5-Butch, a "non-local" isolate, recovery from an infected animal in Germany were used in this study. Both strains were kindly provided by Dr. G. Zielinsky (INTA, Córdoba, Argentina) and previously well characterized (Prieto et al., 1999). In addition, two reference strains, *M. bovis* ehi 70.39 and *M. bovis* bhe 64.9 (Collection of Pasteur Institute, Paris, France) were also used in this research. For the four strains, the piliated wild type (WT) forms along with their nonpiliated, nonreverting TFP(−) variants were obtained and used throughout this work. The nonpiliated variants arise spontaneously upon subculturing each piliated pathogenic strain on Tryptic Soy Agar (Merck, Germany) supplemented with 7% (v/v) defibrinated horse blood (TSAB medium) (Jayappa and Lehr, 1986; Moore and Rutter, 1987, 1989; Sandhu et al., 1974; Wilt et al., 1990). Nonpiliated colonies are distinguished and selected by their morphologic differences. The stability of these variants was confirmed by subsequently culturing on TSAB medium for more than 15 passages. Stock cultures were prepared with cells grown on TSAB for 24 h at 37 °C, and kept at −80 °C in 20% (v/v) glycerol. After thawing, the presence or absence of pili in each stock culture was confirmed by electron microscopy, enzyme-linked immunosorbent assay, and Fourier-transform infrared (FT-IR) spectroscopy (Bosch et al., 2010; Prieto et al., 2003, 2008). Furthermore, at the end of biofilms cultures, the piliation level of sessile cells was controlled by ELISA technique and/or FT-IR spectroscopy. For liquid cultures, planktonic cells were grown in Erlenmeyer flasks under agitation in a rotary shaker at 160 rpm in brain-heart infusion (BHI; Merck, Germany) broth. In all the experiments, cultures were inoculated to give an initial optical density at 650 nm (OD_{650}) of 0.2.

2.2. Biofilm cultures

(1) Microtiter-plate biofilm assay: Biofilm growth in microtiter plates was assayed as described by O'Toole and Kolter (O'Toole and Kolter, 1998b). After 24 h of incubation in BHI broth the cells that had adhered to the walls and/or bottoms of the wells were either washed and stained with crystal violet (CV; 0.1%, w/v) or else scraped, collected, and gently washed with distilled water for FT-IR-spectroscopy analysis (*cf.* below).

(2) Biofilm formation in packed-bed column reactors: Bacteria were grown in glass-column bioreactors (∅ = 3 cm, *h* = 18 cm) packed with polypropylene beads (10 g, ∅ = 4.2 mm, *h* = 2 mm, with an average density of 0.901 g cm^{-3}; Petroken, Argentina) used as growth support, as previously reported (Bosch et al., 2006). The reactors were inoculated with 10 ml of a planktonic culture of *M. bovis* (to an OD_{650} of 0.2). After 4 h of incubation, needed for cell attachment to the beads, the bioreactors were operated under a continuous flow of fresh BHI medium (0.02 ml min^{-1}) at 37 °C (Bosch et al., 2006) and constant aeration (0.1 l min^{-1}). At 24, 48, 72, or 96 h, the bulk-liquid phase of each reactor was collected and the polypropylene beads recovered. The biomass adhered to the beads was quantified by crystal violet (CV) staining as previously described (O'Toole and Kolter, 1998b). When indicated, the bulk-liquid phase was centrifuged and both the pelleted planktonic cells and the resulting supernatant fraction were subjected to immunoblot analysis. When either $MgCl_2$ or NaCl were added to the BHI medium, the salts were used at final concentrations of 0.5, 1.0, or 2.0% (w/v).

(3) Continuous flow chamber culture system: *M. bovis* cells were grown in continuous flow chambers (*L* = 75 mm;

$W = 25$ mm; $H = 3$ mm) on glass coverslips attached to the cell chamber's floor with acrylic adhesive (Serra et al., 2007). For each culture the chamber was inoculated with 5 ml of *M. bovis* cell suspension at an OD_{650} of 0.2. Bacteria were left to attach for 4 h at 37 °C before initiating the flow of nutrients. Fresh BHI broth was pumped through the flow chambers at 0.01 ml min^{-1}. Cultures were incubated at 37 °C for 24, 48, and 72 h. At each of these time points, the adhered cells were stained with SYBR Green I (Molecular Probes, Eugene, USA) and visualized by fluorescence microscopy or confocal laser scanning microscopy (CLSM). When indicated, either $MgCl_2$ or NaCl was added to the BHI medium at final concentrations of 0.5, 1.0, or 2.0% (w/v).

2.3. Fluorescence microscopy

Fluorescence-microscopy images of *M. bovis* grown adhered to surfaces were obtained with a Leica DMLB microscope (Leica Microsystems, Wetzlar, Germany) equipped with a charge coupled device (CCD) camera. Visualization was conducted at 400× or 1000× magnification. The Leica IM50 software was employed to capture and display the images. The bacteria growing attached to the glass coverslip base of the flow chambers were gently washed with phosphate buffer saline solution to remove nonattached or loosely attached cells, fixed with 4% (v/v) paraformaldehyde for 15 min and stained with SYBR Green I (final dilution, 1:500,000) for 30 min.

2.4. Confocal laser scanning microscopy

Confocal microscopy images were obtained on a Leica TCS SP5 spectral confocal inverted microscope (Leica Microsystems, Wetzlar, Germany). *M. bovis* cells were grown for 72 h on the glass-coverslips adhered to the continuous-flow chambers and the attached cells stained with SYBR Green I as described above. The detection of the emitted light was performed by sequential scanning with optimal settings for the use of SYBR Green I (a 488-nm excitation by an argon-laser and a 522-nm long-pass emission). In each experiment, the images were obtained at randomly selected points with a 20× objective of numerical aperture 0.7 and at 1 μm *z*-intervals down through the biofilm. Stacks of *z*-section images were viewed and processed by means of the Las Af Lite version 2.2.1 and/or the Imaris software.

