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ORIGINAL PAPER

Attachment and biofilm formation of *Mycobacterium marinum* on a hydrophobic surface at the air interface

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Abstract Properties of attachment of *Mycobacterium marinum* to hydrophobic surfaces and subsequent biofilm formation were investigated. Binding of *M. marinum* to polypropylene occured under aerobic and anaerobic/microaerophilic conditions. However, aerobic conditions were necessary for biofilms to persist. Highly non-polar organic solvents were found to efficiently remove attached bacteria from the polypropylene surface, indicating strong hydrophobic interactions between the *M. marinum* cell wall and the surface. Increased capsular material, occurring during stationary phase, correlated with a decrease in attachment of cells to polypropylene. A protein of approximately 40 kDa appears to be present in increased amounts during the stationary phase. The protein has been identified by LC MS/MS analysis as alanine dehydrogenase.

Keywords *M. marinum* · Aerobic biofilm · Culture condition · Attachment · Hydrophobic interaction

Introduction

The aquatic microorganism *Mycobacterium marinum* has been isolated from waters along the eastern coast of the United States, including the Chesapeake Bay (Heckert et al. 2001; Rhodes et al. 2001, 2003). This organism is one of several atypical mycobacterial species that causes mycobacteriosis in ectothermic animals, such as fish and frogs (Bonafe et al. 1992; Gluckman 1995; Bercovier and Vincent 2001; Decostere et al. 2004). *M. marinum* is a significant concern for the aquaculture industry, which lacks satisfactory methods for prevention and treatment of infection. In these settings, infection of one fish has often led to destruction of the entire fish stock (Hedrick et al. 1987; dos Santos et al. 2002; Decostere et al. 2004). Dissemination of *M. marinum* and other atypical mycobacteria from fish tanks and environmental reservoirs could also pose serious health problems to immunocompromised humans (Sato 1972; Bruckner-Tuderman and Blank 1985; Patel et al. 1995; Barton et al. 1997; Ghittino et al. 2003).

M. marinum, a close genomic relative of *M. tuberculosis* (Tonjum et al. 1998), belongs to the family *Mycobacteriaceae*, members of which possess an unusually complex cell wall setting them apart from both gram-positive and gram-negative bacteria. The mycobacterial cell wall consists of a peptidoglycan layer covalently attached to a thicker layer of mycolic acid–arabinogalactan complex. The cell wall also contains lipomannan and lipoarabinomannan macromolecules. Enveloping the mycobacterial cell wall is an electron transparent layer of variable thickness that is rich in polysaccharides, glycolipids, proteins and polar molecules that associate, through hydrophobic or electrostatic interactions, with the underlying cell wall (Rastogi and Hellio 1990; Levchenko 1991; Brennan and Nikaido 1995).

Abiotic and biotic surfaces in the environment and in the aquaculture systems can serve as substrates for colonization and growth by *M. marinum*. Recent

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studies indicate that *M. marinum* and other environmental mycobacteria can bind to hydrophobic surfaces and form biofilms in submerged conditions (Ridgway et al. 1984; Schulze-Robbecke 1993; Hall-Stoodley and Lappin-Scott 1998; Bardouniotis et al. 2003; Carter et al. 2003). However, since mycobacteria are aerobic organisms, submerged environmental niches may not sustain *M. marinum* biofilms. Additionally, the interaction of *M. marinum* with the surfaces may be affected by modification of their cell envelope, caused by changes in nutrient and oxygen availability, pH, osmolarity and localized accumulation of secondary metabolites, as has been shown for other bacteria (Krieg et al. 1988; Watson and Watson 1989; Stewart and Olson 1992; Popova and Klitsunova 1998; Castellanosa et al. 2000; Wick et al. 2002; Hassan and Frank 2004). In fact, mycobacteria modify their gene expression (DeMaio et al. 1996; Yuan et al. 1996; Gupta et al. 2002) and increase the thickness of their capsular layer in the late stationary phase of the culture (Cunningham and Spreadbury 1998).

The aim of this study was to characterize the relationship between the cell surface of *M. marinum* and biofilm formation as a function of growth conditions. This was accomplished by: (1) comparing persistence of *M. marinum* as biofilms at hydrophobic surfaces in aerobic and anaerobic/microaerophilic conditions; (2) examining the affinity of the cell wall for the hydrophobic surface; (3) determining the effects of culture conditions on the ability of *M. marinum* to attach to the surface; (4) comparing protein profiles of *M. marinum* from different phases of culture.

Methods

Bacterial strain and culture

The strain used for this study, *M. marinum* 2025R, was isolated from a striped bass (*Morone saxatilis*) in the Animal Research Center II (ARCII) at the Center of Marine Biotechnology of the University of Maryland Biotechnology Institute. The 16S rDNA sequence of this strain is identical to the 16S rDNA sequence from a *M. marinum* strain in the GenBank (accession number AF251565). *M. marinum* 2025R was cultured on 7H11 agar with AODC (oleic acid-albumin-dextrose-catalase) as the enrichment medium and maintained in 7H9 medium supplemented with ADC (albumin-dextrose-catalase) enrichment medium (BD, Franklin Lakes, NJ). All experiments were carried out in Long's minimal medium (Long and Seibert 1926; Wong and Weinzirl 1936) or in Long's medium supplemented with 0.05% (v/v) Tween-80 (T-80) to minimize clump formation. The *M. marinum* used in all experiments was grown in 500 ml sterile screw cap Pyrex flasks (Corning) at 30°C in a shaker incubator at 165 rev/min.

