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ABSTRACT

Alysiella filiformis and *Simonsiella muelleri* belong to the family of the *Neisseriaceae*. They are Gammaproteobacteria found in the oral cavity of warm-blooded vertebrates. An early (1922) ultrastructural study of *A. filiformis* and *S. muelleri* revealed chains of them to be attached by fimbriae to the host epithelial cells. However, up to this study, their reproductive strategies have remained unexplored. Here, by morphometric analysis, we showed that widening of *A. filiformis* not only occurs during division but also predivisionally. Furthermore, by applying a labelled cell wall precursor, we confirmed that septation is longitudinal in both *S. muelleri* and *A. filiformis* and asynchronous in the case of *A. filiformis*, meaning one cell pole septates before the other. Although western blots with anti-*E. coli* FtsZ antibodies did not detect this key bacterial cell division protein, septal and proximal accumulation of the actin homologue MreB in *A. filiformis* suggests that this might direct new PG insertion not only during disperse but also during septal growth. Finally, we showed asymmetrical localization of the DNA to the polar cell half of *A. filiformis*. The fact that oral cavity symbionts are culturable has the tremendous potential to establish a genetically tractable system for the study of bacterial longitudinal fission as an adaption to the symbiotic lifestyle. Importantly, as their abundance correlated to oral health in humans, a better understanding of *A. filiformis* and *S. muelleri* might have biomedical relevance.

INTRODUCTION

SYMBIOSIS

The term „symbiosis” was introduced in 1879 by De Bary to define „the living together of two differently named organisms” (Oulhen et al., 2016). The “two differently named organisms” have to be understood as the host (bigger partner) and the symbiont (smaller partner) (McFall-Ngai & Gordon, 2006). These long-term interactions can be (1) beneficial for both organisms, best known as mutualistic, 2) beneficial for one partner, called commensalism, or (3) parasitic, which is one organism living at the expense of the other.

The characterization of a symbiotic relationship has been addressed by classification criteria such as symbiont location, symbiosis transmission, exchange of commodities between partners. In the case of endosymbiosis, the symbiont lives within the body of the host whereas, in the case of ectosymbiosis, the symbiont stays on the host surface. Allowing its maintenance throughout generations and shaping the evolution of the partners, symbiosis transmission may be horizontal or vertical (Bright & Bulgheresi, 2010).

Horizontal transmission entails two free-living partners who meet and physically contact each other at the right place and at the right time. The vertical route is also referred to as transovarial route as it usually entails incorporating the symbiont into the female germline (Bright & Bulgheresi, 2010). In the case of strict vertical transmission, none of the partners is ever free-living. Importantly for both environmental and vertical transmission, the symbiotic relationship must be perpetuated throughout generations.

REPRODUCTION MODE OF MODEL BACTERIA

As one of the first model bacteria, *E. coli* has been intensively studied in terms of growth and division. After doubling its size, it divides into two equal cells in a process known as binary fission. In this section, I will summarise what is known about its reproductive strategy.

The cytoskeletal proteins MreB, a membrane-associated actin-like protein, and FtsZ, a tubulin-like protein mediating cell growth and division in model bacteria, respectively. MreB coordinates the peptidoglycan (PG) synthesis and polymerization, as well as maintaining *E. coli* cell rod shape (Esue et al., 2005; Ozyamak et al., 2013; Salje et al., 2011), whereas FtsZ forms the Z-ring (proto-ring) at the centre of the cell inserting new PG at the septum as well as recruiting over ten additional, essential division proteins (Rico et al., 2013).

Mesh-like PG is composed of N-acetylglucosamine (GlcNac) and N-acetylmuramic acid (MurNac) linear polymers crossed-linked and strengthen by peptidyl-bridges. For the maintenance of the PG sacculus throughout the cell cycle, synthetases and hydrolases are required. Synthesis of the peptidyl-bridges starts with two D-alanines which are ligated together and then added to a stem tripeptide resulting in a pentapeptide (Typas et al., 2012). Continuous addition of disaccharide subunits to the so-called nascent chain will lead to a mesh-like PG structure. PG growth is controlled by hydrolysis which cleaves covalent bonds between PG allowing constant growth without increasing density (Typas et al., 2012).