2.5. Adhesion to bovine corneal epithelial cells

An established line of cultured bovine corneal-epithelial cells (kindly provided by INTA Castelar, Argentina) was used for adhesion studies of the *M. bovis* strains. Corneal cells were cultured in Dulbecco's modified Eagle's minimum essential medium (Gibco, USA; Annuar and Wilcox, 1985) supplemented with 10% (v/v) foetal bovine serum, 100 μg streptomycin ml^{-1}, and 100 μg penicillin ml^{-1} (Sisti et al., 2002). For adhesion assays, cells were seeded on glass coverslips placed in a 24-well tissue culture plates (Nunc, Roskilde, Denmark), and incubated to 70–80% confluence. The resulting monolayers of corneal cells were incubated at 37 °C for 30 min with the *M. bovis* piliated (WT) and the TFP(−) strains (100 bacteria per cell) in the presence of a rabbit polyclonal antiserum raised against TFP (dilution 1:1000) (Prieto et al., 2003), or a rabbit preimmune serum (dilution 1:1000), in BHI medium. After incubation, the corneal cells with adhered bacteria were washed, fixed with 4% (w/v) paraformaldehyde, washed again, and stained with a 0.1% (w/v) solution of CV. Coverslips were examined by optical microscopy at 1000× magnification. Approximately 50 cells were examined surveyed to calculate the number of adherent bacteria per epithelial cell. All experiments were done at least three times in duplicate.

2.6. Fourier transform infrared (FT-IR) spectroscopy

The material accumulated at the air–liquid interface on the inner wall of the shaken flask cultures after 15 h of growth was *scraped off* and gently washed three times with distilled water to remove culture medium. Suspensions of this material, of that scraped from the microtiter plate well surfaces, and of planktonically grown bacteria – all diluted to approximately a OD_{650} of 8.0 – were placed on ZnSe windows and dried in a desiccator for 45 min (Naumann, 2000). Absorption transmission spectra were acquired between 4000 and 650 cm^{-1} with a FT-IR spectrometer (Spectrum One, PerkinElmer, USA) with a 6 cm^{-1} spectral resolution and the co-addition of 64 scans. To avoid interference from possible biomass variations among the different samples, spectra were vector normalized over the whole spectral range (Naumann, 2000). OPUS software version 4.2 (Bruker Optics GmbH, Ettlingen, Germany) was used for data pre-processing and spectral calculations. Three independent cultures were performed to analyze both the interphase material and the planktonically grown bacteria.

2.7. Antibiotic resistance assays

For determining the minimum bactericidal concentration for planktonically growing bacteria (MBC-P), 200 μl of planktonic cell suspension (OD_{650} of 0.2) were incubated in 96-well microtiter plates with different concentrations of ampicillin, chloramphenicol, gentamicin, and oxytetracycline. After 24 h of incubation at 37 °C the viable colony-forming units were determined by serial-dilution plating on TSAB plates. The MBC-P was defined as the minimal concentration of antibiotic required to kill all planktonic bacteria. The minimum bactericidal concentration against biofilm-grown cells (MBC-B) was obtained as previously described (Mishra et al., 2005). Stated in brief, an overnight culture of *M. bovis* cells was adjusted at an OD_{650} of 0.2 with fresh medium and aliquoted into microtiter plates (200 μl per well). The plates were incubated for 24 h at 37 °C to allow biofilm formation. The supernatants were then removed and fresh medium added containing the antibiotics at increasing concentrations, and the plates were incubated for an additional 24 h. The antibiotic-containing supernatants were next replaced by fresh BHI broth lacking antibiotic and the plates incubated for a final

24 h before transfer of the supernatants to TSAB agar plates for viability analysis. Thus, under these conditions, if any sessile cell remains viable it will grow and seed the fresh medium. The MBC-B is thus defined as the minimal concentration of antibiotic required to kill all of the bacteria grown as a biofilm under the conditions of this assay.

2.8. Immunoblot analysis

For dot-blot analysis, WT *M. bovis* cells were cultured as biofilms in a glass-column system in the presence or absence of 1% (w/v) $MgCl_2$ or NaCl for 72-h. The bacteria recovered from the bulk-liquid phase were then washed by centrifuging and suspended in PBS. The cell-free supernatant from this centrifugation step was filtered through a 0.2-μm-pore-size membrane, and concentrated 100-fold through the use of 10,000 MWCO Amicon Centricon™ filtration devices (Millipore, Billerica, USA). Forty-μl aliquots of cell suspensions or cell-free supernatants were spotted onto activated polyvinylidene-difluoride (Immobilon, Millipore, Billerica, USA) membranes and air dried. The polyvinylidene-difluoride sheets were then processed as previously described (Prieto et al., 2003).

2.9. Statistical analysis

The statistical analyses performed with the SPSS statistical software (version 17.0; SPSS Inc., Chicago, IL, USA) were the two-way ANOVA for time-course evaluations and the Student *t*-test for comparison between groups. Values were considered significantly different if $p < 0.05$.

3. Results

3.1. Attachment of M. bovis to bovine corneal cells

Adherence to host epithelial cells is the initial and critical step of the IBK infectious process. Accordingly, we first examined if the *M. bovis* strains used throughout this study had the ability to attach to bovine corneal epithelial cells. For this purpose we assayed the adhesion of the *M. bovis* WT strains to the cultured bovine corneal-epithelial cells in the presence and absence of anti-TFP antibodies. Fig. 1a and b shows the results obtained for the *M. bovis* 1194 03 piliated cells as being representative of the results we observed with the other clinical and reference strains. Whereas in the presence of preimmune serum, the piliated strains adhered significantly to the bovine corneal epithelial cells to form aggregates, antibodies against TFP inhibited the adhesion of the bacteria to the eukaryotic cells almost completely. Furthermore, when the TFP(−) variants were incubated with the corneal epithelial cells in the presence of the preimmune serum, only a slight adhesion to the cells occurred (Fig. 1c). These results, obtained with wild type *M. bovis* and TFP(−) variants are in accordance with those reported in literature for other clinical isolates (Annuar and Wilcox, 1985) and furthermore indicate that high levels of adhesion to corneal cells *in vitro* are correlated with the presence of TFP.