Biofilm formation

Solid–liquid interface

Mycobacteria from late exponential phase ($OD_{600} \geq 0.4$) were inoculated into 100 µl Long's medium in 96-well polyvinylchloride (PVC) plates and allowed to settle to the bottom of the wells for 1 h. Unbound cells were removed and cells adhering to PVC wells were incubated at 30°C in fresh medium and examined periodically to monitor biofilm development. Images of attached cells and biofilm development were captured via digital camera attached to a light microscope.

Solid–liquid–air interface

About 3-ml aliquots of late exponential phase cells ($OD_{600} \geq 0.4$) were placed in polypropylene tubes with Long's medium with 0.05% T-80 and incubated with shaking at 150 rev/min at room temperature for 1 h. The unbound bacteria were removed and the tubes were washed with 3 ml of water containing 0.05% T-80. The remaining attached cells were incubated in 3 ml of Long's medium supplemented with 0.05% T-80 at 30°C with shaking at 150 rev/min for 17–20 days or until a biofilm could be visualized as a ring at the solid–air interface.

Attachment assay

Mycobacteria attached to the surface of polypropylene were stained using the Ziehl-Neelsen acid-fast staining method (TB Quick Stain kit; Becton Dickinson, Sparks, MD). Tubes were dried at 50°C. To quantify the attached cells, carbolfuchsin retained by the cell wall was measured by dissolving the stained cells in 300 µl of 10% SDS and 95% ethanol (1:1 ratio by vol.). The amount of stain in 100 µl of the dissolved material was quantified in a 96-well polystyrene plate using a microplate reader (Anthus htII, Salzburg, Austria) at OD_{492} nm.

Estimation of strength of attachment to hydrophobic surface

To determine the affinity of the hydrophobic interaction between the *M. marinum* cell wall and polypropylene,

we used a series of reagents with different polarities to dislodge the cells from the surface of polypropylene. Because acid-fast stain is retained by *M. marinum* cell wall after treatment with these solvents (Alavi et al., unpublished observation), the stain can be used to determine the ratio of dislodged cells to those remaining attached after treatment with each reagent. Measurement of the amount of carbolfuchsin in each fraction was carried out as described above. Reagents tested using these methods and their respective polarities were: water (1), ethanol (0.79), dimethylformamide (0.4), acetone (0.36), chloroform (0.26), and xylene (0.07). Mechanical removal of attached cells by scraping was also employed as a measure of maximum removal.

Attachment to polypropylene at different phases of growth

A small inoculum of late exponential phase cells was used to start a 200 ml culture in a 500-ml glass flask. The culture was grown at 30°C at 150 rev/min. At selected time-intervals during culture growth, OD_{600} was recorded and viable counts were determined by plating serial dilutions on 7H11 agar containing OADC. At each time interval, 2 ml-aliquots were also transferred aseptically into polypropylene tubes and incubated at room temperature on a shaker at 150 rev/min for 1 h. After removal of unbound bacteria, attached bacteria were scraped from the tube using a sterile tissue culture scraper (Corning) and placed into 2 ml of fresh medium. The number of bacteria attached to the surface was determined by plating serial dilutions on 7H11 agar. The significance of the difference between the means for data obtained from c.f.u. counts of attached bacteria in exponential and stationary phases was determined by paired *t*-tests. *T*-test computation was performed using PSI-Plot Version 7.01 (Poly Software International, Inc.).

Preparation of capsular material

Glycolipids and other capsular components in exponential and stationary phases of growth were extracted according to Nigou et al. (1999). Briefly, cells from cultures at exponential (OD_{600} = 0.34) and stationary phase (OD_{600} = 0.95) were harvested by centrifugation. Approximately 100 mg from each were treated with 0.75 ml of chloroform/methanol (1:1, v/v). After removing solid material by centrifugation, organic solvents were evaporated in a vacuum incubator at 50°C and the extracted capsular material was dissolved in SDS-PAGE sample buffer. Phase fractionation of capsular material was carried out by treating cell pellets with chloroform/methanol/water (1:1:0.9 by vol.) followed by separation of aqueous and organic phases by centrifugation. Solvents from each phase were again removed by evaporation and the extracted material was dissolved in SDS-PAGE sample buffer.

SDS-PAGE and staining of capsular material

Capsular materials prepared by delipidation were subjected to SDS-PAGE on 8–20% gradient gels. Gels were stained with Alcian blue (0.1 g in 100 ml of 40% ethanol, 5% acetic acid) for staining mucopolysaccharides (Karlyshev and Wern 2001) or Sudan black (0.07 g in ethanol) for staining lipids and phospholipids (Perusse et al. 2001). After staining with Sudan black, gels were treated with a mixture of 0.8% acetic acid and 4% methanol to restore their shape.

