Amino Acids (2012) 42:1913–1926
DOI 10.1007/s00726-011-0917-z

ORIGINAL ARTICLE

On the *Thermus thermophilus* HB8 potential pathogenicity triggered from rhamnolipids secretion: morphological alterations and cytotoxicity induced on fibroblastic cell line

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Received: 5 January 2011 / Accepted: 8 April 2011 / Published online: 25 May 2011

Abstract A limited number of bacterial strains usually grown under nutrient limitation secrete rhamnolipids (RLs), which are recorded as virulence factors that are implicated in the pathogenicity of a microorganism. The non-pathogenic *T. thermophilus HB8* produces extracellular rhamnolipids (*Tth*RLs) under defined cultivation conditions using sunflower seed oil and sodium gluconate as carbon sources. In particular, the secreted *Tth*RLs have been isolated, purified and identified with ATR–FTIR. Their effects on the cells' viability were examined when they were supplemented in a culture of human skin fibroblasts. Purified T*th*RLs triggered a sequence of rapid and pronounced morphological alterations characterized by transformation of fibroblast shape from polygonal to fusiform; retraction with cytoplasm condensation, rounding up, distortion of nuclei and loss of lamellar processes, and finally disruption of membrane. The addition of T*th*RLs in the cultured fibroblasts caused cytotoxicity, in contrast to that of rhamnose that stimulated viability, as it was assessed by MTT test. These results revealed that among the constituents of RLs that are implicated in the cytotoxicity, it has to be attributed to the lipidic chain variation and not to the carbohydrate part. T*th*RLs cytotoxicity on fibroblasts is comparable, and provoked similar effects, to that caused by saponin white, a known surfactant. *Tth*RLs secretion might be a crucial point for the transformation of a non-pathogenic bacterium to a pathogenic one under certain environmental conditions favoring their secretion. RLs secretion in the microorganism's world might be a general route for the passage in the pathogenicity to ensure their survival under nutrient limitation conditions.

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Keywords Rhamnolipids · *Thermus thermophilus* · Fibroblasts · MTT cytotoxicity · Morphological alterations

Abbreviations

RLs	Rhamnolipids
*Tth*RLs	*Thermus thermophilus* rhamnolipids, *Tth*RL_1 and *Tth*RL_2, *Thermus thermophilus* rhamnolipids produced in the presence of sunflower seed oil and sodium gluconate, respectively
HAAs	3-(3-hydroxyalkanoyloxy)alkanoic acids

Introduction

Rhamnolipids (RLs) are surface-active glycolipids intensively investigated and reviewed (Maier and Soberon–Chavez 2000; Nietzsche et al. 2005; Ochsner et al. 1996; Soberón–Chávez 2004; Soberón–Chávez et al. 2005). Although the pathogen *Pseudomonas aeruginosa* has traditionally been considered as the primary RLs-producing microorganism, it was found that a restricted number of bacteria have also as the ability to produce RLs (El-Mawgoud et al. 2010). In accordance with that we recently found that the thermophilic eubacterium *Thermus thermophilus* HB8 secretes RLs growing in the presence of sunflower seed oil or oleic acid. The produced T*th*RLs have been identified using several methodologies, such as the orcinol method, Thin-layer chromatography (TLC) and attenuated total reflection infrared (ATR–FTIR) spectroscopy and LC–(ESI)–MS as well (Pantazaki et al. 2010).

The intrinsic and natural roles of these biosurfactants-RLs as well as their physiological function, for the producing organism, are not precisely defined and remain still a subject for further clarification. RLs physiological roles derived from their well-known physicochemical properties, such as surface activity, wetting ability, detergency, and other amphipathic-related characteristics. Thus, it has been found that indeed, RLs promote the uptake and biodegradation of poorly soluble substrates. Although most of the literature is concerned with the conversion of hydrocarbons and vegetable oils as substrates for production (Syldatk et al. 1985; Passeri et al. 1991; Lang and Wullbrandt 1999) water-miscible substrates, such as glucose or other carbohydrates have also been used (Guerra-Santos et al. 1984; Spoecker et al. 1999). The production of extracellular RLs by *P. aeruginosa* is strictly regulated and maximal RLs synthesis is found under conditions of nitrogen limitation during the stationary phase of growth, using glucose, glycerol, or *n*-paraffins as carbon sources (Mulligan and Gibbs 1989). Factors affecting bio-surfactant production, such as nutrient limitation and the influence of trace element concentration, have been studied extensively in continuous culture (Guerra-Santos et al. 1986).

RLs act as immune modulators and virulence factors, they have antimicrobial activities, and they are involved in surface motility and in bacterial biofilm development (El-Mawgoud et al. 2010). In particular, plethora of biological activities ascribed to their detergent-like properties are referred for *P. aeruginosa* biosurfactants which exhibit low irritancy and even anti-irritating effects, as well as compatibility with human skin (Maier and Soberon-Chavez 2000). Di-RL from *P. aeruginosa* displays differential effects on human keratinocyte and fibroblast cultures (Stipcevic et al. 2005), and is known as the heat-stable hemolysin or glycolipid secreted into the environment by *P. aeruginosa* (Johnson and Boese-Marazzo 1980). In vivo data relate specifically to the treatment of autoimmune diseases and include the effects of di-RL on cellular immunosuppression, oxazolone-induced delayed-type hypersensitivity, immunomodulation and immunorestoration (Piljac and Piljac 1995a). Lastly, the clinical trials with di-rhamnolipid conducted on the treatment of psoriasis, *lichen ruber planus*, neurodermatitis and human wound healing have confirmed excellent ameliorative effects of di-RL when compared with conventional therapy using corticosteroids (Piljac and Piljac 1995b).

In addition, it was suggested that di-RL mode of action could involve a direct cell-receptor binding, creating complexes with serum components, binding to the membrane proteins, passing through the phospholipid bilayer of the cell membrane and interacting with DNA transcriptional and translational machinery (Stipcevic et al. 2005).

More recently, it has been shown that *P. aeruginosa* invades respiratory epithelia reconstituted with primary human respiratory cells due to the RLs production (Zulianello et al. 2006). The mechanism which involves the incorporation of RLs within the host cell membrane followed by tight-junction alterations leads to the suggestion that the junction-dependent barrier of the respiratory epithelium is selectively altered by RLs (Zulianello et al. 2006).

Other remarkable properties of RLs as lysis induction of polymorphonuclear leukocytes (PMNs) or antimicrobial activity have also been referred by (Jensen et al. 2007; Shryock et al. 1984; Van Gennip et al. 2009). Concerning their mechanism of action, it consists of intercalation into the biological membrane and destruction by their permeabilizing effect (Sotirova et al. 2008).

RL secreted from the saprophytic bacterium *Burkholderia pseudomallei* (causative agent of melioidosis, an infectious disease of humans and animals), induced cytopathic changes, which were due to a progressive reorganization of the F-actin network resulting in impaired cell cycle progression and reduced phagocytic function of macrophages. These cytopathic changes are characterized by retraction, rounding up, and finally detachment in phagocytic and non-phagocytic cell lines (Häussler et al. 2003).