3.2. M. bovis forms biofilms on abiotic surfaces

During the growth of the *M. bovis* WT strains in Erlenmeyer flasks we detected a progressive accumulation of biomass on the inner wall of the flasks at the air–liquid interface. Fig. 2a shows the visible thick bacterial ring (indicated by an arrow) formed by *M. bovis* 1194 03 WT strain on the wall of flasks after 15 h of incubation at 160 rpm, which remained firmly adherent and even became thicker during the cultivation period. As expected, *M. bovis* WT strains also grew in the liquid phase as free-swimming cells and as small suspended aggregates. The total amount of biomass accumulated in the liquid phase, however, was lower than that on the flask walls. When the TFP(−) variants were cultivated under the same experimental conditions, a homogeneous cell suspension was obtained; and the bacterial ring was not present (Fig. 2a). The material accumulated on the wall during the growth of piliated WT *M. bovis* cells was scraped from the surfaces aseptically, homogenized to create a uniform cell suspension, serially diluted, and plated in TSAB medium. The confirmed bacterial growth after the serial cultivation of this material indicated that the rings consisted in viable bacteria highly compacted in aggregates (data not shown). The surface-adherence property displayed by WT *M. bovis* strains was suggestive of their ability to form biofilms. In order to demonstrate if this adherent material resembled a typical biofilm population, we gently washed the scraped-

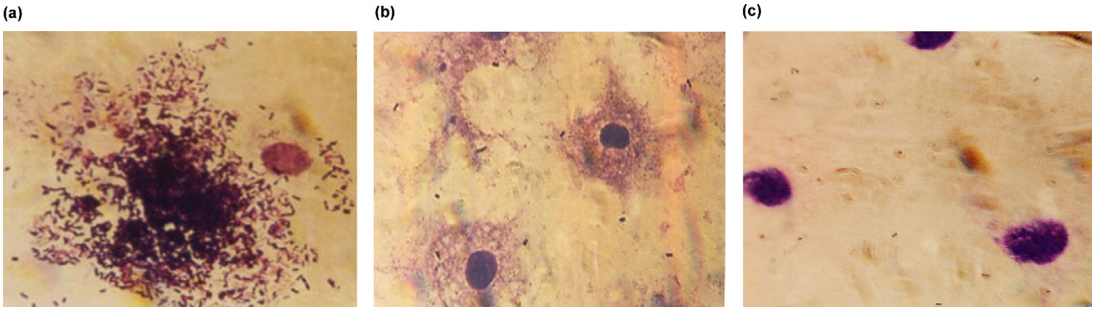

Fig. 1. Optical microscopy showing adhesion of *M. bovis* 1194 03 to bovine cornea-epithelial cells. Wild-type bacterial cells (WT) incubated with bovine corneal-epithelial cells in presence of: (a) preimmune rabbit serum, (b) rabbit polyclonal anti-TFP serum and (c) an *M. bovis* nonpiliated variant TFP(−) population incubated with bovine corneal-epithelial cells (1000× magnification). These results are representative of those obtained in parallel experiments with the clinical isolate *M. bovis* 5-Butch and the two reference strains *M. bovis* ehi 70.39 and *M. bovis* bhe 64.9 strains.

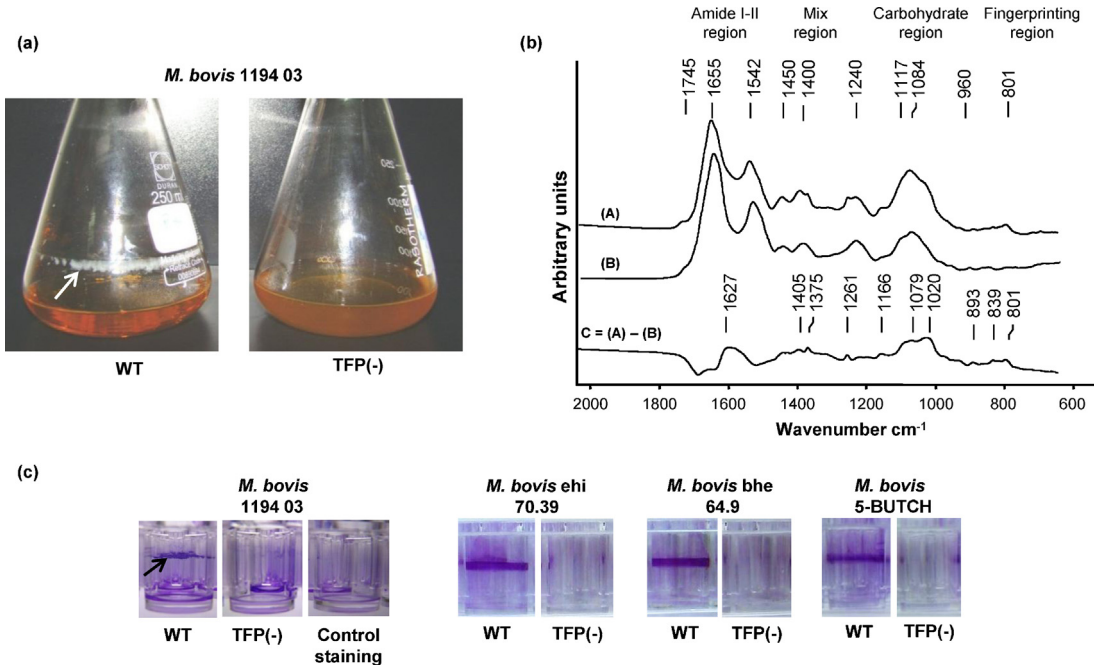

Fig. 2. Bacterial biofilms formed by the different *M. bovis* strains wild type (WT) and their corresponding nonpiliated variants TFP(−) on diverse surfaces. (a) Cultures of *M. bovis* 1194 03 wild-type strain and its TFP(−) variant in Erlenmeyer flasks incubated for 15 h. The interface material adhered to the inner wall of the flask is indicated with a white arrow. (b) Infrared spectrum of the interface material (A), infrared spectrum of planktonically grown cells recovered from the liquid medium (B). The spectra were normalized over the entire spectral range and the difference spectrum (A)–(B) is shown by the curve C. (c) *M. bovis* 1194 03 strain, *M. bovis* 5-BUTCH strain, the *M. bovis* reference strains (ehi 70.39 and bhe 64.9; Collection of Pasteur Institute, Paris, France), and their corresponding nonpiliated variants grown on polystyrene microtiter-well surfaces for 24 h. The CV-stained biofilms are indicated by black arrows.