Protein analysis by two-dimensional gel electrophoresis

Protein profiles of *M. marinum* from exponential and stationary phase cultures were examined by two-dimensional (2-D) gel electrophoresis. Aliquots (5–10 ml) from mid-exponential phase (OD_{600} = 0.29) and late stationary phase (OD_{600} = 0.45, after dropping from OD_{600} = 1) were removed, and cells were harvested and washed once with water containing 0.05% T-80. Cell pellets were dissolved in extraction buffer with the following composition: 5 M urea, 0.625 M thiourea, 2% CHAPS (w/v), 2% SB3-10, 40 mM Tris base and 0.5% 3–10 ampholytes (v/v). The lysates were sonicated for 4–35 s pulses at 50% power. Cell debris was removed by centrifugation and the extract held at –80°C until analysis. The concentration of proteins in the samples was determined using the DC/RC protein assay kit (Bio-Rad Laboratories, Hercules, CA). About 80 μg from each sample was mixed with rehydration buffer with the composition: 8 M urea, 4% CHAPS (w/v), 1 mM DTT and 0.5% 3–10 ampholytes (v/v) and 2-D gel electrophoresis was performed according to Shepard et al. (2000). Gels were stained with Coomassie Blue and scanned with a Kodak EDAS 290 imaging system. Image analysis was carried out with ImageMaster 2-D Elite, version 4.01 (Amersham Bioscience, Piscataway, NJ) according to the manufacturer's instructions. Gels were aligned and matched, and quantitative determination of spot volumes was performed (mode: total spot volume normalization). Normalized spot volumes for triplicate experiments were statistically reproducible.

Protein identification by LC-MS/MS analysis

Digestion and extraction of peptides

Each protein gel spot was excised and destained using the ProteoSilver Plus Destaining (Sigma) following the manufacturer's protocol. In-gel digestion was performed according to a modification of Shevchenko et al. (1996). In short, 50–100 μl of 50 mM ammonium bicarbonate was added to the sample containing 10 ng/μl modified trypsin (Promega, Madison, WI) and incubated at 37°C overnight. Tryptic peptides were extracted twice in 100 μl extraction buffer (0.1% TFA, 50% acetonitrile pH 2.5). The two extractions were combined and dried in a speed-vac device until proper volumes were reached (25–50 μl). Precipitates or suspended particles were removed by centrifugation before samples were loaded for automated LC-MS/MS analysis.

LC-MS/MS analysis

The Surveyor HPLC system (with an autosampler and LC pump, ThermoFinnigan, San Jose, CA) was used for automatic sample loading and flow control. Variable flow was controlled using the divert valve of the LCQ DECA XP ion trap mass spectrometer (ThermoFinnigan). For analysis, 10 μl tryptic peptide sample was injected into a BioBasic C-18-packed nanospray microcapillary column (75 μm × 12 cm packed with 5 μm, 300 Å Magic C18; New Objective), followed by a 20 min buffer A wash with the composition: 0.1% formic acid in water (v/v). Peptides were eluted using a linear gradient of 5–60% methanol over 60 min; flow rate was maintained at 200 μl/min. Separated peptides were automatically analyzed by coupling on line to an ion trap (IT) mass spectrometer (LCQ Deca XP; Thermo Finnigan, San Jose CA, USA). The IT spectrometer was operated in a data-dependent mode at 1.70 kV, where each full MS scan was followed by three MS/MS scans.

The product ions generated from each MS/MS scan were searched against the NCBI-nr database (www.ncbi.nlm.nih.gov) using SEQUEST (ThermoFinnigan, San Jose, CA, USA). This search identified the secreted alanine dehydrogenase of *Mycobacterium leprae* (accession number gi:15826865) as the closest match in the database. All subsequent searches were carried out on the sequence data produced by the *M. marinum* Sequencing Group at the Sanger Institute (http://www.sanger.ac.uk/Projects/M_marinum/). Using the nucleotide sequence of *M. leprae ald* gene as query sequence the databank of the Sanger Institute was searched and the full open reading frame of the *M. marinum ald* gene was identified.

Results and discussion

Biofilm formation at solid–liquid and solid–air interfaces

We compared the persistence of *M. marinum* biofilms at hydrophobic surfaces under both anaerobic/microaerophilic and aerobic conditions. As indicated in Fig. 1, *M. marinum* attached to the bottom of PVC wells began dividing under submerged conditions. However, as cell numbers in aggregates increased, rope-like structures were formed, which eventually detached from the bottom of the wells and floated to the surface. As suggested by Hall-Stoodley and Stoodley (2005), the detachment of mycobacterial aggregates from the surface may be a means for dispersion of *M. marinum* in nature.

We found that *M. marinum* growing in polypropylene tubes with minimal medium in the presence of low concentrations of T-80 tended to bind the tube at the solid–air interface. The attached cells form a ring that could be easily visualized by acid fast staining (Fig. 2). When the attached cells were incubated with fresh medium with gentle shaking, a biofilm ring formed at the liquid–air interface (Fig. 2). This biofilm was encased in an extracellular mucous-like substance and was firmly attached to the surface. Release of large cellular clumps, such as those seen under submerged conditions, could not be detected, although it is possible that smaller aggregates or single cells could be shed from such biofilms. Pellicle formation on the surface of liquid medium, a characteristic of mycobacterial species in standing laboratory cultures, was not seen under these conditions. Surface-attached aerobic biofilms also occur in other opportunistic pathogens and the physiology of bacteria in these biofilms could provide new information about mechanisms of pathogenesis in these organisms (Irie et al. 2004; Mishra et al. 2005). Although *M. marinum* can attach to hydrophobic surfaces in anaerobic and aerobic conditions, our data suggest that they will form persistent biofilms on surfaces under aerobic conditions.