In dividing model rods, initiation of cell division starts with polymerisation of short linear FtsZ protofilaments leading to the formation of the Z-ring at midcell, at the cytoplasmic membrane (Bi & Lutkenhaus, 1991). During preseptal growth, the actin-like protein MreB coordinates PG synthesis and interacts with membrane proteins such as RodZ, MreC, MreD (Addinall & Lutkenhaus, 1996; Den Blaauwen et al., 2014; Li et al., 2007) or other enzymes such as MurG, MreY for proper maintenance of cell rod shape and PG elongation (Mohammadi et al., 2007).

ADHESIVE FIMBRIAE

Besides growing and dividing, many bacteria, including ectosymbionts, are capable to adhere to- and colonize host surfaces. One way to do so is to bear appendages made of long protein polymers on their surface known as pili or fimbriae (Connell et al., 1996; Lukaszczyk et al., 2019) their presence on the surface of many bacteria including *E. coli* has been historically correlated with pathogenicity (Klemm, 1985). Fimbriae confer bacteria the ability to adhere to- and colonize host epithelia. Different groups of these proteins have been characterised, and

their immunogenic role allow them to be exploited as one of the important virulence factors in human immunisation (Klemm, 1985).

In 1961 the K88 adhesin was characterized as one of the virulence factors found in infected piglets' intestinal tract (Ørskov et al., 1961). The protein polymer comprises few subunits from which one is specified as major subunit FaeG (27,540 Da) and others as minor subunits (FaeC, D, E, F, H, I). The stickiness of the adhesin is mainly dependent on its major subunit (Ørskov et al., 1961). Due to the evolutionary pressure on these extracellular appendages which need to interact with an immunologically diverse and everchanging host, fimbrial genes are, notoriously, highly variable (Ørskov et al., 1961). This is the case for the Type 1 fimbriae of uropathogenic *E. coli* which is regulated by a promoter found on an invertible element (Leathart & Gally, 1998).

Fimbriae play also an important role in microbial symbioses by facilitating adherence of bacteria to eukaryotic cell surface (Bright and Bulgheresi, 2010) which will be explained further in the following sections.

LONGITUDINALLY DIVIDING SYMBIONTS

Up to this study, *Ca. T. oneisti* and *Ca. T. hypermnestrae* have been the only rod-shaped Gammaproteobacteria shown to undergo FtsZ-based longitudinal fission (Leisch et al., 2012, 2016). They cover the surface of their marine nematode hosts *Laxus oneistus* (Polz et al., 1994) and *Robbea hypermnestra* (Ott et al., 2014), respectively. Each symbiont ensheaths its respective host by forming a columnar epithelium-like film in which each bacterial cell is attached to the nematode by one pole, which we refer to as proximal pole. Once extracted from tropical, shallow-water sediment, where they abound, they can survive up to weeks in seawater-filled dishes (Pende et al., 2018). However, neither the nematodes, nor *Ca. Thiosymbion* is culturable. Despite *Ca. Thiosymbion oneisti* and *T. hypermnestrae* poor experimental tractability, the key molecular features of their growth and division modes have been described.

Both of these rods divide, non-canonically, by longitudinal division, that is by placing their septation plane along the long cell axis (medially). However, *Ca. T. hypermnestrae* is dividing asynchronously, i.e. invagination starts first at the proximal cell pole followed by subsequent invagination of the distal pole (Leisch et al., 2012). As for the way they grow, both ectosymbionts appear to widen only while they are dividing. Indeed, the tubulin homolog FtsZ colocalises with newly synthesized PG at the growing septum and likely mediates both growth and division (Pende et al., 2018). Consequently, in *Ca. T. hypermnestrae*, not only division

but also cell growth follows a proximal-to-distal pattern, that is the proximal pole is not only the first to divide, but also the first to grow (Pende et al., 2018). Although the actin homologue MreB does not appear to colocalize with new PG insertion, it forms a medial ring-like structure throughout the cell cycle, which is required for proper FtsZ localization and polymerization at the septation plan. Indeed, A22-mediated depolymerization of MreB abolished FtsZ ring formation and incorporation of newly synthesised PG in both dividing and non-dividing *Ca. T. oneisti* and *Ca. T. hypermnestrae* (Pende et al., 2018).