off interface material by centrifuging to remove spent culture medium and analyzed the substance by FT-IR spectroscopy. Fig. 2b shows an infrared spectrum of this material recovered after 15 h of culture (spectrum A) and a spectrum of the bacterial counterparts growing planktonically in the liquid phase (spectrum B). The spectra were normalized over the whole range (4000–650 cm^{-1}) and the difference spectrum calculated (curve C in Fig. 2b). Spectra A and B revealed the typical spectral features of bacteria (Naumann, 2000). The difference spectrum, C, showed a broad band at the region dominated by carbohydrate vibrations (1200–900 cm^{-1}) with shoulders at 1166, 1079, and 1020 cm^{-1} (reflecting C–OH stretching modes and C–O–C and C–O ring vibrations, respectively) along with bands in the 900–800 cm^{-1} region, which is known to be sensitive to glycosidic-type linkages vibrations (Synytsya et al., 2003). Other differences between the A and B spectra involved an increase in the relative intensity of the bands corresponding to the vibrational modes of the carboxylate ion and the C–O–C moiety of esters (1627, 1405, 1372, and 1261 cm^{-1}, respectively) (Serra et al., 2007). These latter bands of increased intensity had previously been assigned to uronic-acid-polysaccharides and some associated spectral bands referred to as biofilm-growth markers (Bosch et al., 2006; Serra et al., 2007). A bacterial-film development on the flask surface at the air–liquid interface under continuous agitation conditions had been reported for several biofilm producing bacteria (Friedman and Kolter, 2004; Russo et al., 2006). Nevertheless, to the best of our knowledge, nothing had been reported about the capacity of *M. bovis* to adhere to surfaces and produce a polysaccharide-enriched matrix. We therefore became interested in examining the capacity of *M. bovis* to produce biofilms on other types of surfaces, such as on microtiter plates (O'Toole and Kolter, 1998b). Consistent with what had been observed in shaken flask cultures, CV staining of the microtiter wells 24 h after inoculation with *M. bovis* WT strains revealed the presence of a biomass ring at the air–liquid interface region of the wells representative of biofilms, which deposition was not observed with the TFP(-) variants (Fig. 2c).

3.3. Requirement of type IV pili for *M. bovis* attachment and mature biofilm formation on abiotic surfaces

As TFP are the major cell surface adhesins of *M. bovis* and have been reported to be essential for establishing ocular infection (Annuar and Wilcox, 1985), we hypothesized that those filaments could also make an essential contribution to *M. bovis* biofilm formation. To test this possibility, we compared the capacity of *M. bovis* WT and corresponding TFP(−) strains to attach and form a biofilm on polypropylene beads packed in column bioreactors. The attachment was assayed under static conditions from 30 min to 4 h and the biofilm growth examined under a continuous flow of nutrients for 72 h. As early as 30 min after inoculation, the amount of biomass adhered to the beads of the two WT clinical strains (*M. bovis* 1194 03 and *M. bovis* 5-BUTCH) was twice that exhibited by their respective TFP(−) variants (Fig. 3a). During the following

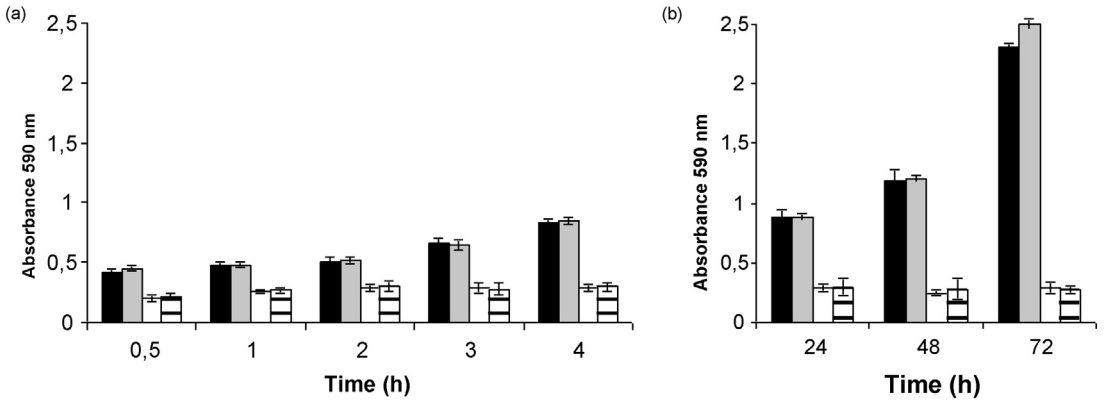

Fig. 3. Attachment and biofilm formation by *M. bovis* clinical strains on polypropylene beads cultivated in glass-column bioreactors. (a) Attachment assayed under static conditions. (b) Biofilm development obtained under a continuous flow of nutrients (*cf.* Section 2). The adhered biomass was quantified by CV staining. The data are the means ± standard deviations of three independent experiments. *M. bovis* 1194 03 wild type (WT) strain (■) its TFP(−) variant (□), and *M. bovis* 5-BUTCH wild type (WT) strain (▩) and its TFP(−) variant (▤).

3.5 h, whereas the WT strains doubled in biomass while attached to the beads, the TFP(−) variants showed no significant increase in the adhered biomass. When the nutrient flow was turned on, the sessile WT biomass started to increase significantly over time for up to 72 h of culture (Fig. 3b). In contrast, under the same experimental conditions, the TFP(−) variants showed only a slight increase in the adherent biomass. A similar behaviour was observed when the reference strains were assayed (data not shown). These results parallel those reported for other bacteria in which attachment and biofilm development are TFP dependent processes (Klausen et al., 2003; Luke et al., 2007).

To gain insight into the role of the TFP in the structural dynamic of biofilm biogenesis, the biofilm development of the *M. bovis* 1194 03 WT strain and the corresponding TFP(−) variant were monitored in continuous-flow chambers. At the 4-, 24-, 48-, and 72-h time points, the biofilms were stained with SYBR Green I and observed by fluorescence microscopy. After 4 h of incubation, the WT cells appeared on the surface forming aggregates (Fig. 4a, upper panel). In contrast, the TFP(−) cells formed no such aggregates and became visibly distributed as single dispersed cells and small clumps (Fig. 4a, bottom panel). At 24 h, the WT strain exhibited an increase in the number of aggregates attached to the substrate, forming microcolonies with compact shapes. As previously reported (O'Toole and Kolter, 1998a), the bacterial migration along the surface observed here for piliated cells may be the result of the twitching motility driven by the TFP. This movement is involved in the rapid colonization of a given surface and allows the development of complex colonial structures. Between the 48 and 72 h of incubation the biofilm of WT cells became structurally complex. Within these two time points, the biofilm consisted in irregular cell aggregates that covered most of the surface. The TFP(−) cells, in contrast, though able to remain attached to the solid surface, increased only slightly in biomass. Moreover, they failed to build up the multicellular aggregates of typical biofilm structures, forming instead a flat carpet.