Affinity of attachment to hydrophobic surfaces

When we examined the affinity of the *M. marinum* cell wall for the hydrophobic surface, using solvents with different polarities, we found that as the polarity of the

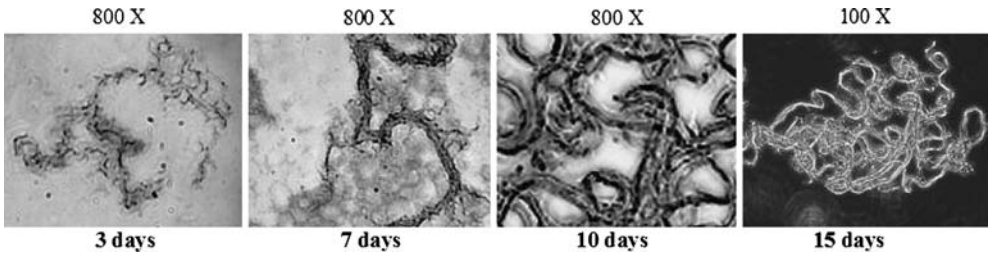

Fig. 1 Attachment and growth of *M. marinum* on the submerged surface of PVC in minimal medium. Attached cells were incubated in a 96-well PVC plate and examined by light microscope at several time intervals. Cell division of the attached cells leads to increase in clump size and eventual detachment from the submerged surfaces The first 3 panels are 800× magnifications while the last panel (detached cord) is magnified 100×

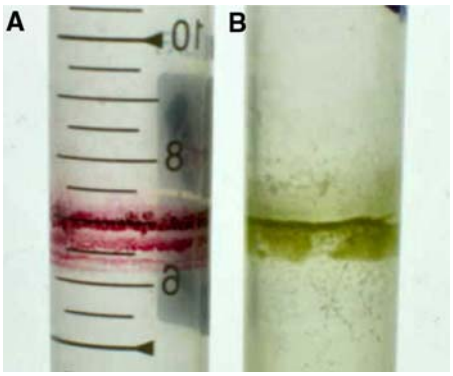

Fig. 2 Attachment to solid surface at interface with air and the resulting biofilm. (**A**) The attached mycobacterial cells at the solid-air interface can be visualized by acid-fast stain. (**B**) When incubated in minimal medium these cells can give rise to an attached biofilm ring at the air interface

reagents decreased, more cells were dislodged from polypropylene (Fig. 3). Xylene with the least polarity (0.07) was the most effective reagent for releasing attached cells from polypropylene, while water with the highest polarity (1) was the least effective reagent. Since successful colonization of surfaces in an aquatic environment depends on the cohesive strength of the initial attachment (Costerton et al. 1987, 1995; Beveridge et al. 1997; Donlan 2002; Soini et al. 2002), *M. marinum* may use strong hydrophobic interactions, which are probably mediated by the lipid components of the cell wall (Ridgway et al. 1984), to attach and colonize surfaces, particularly at the interface with air.

Correlation between the abundance of capsular material and attachment of mycobacteria to hydrophobic surface

Examination of the effect of culture conditions on *M. marinum* cell surface properties revealed that significantly more capsular components of varying sizes were present in cells from the stationary phase than from the exponential phase (Fig. 4a). Nearly all the capsular material detected by the two stains was partitioned into the organic phase suggesting high abundance of lipid-conjugates. The lipid moieties in capsular material are thought to interact with lipid components of the cell wall, while hydrophilic moieties face outward (Draper 1998).

To test the hypothesis that accumulation of capsule on the *M. marinum* cell wall could mask components that interact with hydrophobic surfaces, the number of mycobacteria that attached to the surface was estimated from c.f.u. counts and compared with their total number at different stages of growth. As indicated in

Solvent	Formula	Relative polarity
Scraping		
Xylene	C8H10	0.07
Chloroform	CHCl3	0.26
Acetone	C3H6O	0.36
Dimethylformamide	C3H7NO	0.40
Ethanol	C2H10O	0.79
Water	H2O	1.00

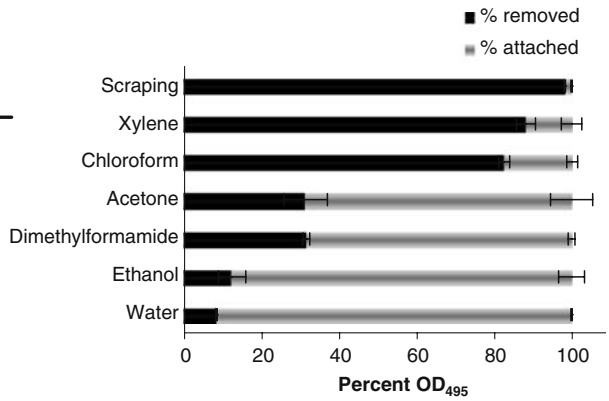

Fig. 3 Estimation of attachment cohesiveness of *M. marinum* cell wall to polypropylene. The attached cells were stained with acid-fast. Reagents with different polarities were then used to dislodge them from the surface. Ratios of dislodged and not-dislodged cells are shown as percent in the bar graphs. Table to the left indicates the relative polarities of the various solvents