In *P. aeruginosa*, the transcription of RLs synthesis gene *rhlAB* is enhanced by the transcriptional activator RhlR, which is controlled by the quorum-sensing (QS) system Rhl (Winzer and Williams 2001; Smith and Iglewski 2003). Although there is no information concerning the existence of functional quorum-sensing system in *T. thermophilus* HB8, the expression of RLs synthesis genes was attested by the identification of the produced RLs using sunflower seed oil and oleic acid as carbon sources (Pantazaki et al. 2010).

Within this work, we present data concerning RLs production as well as their identification by ATR–FTIR from the thermophilic bacterium *T. thermophilus* using sodium gluconate as carbon source. Human skin fibroblasts co-cultivated with two different kinds of purified *Tth*RLs, i.e., $TthRL_1$ and $TthRL_2$, which are originated from *T. thermophilus* cultures grown in different carbon sources and exhibited, pronounced alterations in fibroblasts' morphology. In particular, the characteristic fibroblast-like shape was transformed to a stellate/bipolar form, which was clearly visualized by optical microscopy, and this observed alteration was related to the induced cytotoxicity assessed by MTT test.

Materials and methods

Bacterial strain and media

Thermus thermophilus HB8 (DSM Acc No 579) was grown at 75°C in basal-rich medium (BRM) (Pantazaki et al. 1998) or in (MSM) (Pantazaki et al. 2003). For RLs production in batch cultivation, mineral salt medium (MSM) supplemented with sunflower seed oil from *Helianthus annuus* (Sigma S-5007) (Pantazaki et al. 2010) or sodium gluconate, used each alone as a carbon source. Agar (gellan gum) was obtained from Roeper GmbH for RLs production on agar plates.

Materials

DMSO, MTT kit assay were purchased from Sigma–Aldrich (Munich, Germany). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and other cell culture reagents were obtained from GIBCO. All other reagents used were of the highest purity commercially available. Saponin white derived from BDH biochemicals, product no. 44092.

Methods

RLs production in batch cultivation of T. thermophilus in Erlenmeyer flasks

RLs were produced and purified as previously reported using sunflower seed oil (14 g/l) designated as *Tth*RL_1 (Pantazaki et al. 2010), or in this work using sodium gluconate 1.5% (*w*/*v*) as alternative carbon source and designated as *Tth*RL_2. Sunflower seed oil was selected due to its composition in fatty acids while sodium gluconate was selected since it is a derivative of sugar, glucose. The selection of these two different carbon sources intended to introduce fatty acids or sugars to the RLs biosynthetic pathway. The microorganism was cultivated for RLs production in batch cultivation into 2-l Erlenmeyer flasks containing 600 ml of MSM and supplemented with the pre-mentioned carbon sources, respectively. Flasks inoculated with 5% (*v*/*v*) inocula of a *T. thermophilus* HB8 pre-culture exponentially grown in BRM. Cultivation was incubated on a rotary shaker (at 140 rpm) at 75°C until 80 h. Aliquots of 200 ml were taken from each of these cultures at various cultivation times, and were centrifuged at 10,000 rpm for 15 min at 30°C to separate the biomass from the supernatants that were destined for RLs determination. After harvesting, cells were washed once with 0.9% (*w*/*v*) NaCl to ensure the complete removal of the culture broth, while supernatants were recovered to extract RLs. The culture experiments were performed independently at least three times.

Analytical procedures

Cell dry weight (CDW) per liter of culture broth was determined by centrifuging 8 ml of culture broth at 10,000×*g* for 15 min, washing cell pellets and weighing dry cells as previously reported (Wang and Lee 1997). RLs were extracted from the culture supernatants, as described (Hori et al. 2002) and quantified by the colorimetric orcinol method (Koch et al. 1991; Pesci et al. 1997). Three independent samples were extracted for each cultivation time to monitor the reproducibility of the RLs extraction procedure. RLs concentration was calculated by a coefficient of 3.4, obtained from the correlation of pure RLs/rhamnose (1.0 mg of rhamnose corresponds approximately to 3.4 mg of RLs) (Pearson et al. 1997). Purified RLs were diluted in DMEM immediately before use in the MTT test. The RLs were analyzed by thin-layer chromatography (TLC) on silica gel plates (Kieselgel 60/Kieselgur F254; Merck, Darmstadt, Germany). Samples were spotted on the plates and separated using a mobile phase consisting of 80% (*v*/*v*) chloroform, 18% (*v*/*v*) methanol and 2% (*v*/*v*) acetic acid. TLC plates were sprayed with a thymol spray reagent and RLs appeared as pink spots (Schenk et al. 1995).

Detection of RLs production on agar plates

A well-established semi-quantitative agar plate test was used for the detection of extracellular RLs originally developed (Siegmund and Wagner 1991). In particular, bacteria from overnight cultures in MSM in the presence of 1.5% (*w*/*v*) sodium gluconate were spot-inoculated as 10-μl aliquots onto M8 medium supplemented with $MgSO_4$ (1 mM), glucose (0.2% *w*/*v*), casamino acids (CAA) (0.5% *w*/*v*), cetyl-trimethyl-ammonium bromide (CTAB) (0.02% *w*/*v*) and methylene blue (0.0005% *w*/*v*) and were solidified with agar (1.5% *w*/*v*). Plates were incubated at 37°C for 24 h, followed by incubation at room temperature for additional 24 h. Biosurfactant production results in the precipitation of CTAB and the formation of a dark blue halo that is outlined in white surrounding the colony (Caiazza et al. 2005). In addition, concentrated purified or culture supernatants containing RLs were spotted onto paper filter discs (6.0-mm Whatman AA discs), which were then put onto a layer of the same agar plate. (Typically, 10 ml of a culture supernatant was concentrated down to 100 μl, and drops of 10 μl were applied to the paper discs.)

ATR–FTIR spectroscopy of TthRL$_2$

ATR–FTIR is a FTIR variety, which allows organic substances in aqueous solutions to be determined. ATR–FTIR involves the collection of radiation reflected from the interfacial surface between the aqueous solution and a reflection element (ATR crystal). In this crystal, evanescent waves emanate from the crystal, penetrate the aqueous solution and are absorbed by substances in this solution. When concerning the analysis of biosurfactants several spectroscopic methods for the characterization, identification and quantification have been reported (Leitermann et al. 2008; Pantazaki et al. 2010 and references therein).

FTIR analysis of *Tth*RL$_2$ was performed on a Nicolet 6700 FTIR spectrometer (Thermo Scientific, Massachusetts, USA) with an Orbit Diamond Crystal W (SPA-2 Tower for Nexus) ATR-Dura sampler cell. The sample was dissolved in $CHCl_3$:CH_3OH 2:1 (*v*/*v*) and a small amount (1 droplet) was placed on the ATR diamond, and followed by solvent evaporation before taken the spectra ranging from 600 to 4,000 cm^{-1} using 32 scans and a resolution of 4 cm^{-1}.

Cell culture

Human skin fibroblasts were cultured in DMEM supplemented with 10% fetal bovine serum and antibiotics penicillin and streptomycin. Cell line was maintained in continuous culture at 37°C, in an atmosphere with 5% CO_2, using standard aseptic techniques and cell growth was monitored by determining the cell number/ml with the use of a Coulter counter model ZBI.