In order to define the architectural differences between the mature biofilms formed by the *M. bovis* 1194 03 WT strain and its TFP(−) variant, 72-h-old biofilms developed in flow chamber systems by the two strains were analyzed by confocal laser scanning microscopy (Fig. 4b). Stacks of *z*-section micrographs were collected and analyzed by the Imaris software. The upper panel in Fig. 4b shows top and lateral views of the biofilms formed by each strain, while the bottom panel depicts views of the biofilms reconstructed in three dimensions. As illustrated by the images, the WT biofilm consisted of large cell aggregates that attained a height of up to 150 μm. In contrast, the TFP(−) variant formed a mat of cells over the surface that reached a maximum thickness of only 30 μm. Thus, microscopical analyses demonstrated that the biofilm produced by *M. bovis* WT strain resembled the complex structures composed of microcolony pillars interspersed with water channels produced by other bacterial species (O'Toole et al., 1999).

3.4. Antibiotic tolerance of the *M. bovis* biofilm

The efficacy of antibiotics used against IBK has traditionally been evaluated on the basis of the MBC-P (Zielinski et al., 2000). Herein we compared the minimum bactericidal concentration of ampicillin, chloramphenicol, gentamicin and oxytetracycline for planktonic (MBC-P) and biofilm (MBC-B) cells. Depending on the antibiotic tested, the MBC-B ranged from 256- to over 1000-fold higher than the figure for planktonically grown bacteria (Table 1). Consequently, the MBC-P failed to reflect the bacterial sensitivity to antibiotics shown by the *M. bovis* grown in biofilm communities for any of the four antibiotics tested. These data therefore clearly demonstrated that the biofilm mode of growth enhances the tolerance of *M. bovis* to diverse antibiotics.

3.5. Effect of magnesium chloride on *M. bovis* biofilm

$MgCl_2$ has been reported to disrupt TFP (Annuar and Wilcox, 1985; Gil-Turnes, 1983; Pugh and Hughes, 1970).

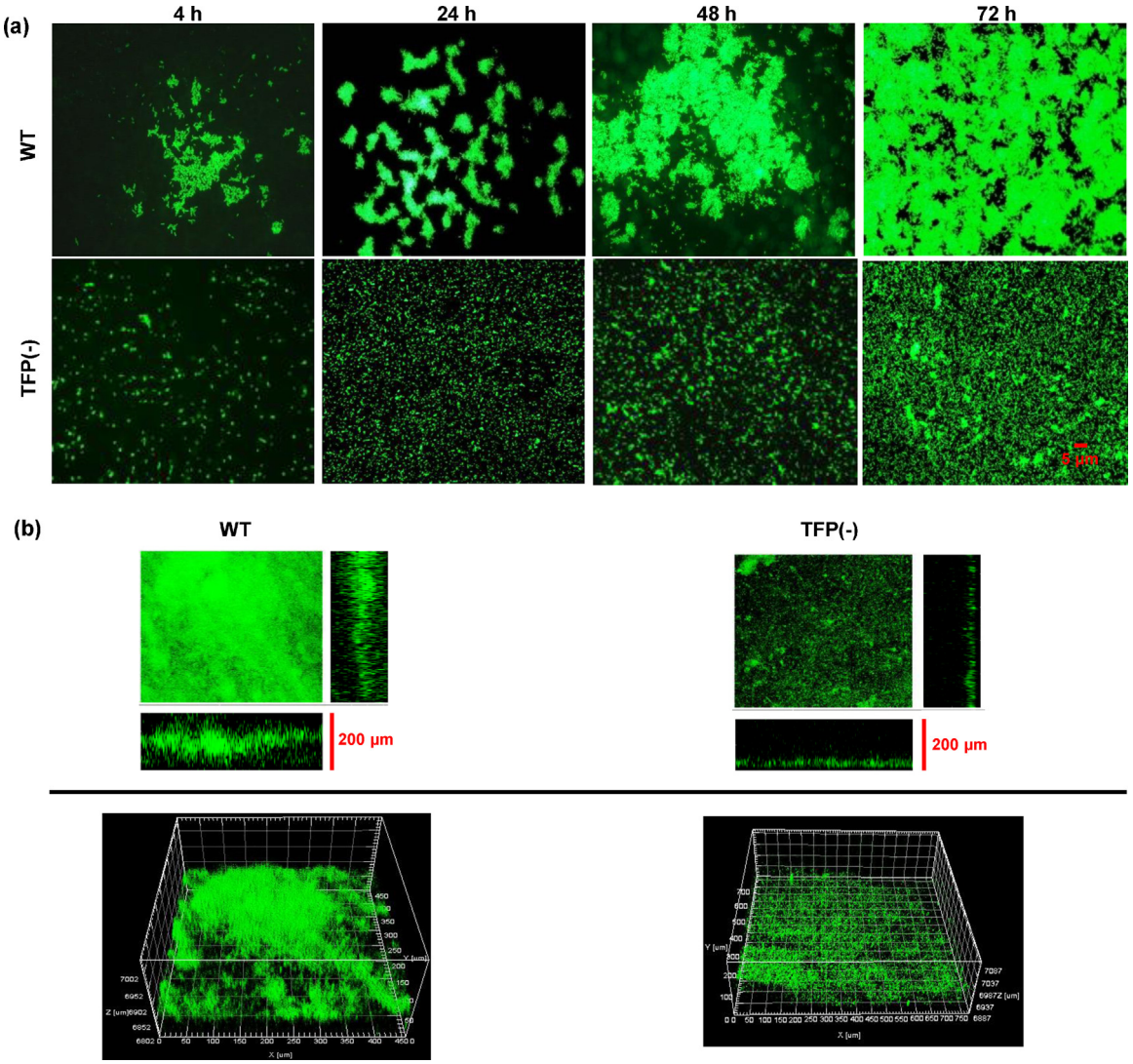

Fig. 4. Microscopical analysis of biofilms formed by *M. bovis* 1194 03 wild type WT strain and its TFP(−) variant. Bacteria were grown in a continuous-flow chamber system as indicated in Section 2. (a) Micrographs of different stages of biofilm formation. Biofilms were stained with SYBR Green I and visualized by fluorescence microscopy (400× magnification). (b) Fluorescence micrographs of 72-h biofilms stained with SYBR Green I and visualized by CLSM microscopy. Images in the upper panel show a middle micrograph, representing the *x*–*y* plane (top view), and adjacent bottom and side micrographs, representing the *x*–*z* and *y*–*z* planes (lateral views), respectively. Scale bar = 200 µm. Images in the bottom panel are volumetric three-dimensional reconstructions of representative *z*-section micrograph stacks of biofilms (200× magnification).