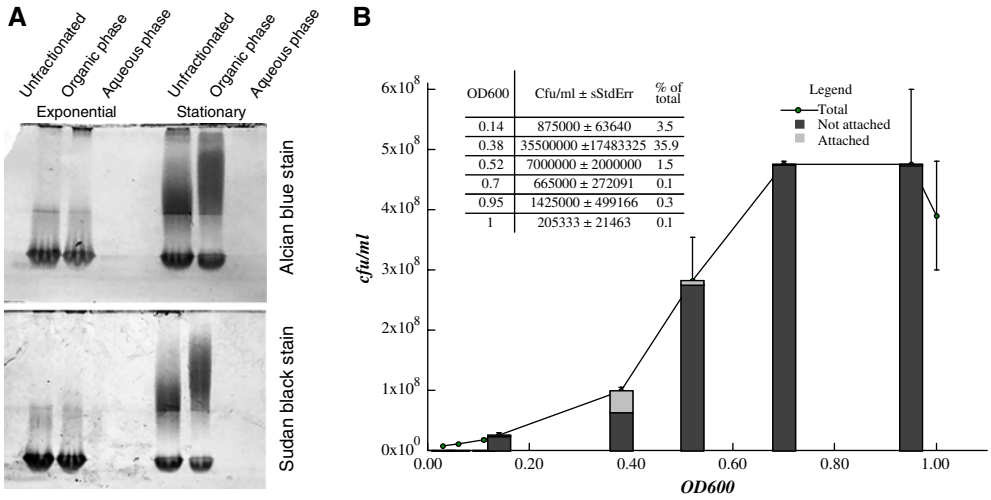

Fig. 4 Correlation between abundance of capsular material on cells in exponential and stationary phases and attachment to polypropylene. (**A**) Capsular components were extracted by chloroform/methanol and an aliquot phase-separated into aqueous and organic fractions. The unfractionated and phase-fractionated material were subjected to SDS-PAGE and then stained with Alcian blue or Sudan black. The lanes contain either total fraction hydrophobic or hydrophilic fraction. (**B**) *M. marinum* growth curve and the stacked bar graph representing the proportion of cells attached to polypropylene at the selected time intervals. The number of attached cells were subtracted from the total number of cells at each stage and plotted as a stacked bar graph. The inset table shows the OD_{600} the number of cells that attached to polypropylene and their percent of the total at the selected time points

Fig. 4b, the number of cells attached to polypropylene was significantly higher ($P < 0.05$) in early and mid-exponential phases, declining sharply thereafter. The lowest rate of attachment was observed when cells were in stationary phase, a time when a thick capsular layer is present. No correlation existed between the number of attached cells and cell density, indicating that cell density was not the major factor influencing the attachment. Since incubation of bacteria with the surface was carried out directly in the presence of the original culture medium, we also examined the possibility that material released from bacteria could inhibit binding of cells in older cultures. The late stationary phase cells were thoroughly washed and incubated with polypropylene in presence of fresh culture medium in parallel with a control aliquot that was incubated in the original medium. The acid-fast staining assay indicated no difference in attachment of the old culture cells in the presence of fresh or spent culture medium ($OD_{492} = 0.035 \pm 0.021$ and $OD_{492} = 0.039 \pm 0.009$, respectively). Therefore, accumulation of the capsule on cells appears to prevent the attachment of *M. marinum* to polypropylene.

Comparative analysis of the most abundant proteins from cells in exponential and stationary phases

We identified proteins that may play a role in protecting *M. marinum* under stress by comparing protein profiles of *M. marinum* from exponential and stationary phases by two-dimensional gel electrophoresis and Coomassie Blue staining. We detected 81 protein spots in stationary phase (Fig. 5a) as compared to 103 spots in exponential phase (Fig. 5b). At least 16 protein spots showed a two-fold or greater level difference between the two phases of culture (data not shown). However, the most noticeable difference was the accumulation of a protein with apparent size of ~40 kDa and PI of 6.7 (arrow) in stationary phase as compared to the exponential phase (>14-fold). MS/MS analysis after in-gel digestion with trypsin indicated sequences related to the alanine dehydrogenase of *M. leprae*.

When the *M. leprae ald* gene sequence was used to search the publicly available *M. marinum* database from the Sanger Institute, we identified the corresponding *M. marinum ald* gene. The amino acid alignment of Ald from *M. marinum*, *M. leprae* and *M. tuberculosis* is presented in Fig. 6. Significant homology exists between the amino acid sequences of *M. marinum*, *M. leprae* and *M. tuberculosis* with the major N-terminal AlaDh_PNT-N (PSSM-id: 16517) and C-terminal AlaDh_PNT_C (PSSM-id: 15323) domains conserved.

Alanine dehydrogenase (PSSM-id:10555) (EC 1.4.1.1) is a membrane or cell-surface associated protein and is involved in amino acid transport and metabolism. This enzyme can catalyse the NAD-dependent reversible conversion of pyruvate into