Experimental treatments for cell morphological observation

Skin fibroblasts cells were seeded for 24 h into a 24-well culture plate (Corning Glassworks, Corning, NY) (1 ml of DMEM-10% FCS per well containing 2×10^5 cells/ml) and pre-warmed to 37°C for 2 h for adhesion. The stock saponin solution (1 mg/ml) was prepared by dissolving saponin white in DMSO and then sample dilutions were prepared in both media (DMSO and DMEM). The evaluation of the effect of DMSO in fibroblast's viability was performed by a control containing 1% DMSO in DMEM. All samples were subjected to various treatments as described below.

Skin fibroblasts were exposed for various time intervals from 24 to 96 h to one of the following conditions: (1) no treatment (in DMEM alone, control) (2) addition of 100–500 μg/ml purified *Tth*RL$_1$, (3) addition of 100–500 μg/ml purified *Tth*RL$_2$ and (4) addition of rhamnose at final concentrations 1.5 and 3 mM, (5) addition of 10 μg/ml saponin (final concentration) and (6) no treatment (medium DMEM supplemented with 1% *v*/*v* DMSO, control).

The experiments were stopped by extensive washing of the fibroblasts with DMEM and metabolically active cells were evaluated using an MTT assay (Sigma) to assess cytotoxicity and viability. To this end, fibroblasts were additionally stained with methylene blue.

MTT assays

Cell proliferation was assessed by monitoring the conversion of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) to formazan. The reduction in MTT is catalyzed by mitochondrial dehydrogenase enzyme and is therefore a measure for cell viability (Mosmann 1983). The MTT assay was used as a cytotoxicity assay for skin fibroblastic cells grown in the presence of the *Tth*RLs. In brief, cells (2×10^5 cells ml^{-1}) were seeded in 96-well plates and treated with varying concentrations (0–500 μg/ml) of *Tth*RLs at various time intervals. The MTT assay was performed as follows: MTT powder was dissolved in PBS at a concentration of 5 mg/ml) and 600 μl of MTT solution were added to each well. Following incubation at 37°C for 4–6 h in a fully humidified atmosphere with 5% CO_2, MTT was taken up by active cells and reduced in the mitochondria to insoluble formazan granules. Subsequently, the medium was discarded and the precipitated formazan was dissolved in isopropanol (400 μl/well) and optical density (OD) of the solution was evaluated using a microplate spectrophotometer (Organon Teknica© Enterprise Ireland) at a wavelength of 490 nm. At least three wells were randomly taken for examination each time. Absorbance values were blanked against DMSO for saponin or DMEM-dependent samples solubilization and the absorbance of cells exposed to medium only (i.e. no RLs added) was taken as 100% cell viability. Values shown are the standard deviation of at least three measurements.

Results

Analysis of time profile of RLs production in batch cultivation using sodium gluconate as carbon source

RLs used in this study were produced for the first time, and identified *Tth*RL$_1$, when *T. thermophilus* HB8 grown in two immiscible substrates as carbon sources, sunflower seed oil or oleic acid composed from fatty acids (Pantazaki et al. 2010). In addition, RLs production was attempted in the present work using a soluble carbon source sodium gluconate, which is very different from the fatty acids used in the previous study. Sodium gluconate has glucose as

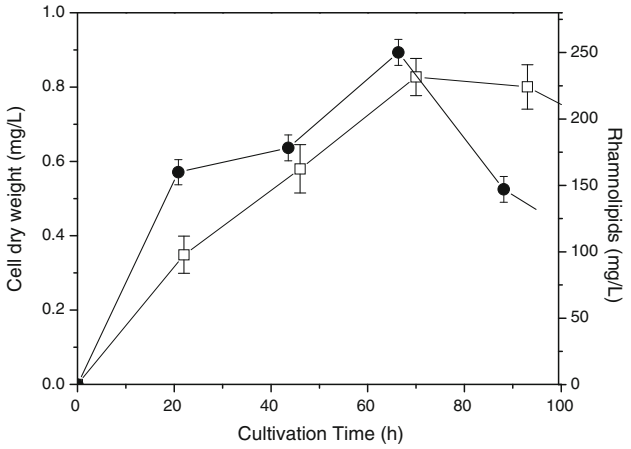

Fig. 1 Profiles of cell growth measured as cell dry weight (CDW) (*squares*) and rhamnolipids production (*filled circles*) during cultivation time of a typical *T. thermophilus* HB8 fermentation using sodium gluconate (1.5% *w/v*) as sole carbon source

metabolic precursor, in which it is transformed via Entner–Doudoroff pathway by glucose dehydrogenase with the $NAD(P)^+$ reduction. In fact, there have been several reports on RLs production from glucose produced by *Pseudomonas* (Guerra-Santos et al. 1984; Spoecker et al. 1999). To clarify the dependence of RLs synthesis by *T. thermophilus* HB8 on the cultivation time using sodium gluconate as carbon source, the profiles of CDW and of RLs production were determined during cultivation time (Fig. 1). *T. thermophilus* HB8 growth was satisfactory on sodium gluconate and CDW reached a maximal value of 0.9 g/l after 72 h of cultivation. RLs productivity, in the presence of 1.5% (*w/v*) sodium gluconate attainted its maximal value, which was as high as 250 mg/l approximately during 72–90 h of cultivation (Fig. 1). The carbon flux is crucial for RLs production and RLs synthesis continued as long as carbon sources became exhausted. Prolonged incubation decreases RLs productivity due to RLs consumption by the cells to ensure their survival since nutrient limitation is advanced and crucial under these circumstances or probably due to progressive cell death.

Detection of RLs production on agar plates

The capability of *T. thermophilus* HB8 cells to produce RLs in the presence of sunflower seed oil or sodium gluconate was monitored on agar plates. Two suspensions of cells originated from the respective cultures grown overnight, were spotted onto the agar plates (Fig. 2, plate a, spot 1, culture in the presence of sunflower seed oil), and (Fig. 2, plate b, spot 1′, culture in the presence of sodium gluconate). In addition, a supernatant derived from a *T. thermophilus* culture grown in the presence of sodium gluconate (plate a, spot 2), and as sample of reference MSM alone (plate b, spot 2′) were spotted onto filters. Supernatant was previously determined for the presence of RLs by the colorimetric orcinol method. Then, filters were placed onto the agar plates and all plates were incubated at 70°C for 72 h. These results revealed that *T. thermophilus* cells secreted detectable levels of rhamnolipids, as visualized by the blue halos formatted in the plate assay (Fig. 2). The slightly anionic rhamnolipid molecules (biosurfactants) form an insoluble ion pair with the cationic tenside CTAB and the basic (cationic) dye methylene blue included in mineral agar plates. This complex was visualized in the form of a blue precipitate surrounding the rhamnolipids producing colonies. This specific method for anionic biosurfactants was originally developed for the selection of RL-producing strains.