Table 1
Antibiotic sensitivity of planktonically and biofilm grown cells of the *M. bovis* 1194 03 WT strain.

Antibiotic	MBC-P[a] (µg ml^{-1})	MBC-B[b] (µg ml^{-1})	MBC-B/MBC-P
Ampicillin	0.25	64	256
Chloramphenicol	0.5	512	1024
Gentamicin	0.5	256	512
Oxytetracycline	1	1024	1024

Data are means from three independent experiments.
[a] MBC-P, minimum bactericidal concentration for planktonically grown cells.
[b] MBC-B, minimum bactericidal concentration for biofilm-grown cells.

Since our findings showed that TFP played a major role in bacterial attachment and subsequent biofilm formation, we put forward whether $MgCl_2$ may inhibit *M. bovis* biofilm formation. For this purpose we monitored the biofilm formed in glass column reactors by the *M. bovis* 1194 03 WT strain growing under continuous flow conditions in BHI broth in both the presence and the absence of either $MgCl_2$ (1% [w/v]) or NaCl (1% [w/v]), with the latter being used as an inactive salt control (Gil-Turnes and Ribeiro, 1985; Pugh and Hughes, 1970). The results demonstrated that in the presence of $MgCl_2$, the amount of adhered biomass after 72 h of growth was less than one-third of the quantity formed in $MgCl_2$-free cultures or in

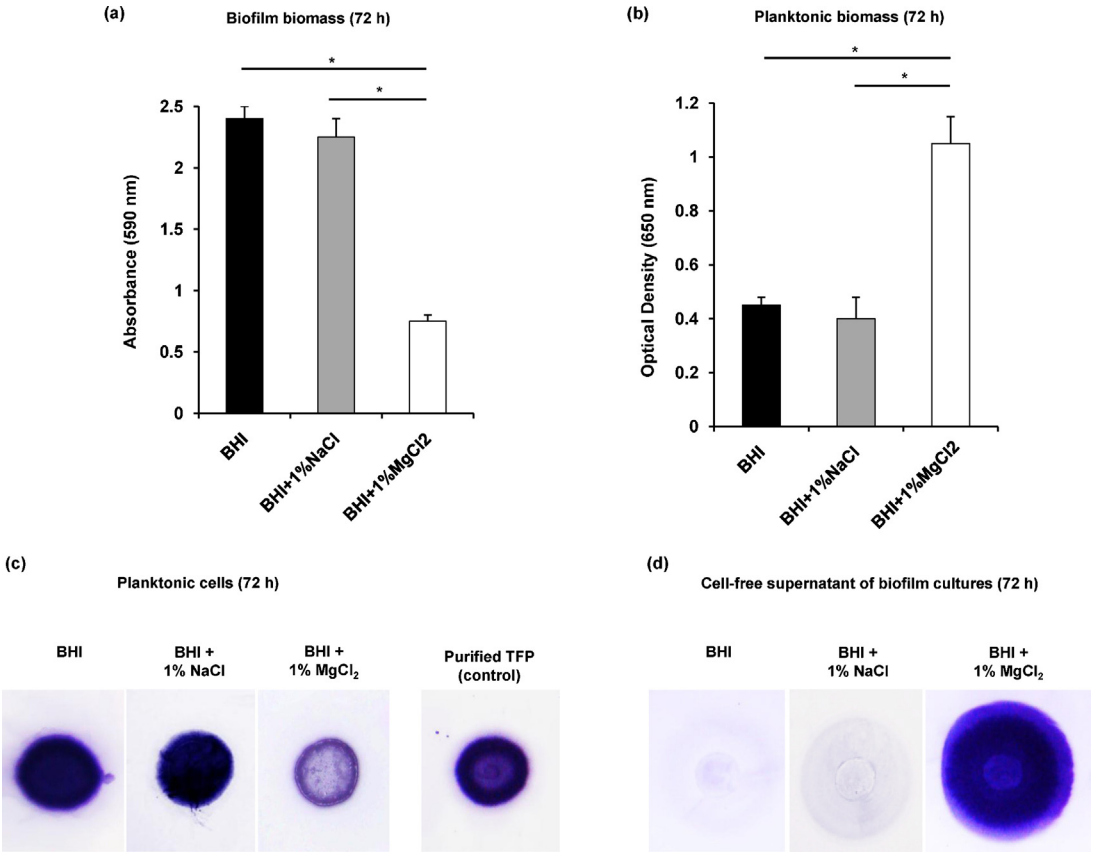

Fig. 5. Cultivation of *M. bovis* 1194 03 wild type WT strain on polypropylene beads in glass-column bioreactors under continuous-flow conditions for 72 h in the presence or absence of 1% (w/v) $MgCl_2$ or NaCl (used as a salt control): (a) Biofilm biomass quantified by CV staining; (b) planktonic biomass in the bulk-liquid phase present at the end of the cultures, determined by OD_{650}. Asterisks indicate significant differences among the corresponding groups ($p < 0.05$, *t*-test). Dot-blot analysis to detect TFP in the bulk-liquid phase of the biofilm culture, either (c) adhered to planktonic cells, or (d) free in the cell-free supernatant fraction. Immunoblots were performed with a rabbit polyclonal serum against *M. bovis* TFP.

presence of NaCl (Fig. 5a). In contrast, the concentration of planktonic cells in the bulk liquid phase once the flow was stopped after those 72 h of culture was approximately 2.5-fold higher with $MgCl_2$ present than with NaCl or with both of those salts absent (Fig. 5b).

We next checked whether these results were a consequence of the loss of the TFP from the bacterial cell surface as a result of the presence of $MgCl_2$ in the culture medium. To investigate that possibility, we compared by immunoblotting the piliation level of the planktonic cells obtained from the bulk liquid phase of the biofilm continuous-flow culture systems after 72-h incubation in the presence or the absence of either $MgCl_2$ or NaCl. The analysis revealed that in the presence of $MgCl_2$ the planktonic cells showed significantly less piliation level than the cells obtained from the BHI medium either free of $MgCl_2$ or containing NaCl (Fig. 5c). In order to confirm this effect of $MgCl_2$, we removed the planktonic cells from the bulk liquid phase of the cultures and evaluated the amount of free type IV pili in the supernatant by immunoblotting. As expected, free TFP were detected in high abundance in the supernatants of the $MgCl_2$-containing cultures but were only slightly detected or almost undetected in the respective BHI supernatants from the cultures containing NaCl or no salt at all (Fig. 5d). These results, taken as a whole, strongly suggested that the removal of TFP of *M. bovis* cells through the action of $MgCl_2$ has a high impact on the colonization of the abiotic surface here assayed.