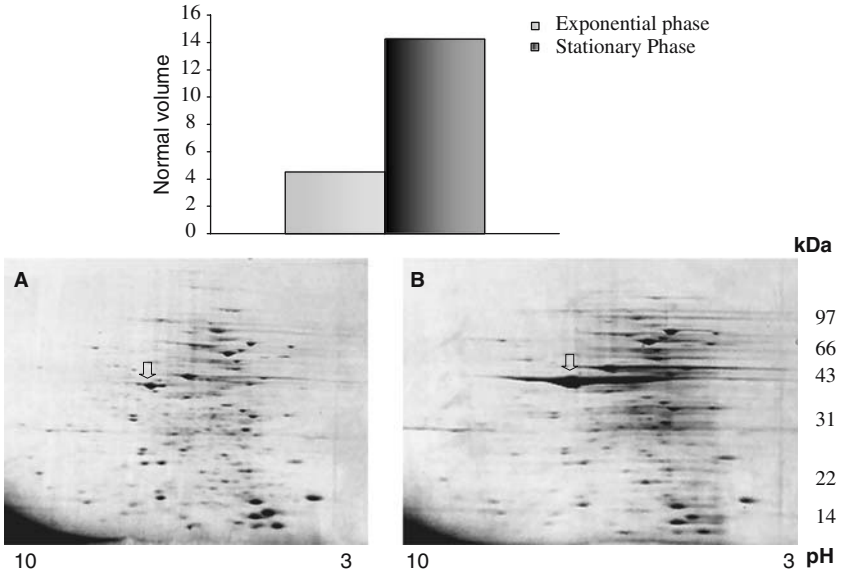

Fig. 5 Profiles of most abundant *M. marinum* proteins in exponential and stationary phase. Proteins from cells in the exponential (**A**) and the stationary (**B**) phases of growth were analysed by two-dimensional gel electrophoresis and visualized by Coomassie blue staining. The arrow indicates the most prominently over-expressed spot in the stationary phase. The difference in the intensity of this spot is presented as normal volume in the bar graph at the top

alanine concomitant with translocation of a proton across the membrane. In other mycobacteria, alanine dehydrogenase is upregulated during anaerobic conditions and is required for sustaining anaerobic growth (Feng et al. 2002; Starck et al. 2004). We therefore suggest that increased accumulation of this protein during stationary phase in *M. marinum* may also be an upregulation event. Evidence also suggests that mycobacterial alanine dehydrogenase is active as a secreted enzyme and, as such, may be involved in cell

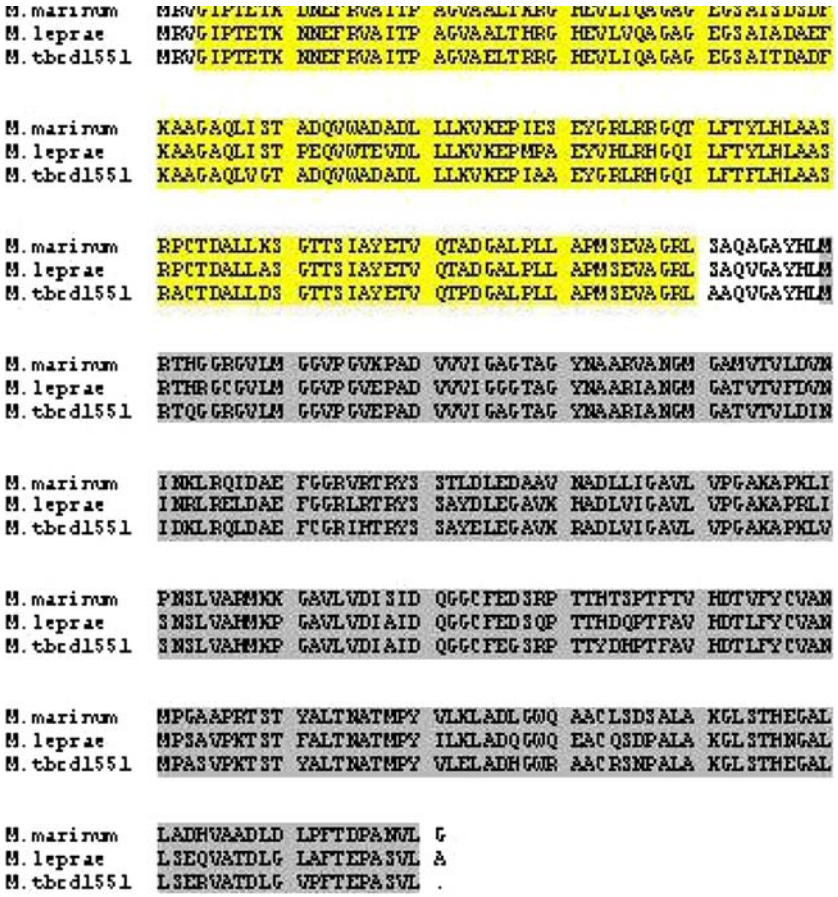

Fig. 6 Alignment of mycobacterial alanine dehydrogenases. Amino acid sequences of the alanine dehydrogenases from *M. marinum*, *M. tuberculosis* and *M. leprae* were aligned by ClustalX 1 81. The two domains AlaDh_PNT-N (PSSM-id: 16517) and AlaDh_PNT_C (PSSM-id: 15323) are indicated by shaded background

wall synthesis (Andersen et al. 1992; Ortalo-Magne et al. 1995; Raynaud et al. 1998). Current experiments in our laboratory are being conducted to fully elucidate the role of this enzyme in *M. marinum* stress responses.

Conclusions

Environmental mycobacteria are generally found in the water column, in sediments (particulate) or in shallow stagnant water (pellicles). Our results suggest that the solid–air interface may be an aerobic ecological niche for persistence of this bacterium in the environment. Although *M. marinum* binds firmly to abiotic hydrophobic surfaces under both aerobic and anaerobic/microaerophilic conditions, attachment leads to the development of a cohesive and persistent biofilm only under aerobic conditions. Initial attachment is mediated by cell wall components, which could be masked by accumulation of the capsular material on the cell wall. We also found that *M. marinum* alanine dehydrogenase accumulates significantly in the stationary phase cells. These data suggest that fortification of the physical barrier between the cytoplasm and extracellular environment, by increase in the thickness of the cell wall and capsular layer, may be a strategy for *M. marinum* to adapt to stress conditions.