Characterization of $TthRL_2$ by attenuated total reflection Fourier transform infrared (ATR-IR/IR) spectroscopy

The molecular composition of the crude biosurfactant produced by *T. thermophilus* HB8 was evaluated by ATR–FTIR and conventional FTIR spectroscopy. Figure 3 represents the spectra of the freeze-dried extracellular biosurfactant derived from a *T. thermophilus* culture grown in sodium gluconate. The broad negative bands at about 3,300 and 1,654 cm^{-1} resulted from a higher water concentration in the reference spectrum (pure water) relative to the aqueous sample, and are attributed to hydrogen bonding and O–H stretching of water and the 3,400 cm^{-1} (for O–H bonds) confirming the presence of glycolipid moieties. The double bands present at around 2,965, 2,921 (CH band: CH_2–CH_3 stretching), 2,855 and 1,401 cm^{-1} that include (–CH_3) symmetric deformation vibrations (C–H) bending vibrations of CH_3 and CH_2 groups and CH_2–CH_3 stretching vibrations are characteristic of polysaccharides or are derived from symmetric C–H stretching vibrations of aliphatic groups, like those represented in the hydroxydecanoic acid chain tails of rhamnolipid 3 (Leitermann et al. 2008). A C=O stretching band at 1,718 cm^{-1} is characteristic of ester bonds and carboxylic acid groups. (The absorption band at about 1,654 cm^{-1} originated from carboxylic groups while the absorption bands of higher frequencies 1,718 cm^{-1} are assigned to the ester groups.) In the fingerprint region of the spectrum, the area between 1,200 and 1,460 cm^{-1} represents C–H and O–H deformation vibrations, typical for carbohydrates as in the rhamnose units of the molecule, while 1,255 cm^{-1} (PI band: phosphates) and between the 1,102–1,056 cm^{-1} were attributed to ethereal and the hydroxylic C–O stretch vibrations of carbohydrates (PII band: polysaccharides). In addition, the appearance of absorption bands at about 1,654 cm^{-1} (AmI band: C=O stretching in proteins),

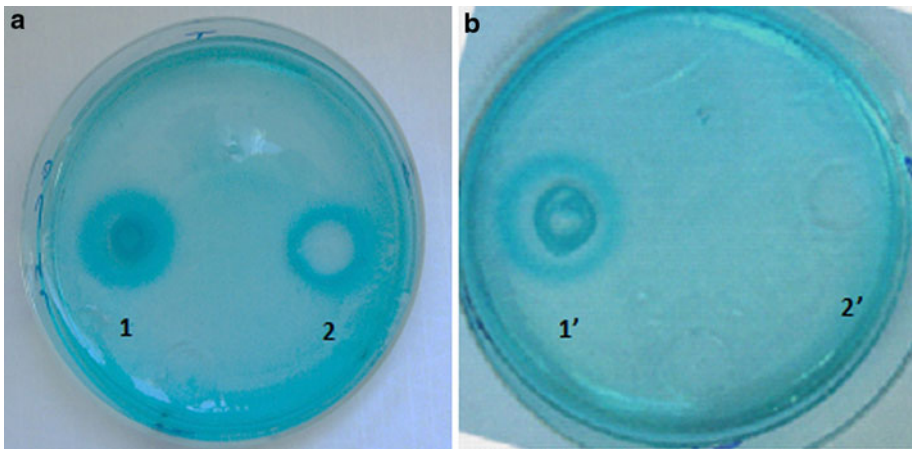

Fig. 2 Representative images of *T. thermophilus* HB8 that secreted detectable levels of RLs as visualized by the *dark blue halo* in the plate assay. *Plates* **a** and **b** (*1* and *1′*): *T. thermophilus* cells were grown in MSM in the presence of 14 g/l sunflower seed oil (**a***1*) or 1.5% (*w*/*v*) sodium gluconate (**b***1′*) for 72 h at 70°C and cells suspension were spot-inoculated as 10-µl aliquots onto a solidified with agar (1.5% *w*/*v*) plate. *Plate* **a** and **b** (*2* and *2′*): Culture supernatant or concentrated purified RLs from *T. thermophilus* cultivated in the presence of 1.5% (*w*/*v*) sodium gluconate (**a***2*), and MSM alone (**b***2′*) for 72 h at 70°C, and were spotted onto paper filter discs (6.0-mm Whatman AA discs), which were then putted onto a layer of the same agar plate

1,551 cm^{-1} (AmII band: N–H bending in proteins) is indicative for presence of proteins in the samples. All spectra showed essentially the same adsorption bands, and only the relative areas under the various absorption bands were different.

*Tth*RLs attack fibroblasts changing their morphology

Morphology of untreated cells

Skin fibroblasts allowed proliferating in culture without any treatment, showed the expected morphological characteristics. In particular (Fig. 4, upper panel), a homogenous population with a branched cytoplasm surrounding an elliptical speckled nucleus is observed. Although disjointed and scattered, they locally align in parallel clusters. Cells appear flattened and demonstrated the longitudinally arrayed cell processes (Fig. 4a, b, upper panel), with elevated central, nuclear regions (culture of reference). The nucleus (N) contained one to three nucleoli (n) and the contour integrity of their nuclear membrane remained unaffected. The fine chromatin network was regularly scattered as clumps within the nucleus with a classical weak condensation around the nucleolus. Their parallel clusters and the nucleus with 2–3 nucleoli as well can also be clearly distinguished after staining with methylene blue (Fig. 4a, b, inset, upper panel).

Morphology of fibroblasts co-incubated with TthRLs

Upon incubating the fibroblasts with purified *Tth*RLs a sequence of rapid and dramatic alterations in cell morphology was triggered which was observed with phase-contrast microscopy. The extent of the morphological changes could be detected in the cells as early as 24 h of incubation with *Tth*RLs. These alterations consisted of transformation of fibroblast shape from polygonal to fusiform, retraction with cytoplasm condensation and loss of lamellar processes, and appearance of groups of round, swollen, and highly retractile degenerating cells, appeared in a fairly regular pattern throughout the monolayer. Figure 4 (middle panel) shows some characteristic cell transformation of fibroblast shape in response to treatment with *Tth*RLs. Fibroblasts varied in outlines and in the early phase of alterations retract their dendritic network of extensions, which had thickened and simplified giving the cells from fusiform to a more stellate or bipolar appearance (Fig. 4a, b, middle panel). This retraction of cell extensions might well be attributed to the detergent-like activity of RLs which is well known that reduces the surface tension. In response to that cells begin to resist and to develop isometric tension making by this way the transition from dendritic to stellate/bipolar morphology.

In fibroblasts derived from the same culture, but showing a more advanced stage of degeneration, an enlargement of the cell nuclei diameter was observed, which in most cases appears distorted to have been forced aside to the edges of the cell by a cytoplasmic clump of material surrounding the nucleoli (Fig. 4b, c, middle panel), while the outlines of the cell nucleus are obscure by the paranuclear mass (Fig. 4d, middle panel). The two nuclei (n) at upper right can be clearly distinguished into the nucleus (Fig. 4c, middle panel), while in some cells, it was difficult to distinguish the nucleolus because of the shriveling of the nucleus and the span of existence of the vacuoles that cover almost the