In order to test whether $MgCl_2$ could also disassemble a preformed *M. bovis* biofilm we assayed the effect of this salt on *M. bovis* mature biofilm. BHI medium supplemented with 0.1, 1.0, or 2.0% (w/v) $MgCl_2$ or 1% (w/v) NaCl, or without supplements, was pumped for 24 h through continuous-flow column bioreactors, where 72-h *M. bovis* biofilms had been previously grown. The biomass adhered to the polypropylene beads was then quantified by staining with CV at 0, 12, and 24 h after starting the flow with the salt-containing medium (Fig. 6a). The results showed at 12 and 24 h significant differences in the amount of adhered biomass between cultures supplemented with $MgCl_2$ and NaCl as well as between $MgCl_2$-containing cultures and cultures incubated with BHI medium alone ($p < 0.05$). The results clearly reflect a time- and dose-dependent disruptive effect of $MgCl_2$ on the *M. bovis* biofilm. Under these assay conditions, the maximum effect of $MgCl_2$ in reducing biofilm biomass was

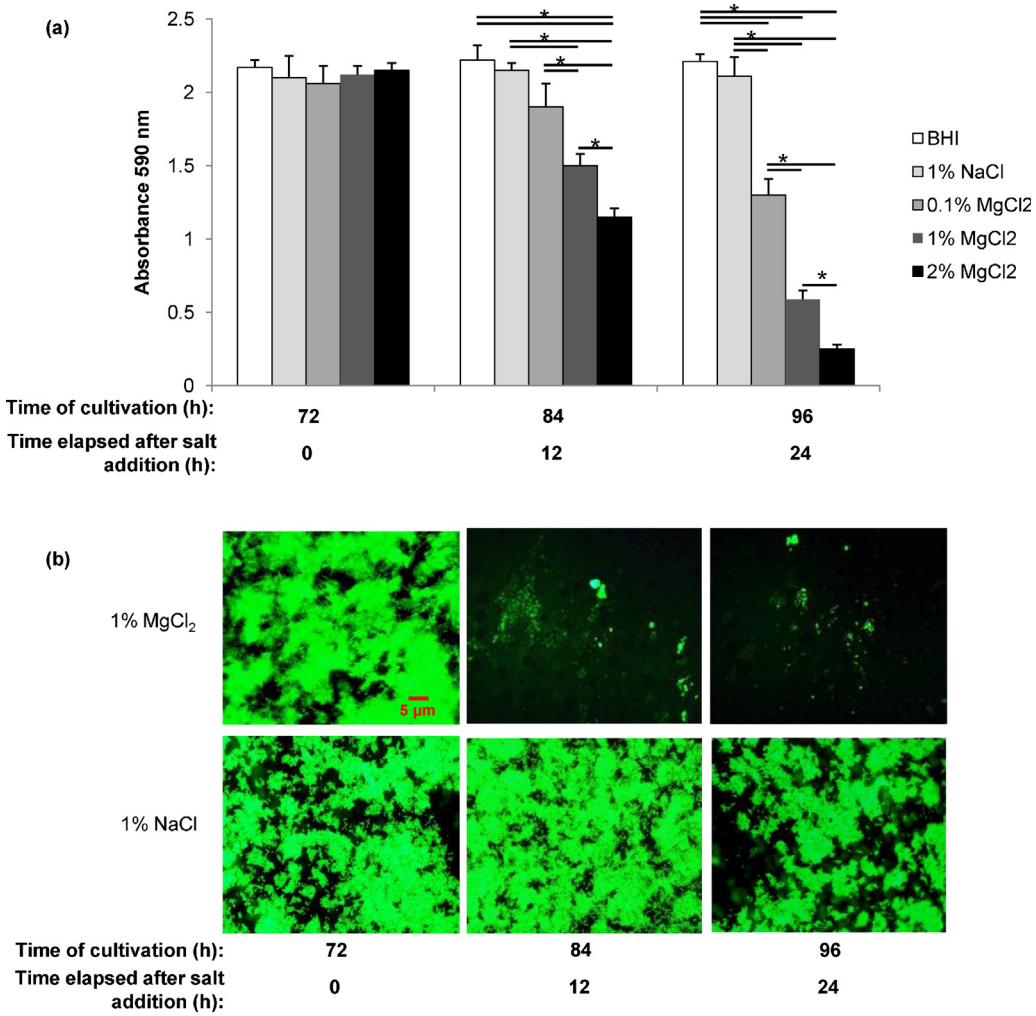

Fig. 6. Effect of $MgCl_2$ on pre-established *M. bovis* biofilms. Seventy two-h-old preformed *M. bovis* 1194 03 wild type (WT) strain biofilms were produced on polypropylene beads in column bioreactors operated under continuous-flow conditions in BHI medium. (a) Biofilm treated with BHI medium supplemented with various concentrations of $MgCl_2$ and 1% (w/v) NaCl (used as salt control). Adhered biomass obtained after 12 and 24 h of the salt addition was quantified by CV staining. The data are the means ± standard deviations of three independent experiments. An asterisk indicates significant differences between the conditions being compared (p value < 0.05; Student t-test). (b) Fluorescence micrographs of 72-h preformed biofilms grown on the base of the flow chamber system, incubated under a continuous-flow of nutrients, and treated for 12 and 24 h with 1% (w/v) $MgCl_2$ or NaCl. Biofilms were stained with SYBR Green I (400× magnification).

observed after 24 h at a concentration of 2% (w/v). In contrast, no significant changes in the adherent biomass ($p > 0.05$) were observed between *M. bovis* biofilms treated with BHI medium alone and BHI supplemented with NaCl. With the purpose of directly visualizing the effect of $MgCl_2$ on mature biofilms, we pumped BHI broth containing either 1% (w/v) of this salt or NaCl, as a control, for 24 h into 72-h-old *M. bovis* biofilms that had been produced in flow chamber systems. At interval times of 12 h after the addition of the salts we visualized the biofilms adhered to the surfaces by fluorescence microscopy using SYBR-Green-I staining. Only cell aggregations and dispersed single cells remained on the chamber surface after 24 h of $MgCl_2$ treatment. In contrast, the treatment with NaCl under the same experimental conditions did not modify the mature-biofilm structure (Fig. 6b).