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References

Andersen AB, Andersen P, Ljungqvist L (1992) Structure and function of a 40,000-molecular-weight protein antigen of *Mycobacterium tuberculosis*. Infect Immun 60:2317–2323

Bardouniotis E, Ceri H, Olson ME (2003) Biofilm formation and biocide susceptibility testing of *Mycobacterium fortuitum* and *Mycobacterium marinum*. Curr Microbiol 46:28–32

Barton A, Bernstein RM, Struthers JK, O'Neill TW (1997) *Mycobacterium marinum* infection causing septic arthritis and osteomyelitis. Br J Rheumatol 36:1207–1209

Bercovier H, Vincent V (2001) Mycobacterial infections in domestic and wild animals due to *Mycobacterium marinum*, *M. fortuitum*, *M. chelonae*, *M. porcinum*, *M. farcinogenes*, *M. smegmatis*, *M. scrofulaceum*, *M. xenopi*, *M. kansasii*, *M. simiae* and *M. genavense*. Rev Sci Tech 20:265–290

Beveridge TJ, Makin SA, Kadurugamuwa JL, Li Z (1997) Interactions between biofilms and the environment. FEMS Microbiol Rev 20:291–303

Bonafe JL, Grigorieff-Larrue N, Bauriaud R (1992) Atypical cutaneous mycobacterium diseases. Results of a national survey. Ann Dermatol Venereol 119:463–470

Brennan PJ, Nikaido H (1995) The envelope of mycobacteria. Annu Rev Biochem 64:29–63

Bruckner-Tuderman L, Blank AA (1985) Unusual cutaneous dissemination of a tropical fish tank granuloma. Cutis 36:405–6, 08

Carter G, Wu M, Drummond DC, Bermudez LE (2003) Characterization of biofilm formation by clinical isolates of *Mycobacterium avium*. J Med Microbiol 52:747–752

Castellanosa T, Ascenciob F, Bashana Y (2000) Starvation-induced changes in the cell surface of *Azospirillum lipoferum*. FEMS Microbiol Ecol 33:1–9

Costerton JW, Lewandowski Z, Caldwell DE, Korber DR, Lappin-Scott HM (1995) Microbial biofilms. Annu Rev Microbiol 49:711–745

Costerton JW, Cheng KJ, Geesey GG, Ladd TI, Nickel JC, Dasgupta M, Marrie TJ (1987) Bacterial biofilms in nature and disease. Annu Rev Microbiol 41:435–464

Cunningham AF, Spreadbury CL (1998) Mycobacterial stationary phase induced by low oxygen tension: cell wall thickening and localization of the 16-kilodalton alpha-crystallin homolog. J Bacteriol 180:801–808

Decostere A, Hermans K, Haesebrouck F (2004) Piscine mycobacteriosis: a literature review covering the agent and the disease it causes in fish and humans. Vet Microbiol 99:159–166

DeMaio J, Zhang Y, Ko C, Young DB, Bishai WR (1996) A stationary-phase stress-response sigma factor from *Mycobacterium tuberculosis*. Proc Natl Acad Sci USA 93:2790–2794

Donlan RM (2002) Biofilms: microbial life on surfaces. Emerg Infect Dis 8:881–890

dos Santos NM, do Vale A, Sousa MJ, Silva MT (2002) Mycobacterial infection in farmed turbot *Scophthalmus maximus*. Dis Aquat Organ 52:87–91

Draper P (1998) The outer parts of the mycobacterial envelope as permeability barriers. Front Biosci 3:D1253–1261

Feng Z, Caceres NE, Sarath G, Barletta RG (2002) *Mycobacterium smegmatis* L-alanine dehydrogenase (Ald) is required for proficient utilization of alanine as a sole nitrogen source and sustained anaerobic growth. J Bacteriol 184:5001–5010

Ghittino C, Latini M, Agnetti F, Panzieri C, Lauro L, Ciappelloni R, Petracca G (2003 Emerging pathologies in aquaculture: effects on production and food safety. Vet Res Commun 27:471–479

Gluckman SJ (1995) *Mycobacterium marinum*. Clin Dermatol 13:273–276

Gupta S, Pandit SB, Srinivasan N, Chatterji D (2002) Proteomics analysis of carbon-starved *Mycobacterium smegmatis*: induction of Dps-like protein. Protein Eng 15:503–512

Hall-Stoodley L, Lappin-Scott H (1998) Biofilm formation by the rapidly growing mycobacterial species *Mycobacterium fortuitum*. FEMS Microbiol Lett 168:77–84

Hall-Stoodley L, Stoodley P (2005) Biofilm formation and dispersal and the transmission of human pathogens. Trends Microbiol 13:7–10

Hassan AN, Frank JF (2004) Attachment of *Escherichia coli* O157:H7 grown in tryptic soy broth and nutrient broth to apple and lettuce surfaces as related to cell hydrophobicity surface charge and capsule production. Int J Food Microbiol 96:103–109

Heckert RA, Elankumaran S, Milani A, Baya A (2001) Detection of a new *Mycobacterium* species in wild striped bass in the Chesapeake Bay. J Clin Microbiol 39:710–715

Hedrick RP, McDowell T, Groff J (1987) Mycobacteriosis in cultured striped bass from California. J Wildl Dis 23:391–395

Irie Y, Mattoo S, Yuk MH (2004) The Bvg virulence control system regulates biofilm formation in *Bordetella bronchiseptica*. J Bacteriol 186:5692–5698

Karlyshev AV, Wern BW (2001) Detection and initial characterization of novel capsular polysaccharide among diverse *Campylobacter jejuni* strains using Alcian Blue dye. J Clin Microbiol 39:279–284