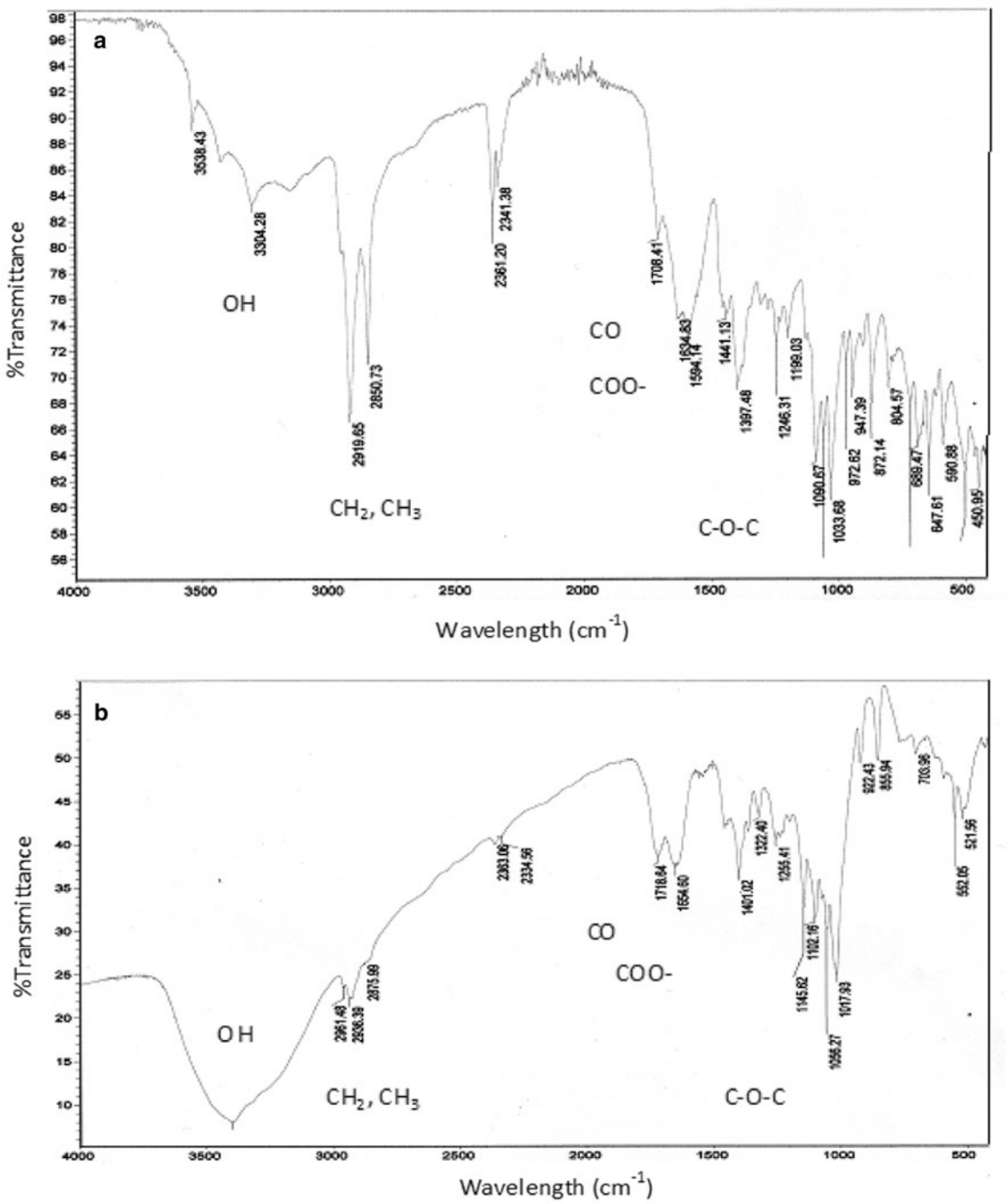

Fig. 3 Rhamnolipids IR-spectrum. **a** ATR–FTIR and **b** FTIR spectra of an aqueous solution of rhamnolipid isolated from the supernatant of a *T. thermophilus* HB8 culture grown in sodium gluconate as carbon source. The allocation of conspicuous absorption to the corresponding characteristic group absorption from literature was attempted

cytoplasm. Characteristic vacuoles are seen in the peripheral zone of the cytoplasm (v) (Fig. 4c, d, middle panel). The cell shows a well defined cytoplasmic mass (cm) (Fig. 4c, middle panel). Clear, filamentous areas are seen in the cytoplasm. They appear to arise in the central zone of the cytoplasm and they constantly change their shape and location, moving towards the clear peripheral zone. The cell has lost contact with its neighbors and exhibits thread-like cytoplasmic extensions which contain dense swellings. In culture, at a slightly more advanced stage, the cell has begun to have an unusual round shape and presented clear cytoplasmic vacuolization (Fig. 4c–f, middle panel). The