4. Discussion

Biofilms have been implicated in many infectious diseases. A biofilm is a complex community-based mode of existence that microbes establish over abiotic or living surfaces. Biofilms represent a bacterial lifestyle that provides protection from environmental stress and confers upon pathogens several advantageous traits linked to virulence and resistance to environmental stress, host defences, and antimicrobial compounds (O'Toole and Kolter, 1998b; O'Toole et al., 1999). Such characteristics ultimately facilitate the establishment of persistent infections or a carrier state in affected host. The results obtained from studies on experimentally infected animals and/or from clinical trials have been supporting a current hypothesis that many airway

infections are associated with the persistence of bacteria in biofilms. For some airway pathogens like *Pseudomonas aeruginosa*, *M. catarrhalis*, *Streptococcus pneumoniae*, and *Haemophilus influenzae* the upper respiratory tract mucosa, such as nasopharyngeal surfaces or paranasal sinuses, constitute propitious niches or reservoirs for the persistence of bacteria within biofilm communities (Armbruster et al., 2010; Hansen et al., 2012; Mainz et al., 2009). Thus, in view of these observations and the well documented findings that: (i) *M. bovis* is normally isolated from the conjunctivas and nasal secretions of animals without any sign of infection (Pugh et al., 1980), (ii) cattle are the principal known reservoir for *M. bovis* bacteria, (iii) the same bacterial serotype can remain on a farm producing intermittent infections over a period of years (Pugh et al., 1980), (iv) IBK treatment with several antibiotics may fail to eliminate the carrier state of cattle (McConnel et al., 2007; Sargison et al., 1996) and (v) increasing evidence demonstrating that bacterial biofilms participate in ocular infections by direct colonization of eye surfaces (Murugan et al., 2010; Zegans et al., 2002); we hypothesize here that biofilm formation might constitute the mechanism whereby *M. bovis* is able to adhere and growth on the eye and/or nasal cavity of cattle and eventually persist in such niches. As a first approach to address our hypothesis, we demonstrated that *M. bovis* was able to adhere to abiotic surfaces and grow in a self-produced matrix. We verified by FT-IR spectroscopy that the interface material adhered on the inner wall of the Erlenmeyer flask or the microtiter wells consisted mainly of bacteria embedded in a polysaccharide-enriched matrix (Fig. 2), that might be contributing to the biofilm architecture as was reported for many other Gram-negative bacteria. We therefore applied more complex biofilm cultivation systems such as column reactors packed with polypropylene beads or flow cell chambers operated under a continuous flow of nutrients, which enabled the monitoring of the bacterial biofilm growth under hydrodynamic conditions. We were able to determine and compare the biofilm structural characteristics of the WT strains and the corresponding TFP(−) variants in order to provide evidence supporting the notion that *M. bovis* biofilm formation represents a TFP mediated mechanism (Figs. 3 and 4). In our experimental conditions we found that the differences in biofilm structure between the WT strain and the TFP(−) variant suggested that the combination of adhesion and migration over the surface mediated by TFP are relevant to the development of *M. bovis* mature biofilms, as had been reported for other Gram-negative bacteria (Luke et al., 2007; O'Toole and Kolter, 1998a). Interestingly, the far less pronounced biofilm exhibited by TFP(−) cells strongly resembled the results reported by Luke et al. (2007) for *M. catarrhalis* biofilms. Those investigators compared the ability of the WT strain to that of its *pilA* mutant (*i.e.*, *tfp*$^{-}$) to form biofilms, likewise using BHI medium in a flow chamber system, and reported differences between biofilms formed by piliated and nonpilited cells (average depths of 104 μm and 72 μm, respectively).

It has widely been demonstrated that *M. bovis* is susceptibility to a variety of antibiotics *in vitro* (Zielinski et al., 2000; Shryock et al., 1998), it has also been shown that different antimicrobial therapies – administrated as subconjunctival injection, topical application and/or systemic administration – can be successfully applied to control IBK outbreaks (Angelos et al., 2000; Lane et al., 2006; Zielinski et al., 2000). However, it was also reported that some antibiotics failed to effectively eradicate the pathogen within a season (Angelos et al., 2000; Lane et al., 2006; Zielinski et al., 2000) or were not always successful in eliminating the carrier state in cattle (McConnel et al., 2007). Thus, although the reemergence of the IBK in the fields in several countries is undoubtedly the result of a low vaccine protection coverage, the persistence of *M. bovis* in the field might also be promoted by the establishment of a biofilm lifestyle on eyes and/or nasal cavities in cattle—which should be analyzed in future studies. Our results presented in Table 1 in which biofilm bacteria presented up to 1000-fold more tolerant and/or resistant to antibiotics than planktonic cells might also be consistent with the difficulties encountered to apply an antibiotic therapy which enables the elimination of relapses and/or the carrier state of IBK.

The dominant role that TFP played in *M. bovis* biofilm development through participation in both surface attachment and biofilm formation accordingly prompted us to investigate the use of an alternative non-antibiotic agent able to interfere with biofilm generation and maintenance through a specific interaction with the TFP (Fig. 5). Considering the TFP-disruptive effect reported for $MgCl_2$, we assayed that salt *in vitro* as a potential drug for biofilm disruption. Our results showed that, in general, $MgCl_2$ not only significantly reduced *M. bovis* attachment to and colonization of abiotic surfaces, but also caused the disassembly of pre-established or mature biofilms (Fig. 6). The data presented here therefore provide the first evidences of the fitness of *M. bovis* to form biofilm. These results could lead to a novel strategy in IBK prevention and treatment that targets both the biofilm formation and disassembling. Ongoing results from our laboratory show promising possibilities for $MgCl_2$ when it is used in combination with antibiotics to disrupt biofilm and kill bacteria.

5. Conclusion

In this report, we demonstrated for first time that *M. bovis* is able to grow as biofilm on different abiotic surfaces under diverse culture conditions and that the TFP – one of the principal virulence factor of *M. bovis* – were critical for both the attachment and the subsequent stages of development and maintenance of biofilm architecture. In agreement with the hallmark feature of biofilm related infections, we found that the *M. bovis* biofilm cells were more resistance to antibiotic than their planktonic counterparts. Most relevantly, we further demonstrated that $MgCl_2$, by removing the TFP, not only prevented biofilm formation but also disassembled preformed *M. bovis* biofilms. Our *in vitro* results constitutes a new

approach that could help in the understanding of *M. bovis* colonization process in cattle eye/or nasal cavities.

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