Krieg DP, Bass JA, Mattingly SJ (1988) Phosphorylcholine stimulates capsule formation of phosphate-limited mucoid *Pseudomonas aeruginosa*. Infect Immun 56:864–873

Levchenko TN (1991) [Ultrastructural organization of tuberculosis mycobacteria]. Probl Tuberk 12:42–46

Long ER, Seibert JS (1926) The chemical composition of the active principle of tuberculin I A non-protein medium suitable for the production of tuberculin in large quantity. Amer Rev Tuberc 13:393–397

Mishra M, Parise G, Jackson KD, Wozniak DJ, Deora R (2005) The BvgAS signal transduction system regulates biofilm development in *Bordetella*. J Bacteriol 187:1474–1484

Nigou J, Gilleron M, Puzo G (1999) Lipoarabinomannans: characterization of the multiacylated forms of the phosphatidyl-myo-inositol anchor by NMR spectroscopy. Biochem J 337:453–460

Ortalo-Magne A, Dupont M, Lemassu A, Andersen AB, Gounon P, Daffe M (1995) Molecular composition of the outermost capsular material of the tubercle bacillus. Microbiology 141:1609–1620

Patel S, Duke O, Harland C (1995) Septic arthritis due to *Mycobacterium marinum*. J Rheumatol 22:1607–1608

Perusse M, Pascot A, Despres J-P, Couillard C, Lamarche B (2001) A new method for HDL particle sizing by polyacrylamide gradient gel electrophoresis using whole plasma. J Lipid Res 42:1331–1334

Popova TE, Klitsunova NV (1998) [Capsule formation in *Pasteurella multocida* serovar A]. Zh Mikrobiol Epidemiol Immunobiol 6:11–16

Rastogi N, Hellio R (1990) Evidence that the capsule around mycobacteria grown in axenic media contains mycobacterial antigens: implications at the level of cell envelope architecture. FEMS Microbiol Lett 58:161–166

Raynaud C, Etienne G,Peyron P, Laneelle MA, Daffe M (1998) Extracellular enzyme activities potentially involved in the pathogenicity of *Mycobacterium tuberculosis*. Microbiology 144:577–587

Rhodes MW, Kator H, Kotob S, van Berkum P, Kaattari I, Vogelbein W, Quinn F, Floyd MM, Butler WR, Ottinger CA (2003) *Mycobacterium shottsii* sp nov a slowly growing species isolated from Chesapeake Bay striped bass (*Morone saxatilis*). Int J Syst Evol Microbiol 53:421–424

Rhodes MW, Kator H, Kotob S, van Berkum P, Kaattari I, Vogelbein W, Floyd MM, Butler WR, Quinn FD, Ottinger C, Shotts E (2001) A unique *Mycobacterium* species isolated from an epizootic of striped bass (*Morone saxatilis*). Emerg Infect Dis 7:896–899

Ridgway HF, Rigby MG, Argo DG (1984) Adhesion of a *Mycobacterium* sp to cellulose diacetate membranes used in reverse osmosis. Appl Environ Microbiol 47:61–67

Sato N (1972) [Pathogenic bacteria common to man and fish—*Mycobacterium marinum* isolated from skin granuloma in man]. Nippon Saikingaku Zasshi 27:775–779

Schulze-Robbecke R (1993) [Mycobacteria in the environment]. Immun Infekt 21:126–131

Shepard JL, Olsson B, Tedengren M, Bradley BP (2000) Protein expression signatures identified in *Mytilus edulis* exposed to PCBs copper and salinity stress. Mar Environ Res 50:337–340

Shevchenko A, Wilm M, Vorm O, Mann M (1996) Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. Anal Chem 68:850–858

Soini SM, Koskinen KT, Vilenius MJ, Puhakka JA (2002) Effects of fluid-flow velocity and water quality on planktonic and sessile microbial growth in water hydraulic system. Water Res 36:3812–3820

Starck J, Kallenius G, Marklund B, Andersson DI, Akerlund T (2004) Comparative proteome analysis of *Mycobacterium tuberculosis* grown under aerobic and anaerobic conditions. Microbiology 150:3821–3829

Stewart MH, Olson BH (1992) Physiological studies of chloramine resistance developed by *Klebsiella pneumoniae* under low-nutrient growth conditions. Appl Environ Microbiol 58:2918–2927

Tonjum T, Welty DB, Jantzen E, Small PL (1998) Differentiation of *Mycobacterium ulcerans*, *M. marinum* and *M. haemophilum*: mapping of their relationships to *M. tuberculosis* by fatty acid profile analysis DNA–DNA hybridization and 16S rRNA gene sequence analysis. J Clin Microbiol 36:918–925

Watson DL, Watson NA (1989) Expression of a pseudocapsule by *Staphylococcus aureus*: influence of cultural conditions and relevance to mastitis. Res Vet Sci 47:152–157

Wick LY, Ruiz de Munain A, Springael D, Harms H, de MA (2002) Responses of *Mycobacterium* sp LB501T to the low bioavailability of solid anthracene. Appl Microbiol Biotechnol 58:378–385

Wong S, Weinzirl J (1936) An inexpensive synthetic medium for growing *Mycobacterium tuberculosis*. Amer Rev Tuberc 33:577–586

Yuan Y, Crane DD, Barry CE 3rd (1996) Stationary phase-associated protein expression in *Mycobacterium tuberculosis*: function of the mycobacterial alpha-crystallin homolog. J Bacteriol 178:4484–4492