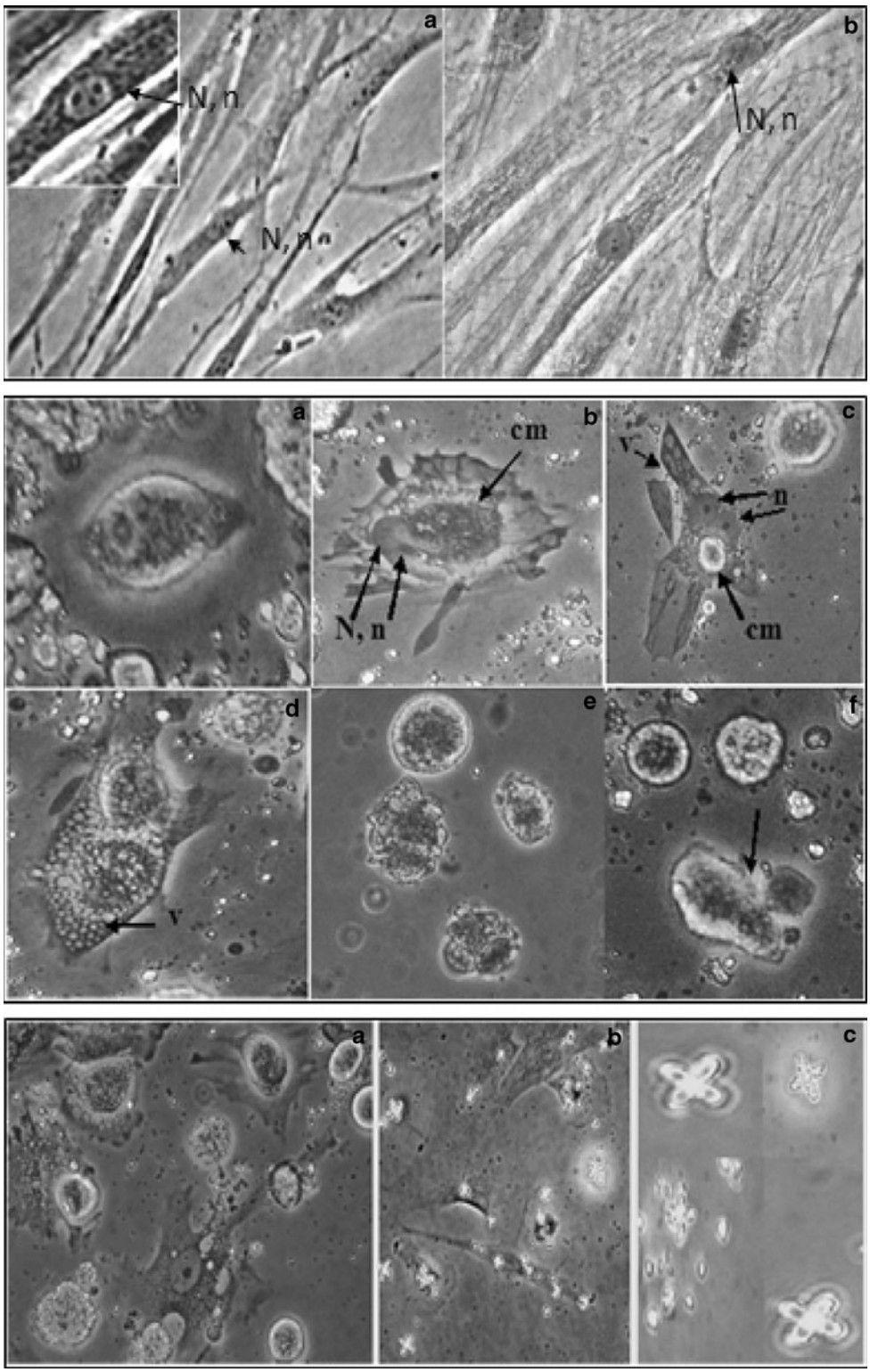

Fig. 4 Phase-contrast micrographs of cultured human dermal fibroblasts. (*Upper panel*, **a**, **b**) Untreated fibroblasts (**a**), and *inset*: detail from the same image (**a**); magnifications (×40). **b** The same preparation was stained with methylene blue (×40). (N); represents nucleus containing two or three nucleoli (n), which were well distinguished. (*Middle panel*, **a**–**f**) Fibroblasts treated with *Tth*RLs. Fields from the culture preparation showing fibroblastic cells subjected to morphological changes in response to treatment with 500 μg/ml of *Tth*RL_2 after 48 h. The cultures were observed at ×40 magnification (v and cm represent vacuoles and cytoplasmic mass, respectively). (*Bottom panel*, **a**–**c**) Fibroblasts treated with saponin (10 μg/ml) for comparison (**a**, **b**), and (**c**). Domain from image **b**

nucleus is masked by the cytoplasmic paranuclear mass. Finally, the cells showed an unusual round morphology, swollen nucleus, suggestive of oncosis, and disintegrated (disrupted) plasma membranes. A field from the same preparation containing a cell, with a disrupted membrane is shown in Fig. 4f, middle panel. The extent of all these alterations was visualized by observing various fields from the preparation after methylene blue staining (Fig. 5).

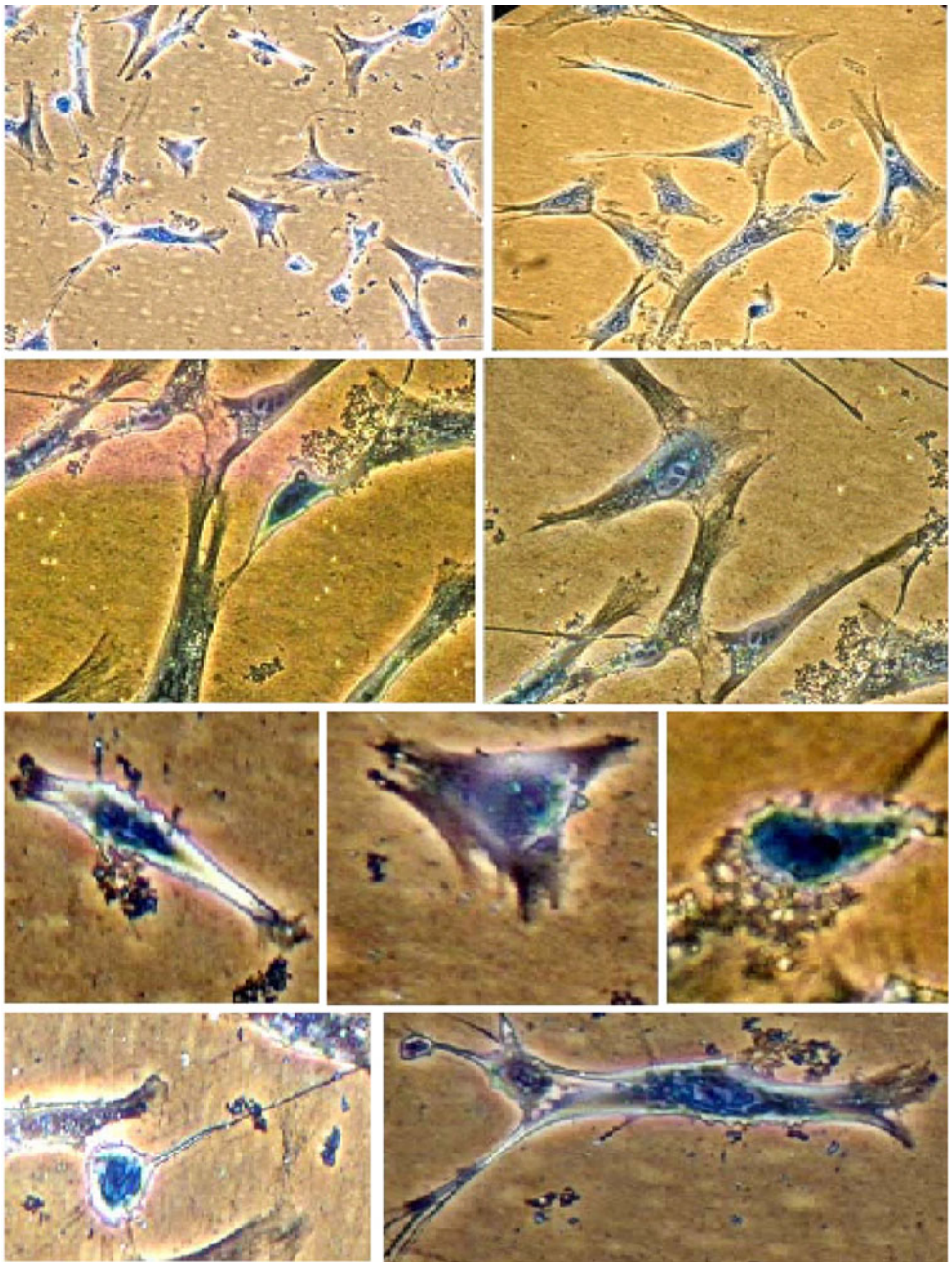

Fig. 5 Phase-contrast micrographs of cultured human dermal fibroblasts. Fields from culture preparation show cells subjected to morphological changes after exposure to *Tth*RL_1 and *Tth*RL_2 for 48 h and stained with methylene blue

Morphology of saponin white-treated fibroblasts

Saponin, a well-known biosurfactant was used as a positive sample of reference in the fibroblast investigations described above. Figure 4 (bottom panel), down has included images of fibroblasts treated for 24 and 48 h with saponin white (10 μg/ml) for comparison (Fig. 4a–c). When the culture medium of fibroblasts was supplemented with saponin, fibroblasts exhibited an altered morphology. In particular cells were shriveled and most of them appeared fully rounded and therefore lacking major processes due to the eccentric nucleus. It has to be addressed that a similar observation was taken for fibroblasts undergoing treatment—as mentioned before—with *Tth*RLs. It is also notable that fibroblasts, exposed during a prolonged time to saponin had a cross-shaped appearance, as it can be seen (Fig. 4c, bottom panel), resulting from their retraction effect which seems to constitute an intermediate transition step before rounded cell and might be attributed to its surfactant properties. These fibroblasts varied in outline from fusiform to stellate with 4–5 broad tapering processes. Similar forms were formatted after fibroblasts treatment with prostaglandin E2 (Smith et al. 1994).

Morphology of rhamnose-treated fibroblasts

In several systems, rhamnose promotes the binding of polysaccharides to specific receptors in the membrane (Soell et al. 1995). To elucidate whether rhamnose promoted the insertion of RLs within the membranes of fibroblastic cells, or the lipidic chain that composes *Tth*RLs is the responsible constituent for the influence on the fibroblast's morphology, we investigated the morphological changes caused by unconjugated α-L-rhamnose by exposing cells to L-rhamnose for the same time intervals. In particular, when the culture medium of fibroblasts was supplemented with rhamnose (1, 2 and 3 mM), fibroblasts demonstrated the same typical and longitudinal shape of untreated cells suggesting that rhamnose did not alters fibroblast's morphology (data not shown).

MTT assay

Cytotoxicity of the purified *Tth*RLs (1 and 2) was studied by means of a colorimetric microculture assay (MTT assay) in fibroblastic cell line originating from human skin tissue. (Treatment i) Fibroblasts were cultivated in DMEM without any treatment and this cell viability represents the 100% (Control) (Fig. 6). (Treatment ii and iii) To assess whether un-conjugated rhamnose as a major constituent of the RLs promoted itself inhibition of fibroblasts' viability (cytotoxicity), cells were exposed to rhamnose for the same time intervals. Thus, when the culture medium of fibroblasts was supplemented with rhamnose (1.5 and 3 mM), it was demonstrated that viability of fibroblasts stimulated and reached approximately at 110 and 116%, respectively. (Treatment iv and v) When fibroblastic cells were exposed for 24 h to purified *Tth*RL_2, diluted in DMEM at final concentrations 200 and 500 μg/ml, viability of fibroblastic cells decreased drastically to 80 and 66%, respectively. (Treatment vi and vii) Similarly purified *Tth*RL_1, diluted in DMEM at final concentrations 200 and 500 μg/ml viability of fibroblastic cells decreased drastically to 87 and 37%, respectively. Treatment (viii). Additionally, cell viability of fibroblastic cells was also assessed when cells were supplemented with saponin white, which was used as a positive control at a final concentration of 10 μg/ml in DMSO. Under these conditions viability of fibroblastic cells reached barely to 32% compared with the control performed in DMEM. Treatment (vi). Since saponin was diluted in DMSO, a control culture was performed in the corresponding concentration of 1% (*v*/*v*) DMSO. Therefore, fibroblasts' viability after exposing to 1% (*v*/*v*) DMSO was reduced to 84% compared to the culture of reference that was performed in DMEM (100%). Conclusively, these results attest that *Tth*RLs affect viability of fibroblastic cells evidently at the concentrations tested.

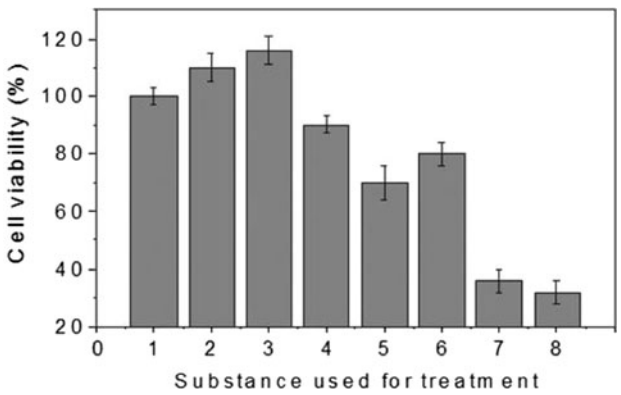

Fig. 6 Cytotoxic activity of *Tth*RLs measured by the MTT assay on skin fibroblasts cells over an incubation period of 24 h. *Bar 1* fibroblasts without treatment (control). *Bars 2 and 3* fibroblasts treated with rhamnose (1.5 and 3 mM, respectively). *Bars 4 and 5* fibroblasts treated with *Tth*RL_2 (100 and 500 μg/ml), respectively. *Bars 6 and 7* fibroblasts treated with *Tth*RL_1 (100 and 500 μg/ml, respectively. *Bar 8* fibroblasts treated with saponin white (at a final concentration of 10 μg/ml)

Discussion

The thermophilic and non-pathogenic bacterium *T. thermophilus* HB8 could be classified, for the first time, as it is shown within our work (Pantazaki et al. 2010; this work), among the microorganisms that secretes rhamnose-containing glycolipid biosurfactants called *Tth*RLs. Until now, almost only *Pseudomonas aeruginosa*, which is a major pathogen among immunocompromised or critically ill individuals, and a few other bacterial species, held conspicuous place in RLs production (El-Mawgoud et al. 2010). The virulence of *P. aeruginosa* is multi-factorial and includes a wide variety of cell-associated and extracellular proteins or other exo-products (Lazdunski 1998; Kipnis et al. 2006), including RLs which were implicated as immunomodulators and virulence factors (El-Mawgoud et al. 2010).

We have previously demonstrated that *T. thermophilus* HB8 grown in MSM in the presence of fatty acids (Pantazaki et al. 2010), and in this work in the presence of sodium gluconate secreted large amounts of RLs, when bacterial cells reached at high cell densities at the late stationary phase after a prolonged cultivation. At this phase of growth where RLs are secreted it was shown that carbon sources used as well as nutrients became exhausted in most cases (Pantazaki et al. 2010). It is also remarkable that the microorganism did not secrete RLs when grown in rich medium. In *P. aeruginosa* the so-called quorum sensing (QS response) is a bacterial generic reply to a chemical signal produced by the same cells and in this way bacteria modulate the transcription of genes important for their

survival at high densities. QS response in *P. aeruginosa* constitutes a novel generic regulon that integrates and responds to nutritional factors and stress conditions in addition to bacterial density (Soberón-Chávez et al. 2005) and RLs production at transcriptional level regulated by the QS response.

In this work, we demonstrated a previously unknown effect of *Tth*RLs on fibroblasts concerning their dramatic morphological changes in vitro which might have physiological relevance. In particular, our experimental data shows that morphological alterations of fibroblasts attributed to *Tth*RLs were accompanied by strong cytotoxicity, as revealed by MTT test. Thus, the observed morphological alterations (transformation) elicited by *Tth*RLs were accompanied by decrease in the MTT reduction activity, reflecting a dysfunction in the cell metabolic activity. It has to be addressed that the aforementioned dysfunction usually occurs in both early oncosis and apoptosis, while the loss of cell membrane integrity is a common in vitro event in necrosis secondary to both oncosis and apoptosis (Majno and Joris 1995). These effects are comparable with that caused by 10 μg/ml of saponin white, well known for its hemolytic activity and cytotoxicity. In brief, the resulting cytotoxicity, as determined by MTT test, reveals the duality and/or complementarity of morphological alterations, especially in plasma membrane alteration affecting the action on membrane receptors for apoptosis.

Disruption of plasma membrane integrity is characteristic of oncotic cells but also of late apoptotic cells (Majno and Joris 1995). The proposed term "oncosis" used in cell death is accompanied by cellular and organelle swelling, cytoplasmic vacuolization, blebbing and disintegration of the cell membranes. In contrast, apoptosis is characterized morphologically by cell shrinkage and by condensation of chromatin within the limits of near-to-intact plasma membranes. The nucleus may also break up and the cell emits processes (apoptotic bodies) that often contain pyknotic nuclear fragments (Majno and Joris 1995).

For comparison we near the effect of *P. aeruginosa* di-rhamnolipids (di-RLs), referred to as BAC-3, on fibroblasts which were recorded only as a cytotoxic effect as follows: at high concentrations, such as 0.5 and 1 mg/ml of di-RLs showed a cytotoxic effect on fibroblasts, since shortly after the treatment with di-RLs the cell membranes were disrupted and the cytoplasmic structures appeared to dissolve, suggesting the process of cell necrosis (Stipcevic et al. 2005). Analytically, at 50–200 μg/ml, di-RLs significantly inhibited proliferation of fibroblasts grown in standard DMEM culture medium and in accordance with proliferation results at higher concentrations (0.5 and 1 mg/ml), di-RLs killed the cells. Di-RLs concentration of 200 μg/ml showed a 61% decrease ($p < 0.01$) in cell viability resembling the apoptotic effect of 396 J/m2 UVB irradiation. At concentration of 100 μg/ml and lower, di-RLs decreased the cell viability dose dependently. It is notable that at 50 μg/ml, an increase in caspase activity (91%) when compared with control, suggesting that programmed cell death (apoptosis) reached its peak while higher or lower concentrations were less efficient (Stipcevic et al. 2005). It was also reported that di-rhamnolipid mode of action could involve a direct cell-receptor binding, creating complexes with serum components, binding to the membrane proteins, passing through the phospholipid bilayer of the cell membrane and interacting with DNA transcriptional and translational machinery (Stipcevic et al. 2005). Similar results show that *Burkholderia pseudomallei* rhamnolipid, which is secreted in large amounts when bacterial cells are cultured at high cell densities, exerts a cytotoxic effect on eukaryotic cells (Häussler et al. 1998), probably by directly perturbing cell membranes at concentrations in the range of micrograms per milliliter. Structural and functional cellular changes induced by extracellular *B. pseudomallei* rhamnolipid was also demonstrated and characterized by retraction, rounding up, and, finally, detachment in phagocytic and non-phagocytic cell lines (Häussler et al. 2003).

Recently, a search was performed for virulence factors secreted by *P. aeruginosa* that affect the structure of human airway epithelium in the early stages of infection and the mechanism by which the pathogen *P. aeruginosa* initially modulates the paracellular permeability of polarized respiratory epithelia was investigated. Purified *P. aeruginosa* rhamnolipids, applied on the surfaces of the epithelia, were sufficient to functionally disrupt the epithelia and to promote the paracellular invasion of rhamnolipid-deficient *P. aeruginosa*. This study provides direct evidence for a hitherto unknown mechanism whereby the junction-dependent barrier of the respiratory epithelium is selectively altered by RLs (Zulianello et al. 2006). In addition, it was found that only bacterial strains secreting RLs were efficient in modulating the barrier function of an in vitro-reconstituted human respiratory epithelium without affecting cell viability at concentration of RLs as low as 150 μg/ml. This discrepancy concerning cell viability when compared with this work might be attributed to the low RLs concentration used (150 μg/ml) (Zulianello et al. 2006), against the higher concentration of *Tth*RLs used in this work (100 and 500 μg/ml), which is consistent with other reports (Stipcevic et al. 2005).

A plethora of examples will be numerated to argue that the morphological alterations are attributed to the *Tth*RLs and their bio-surfactants properties. Analogous substances with RLs are saponins, which are triterpene or steroid glycosides that possess detergent-like properties found in a wide variety of plants and certain marine organisms exhibiting many biological activities, such as hemolytic,

antiviral, fungicidal, molluscicidal, pharmacological or cytotoxic. The lysis of erythrocytes by saponins is the result of interactions with membrane cholesterol (Hostettmann and Marston 1995; Voutquenne et al. 2002; Takechi and Tanaka 1995) forming pits and holes (Segal and Milo-Goldzweig 1978; Nakamura et al. 1979; Seeman 1974), which produce a destabilization of the membrane by a micellar type-arrangement (Glauert et al. 1962). Saponins can also increase the permeability of cell membranes without destroying them (Jacob et al. 1991). Saponins could also affect the interaction between trans-membrane proteins, such as Na^+/K^+-ATPase and proteins of the cytoskeleton related to the cell membrane (Haruma et al. 1995; Baumann et al. 2000). It was also found that the cell responses pointed out that the different saponins might block the cell cycle at different steps (Trouillas et al. 2005) and/or that the mechanisms were initiated with varied delay.

Our results showed also that rhamnose, one of the RLs components did not provoke neither morphological transformation, nor cytotoxicity similar to that found for T*th*RLs, evidence that directs to the implication of the lipidic chain constituent of the RLs to trigger these effects. The results also confirm that T*th*RL_1 and T*th*RL_2 affect fibroblasts in different ways since these mixtures of RLs (Pantazaki et al. 2010) are derived from different carbon sources and have different composition of RLs (mono-RLs and di-RLs). The same concentrations of T*th*RL_1 and T*th*RL_2 had different cytotoxicity in MTT test and this might be strongly attributed to the lipidic chain variation and to the composition of RLs.

This is consistent with other studies that reported that the same glycoside chain can also have a different influence when the aglycone is only slightly modified (carboxylic acid or methyl ester). The hydrophobic aglycone backbone could probably intercalate into the hydrophobic membrane bi-layer, while the glycoside chain could interact with the polar group. The methyl ester saponins were generally more hemolytic and less cytotoxic than their corresponding-free carboxylic acid (Chwalek et al. 2006). Hemolytic activity of hederagenin saponins has been linked to the structure of the aglycone and also depends on the sugar moiety of the saponin (Chwalek et al. 2006).

Rhamnolipid is known to be produced at high bacterial densities in vitro for modulating swarming behavior, while HAAs the most common component of RLs required for surface wetting (Caiazza et al. 2005). Owing to their surface wetting properties might affect cell adhesion. Similar modulation of fibroblast morphology and adhesion during collagen remodeling was also observed and the cells retract their dendritic network of extensions in response to growth factor lysophosphatidic acid (Tamariz and Grinnell 2002).

This study provides strong evidence for the involvement of the *Tth*RLs in the morphological alterations leading in disruption of the host cell membrane and cytotoxicity and for the occurrence of *Tth*RLs secretion in both cases tested under high cell density where nutrients were exhausted. In the opportunistic pathogenic bacterium *P. aeruginosa* only bacterial strains secreting RLs was implicated in the virulence (El-Mawgoud et al. 2010; Ron and Rosenberg 2001). Thus, it was suggested that *Tth*RLs secretion might be a crucial point to be transformed a non-pathogenic bacterium to a pathogenic one under certain environmental conditions favoring RLs secretion. The constructive perspectives of this work are to elucidate whether RLs secretion in the microorganism's world in symbiosis with mammalian cells might be a general route for the passage in the pathogenicity as a result to ensure their survival under nutrient limitation.

Acknowledgments The authors declare that they have no conflict of interest.

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