As mentioned above, *Ca. Thiosymbion oneisti* and *T. hypermnestrae* are not genetically tractable, i.e. to unravel the molecular basis of this organisms, isolation in pure culture must be achieved and genetic tools need to be developed.

HUMAN MICROBIOME

In the early 1680s, Antonie van Leewenhoek was one of the first who compared his oral and fecal microbiota and recognised differences in microbe composition between these two habitats, as well as between healthy versus sick individuals (Dobell, 1920; Van Leeuwenhoek, 1683). He isolated samples from his mouth and stool and, by using his handmade microscope, observed the most abundant bacteria (cocci and fusiform bacteria, as well as spirochetes in the oral cavity). More than two centuries after van Leewenhoek's seminal observations, thanks to the advent of high throughput sequencing techniques, the scientific community could indentify and begin to characterize the physiology of the microbes associated with humans and who may, therefore, impact their health (Peterson et al., 2009; Qin et al., 2010). "Human microbiome" collectively refers to the genomes of microbes (bacteria, fungi, viruses, protozoa) that colonize internally or externally the human body depending on host environment, age, sex, lifestyle, immune system, physiology and pathobiology (Hollister et al., 2014; Turnbaugh et al., 2007). Each individual comprises 10-100 trillion of symbiotic microbial organisms, the largest number of which is found in the gastrointestinal tract. Nevertheless, a significant number thrives on the skin, mouth, nose, vagina and on other human body parts (Turnbaugh et al., 2007). This host-bacteria relationship is known to provide advantages for both partners. As a consequence of the interaction, the host may gain additional, microbe-derived immune protection, as well as maintainence of immune homeostasis. The symbiont itself metabolizes and utilizes nutrients that the host may not degrade (Round & Mazmanian, 2009). After the gut, the oral cavity microbiota, comprising over 700 bacterial species, is the bodypart with the second highest bacterial diversity (Deo & Deshmukh, 2019).

ORAL CAVITY MICROBIOTA

In 1674 Antonie van Leeuwenhoek was the first to discover microbes, while examining his own dental plaque, which he described as “little living animalcules prettily moving” (Patil et al., 2013). With the advancement in microscopy, it was discovered that the microbiota also covers the tonsils, tongue, gingival sulcus, soft and hard palate, and acts like a bridge which connects the outside and inside of the digestive tract and the respiratory tract (Gao et al., 2018). This habitat comprises a high diversity in microorganisms and provides a suitable space for the microbial colonization (Zaura et al., 2014). The colonisation of the oral cavity by microorganisms begins at, or very shortly after, the birth of a child with so-called pioneer species (Deo & Deshmukh, 2019). The pioneer species include species as *Streptococcus salivarius* followed mainly by aerobes like *Neisseria*, *Lactobacillus*, *Actinomyces* and *Veillonella* (Deo & Deshmukh, 2019; Patil et al., 2013). The species composition of the oral cavity microbiota is affected by- and vulnerable to environmental perturbation (Demmitt et al., 2017). Different microbial communities colonize different oral cavity surfaces during the human lifetime. It begins with passive bacterial transmission from mothers breastmilk, then by intake of water and other nutritional sources, and later on during teeth growth (Batabyal et al., 2012; Marsh, 2000; Sowmya, 2016).

With a total of 392 taxa (and at least one reference genome) (McLean, 2014) belonging to 185 genera and 12 phyla, the oral cavity has one of the most studied microbiomes (Zhao et al., 2017). From all identified species 54% are named and cultivable, 14% are not named but cultivable and 32% of them are uncultivable as of yet (Zhao et al., 2017). The above-mentioned 12 phyla are: *Firmicutes*, *Fusobacteria*, *Proteobacteria*, *Actinobacteria*, *Bacteroidetes*, *Chlamydiae*, *Chloroflexi*, *Spirochaetes*, *SR1*, *Synergistetes*, *Saccharibacteria* and *Gracilibacteria* (Marsh, 2000; Perera et al., 2016). All these microbial genera were shown to be important for the human body for their metabolic and immunological functions (Kilian et al., 2016). These include digestion of nutrients, energy generation and maturation of the host immune system (Kilian et al., 2016). It is known for oral cavity symbionts to provide control over the fat storage, to regulate colonisation resistance and, potentially, disease development (Donohoe et al., 2011; Krajmalnik-Brown et al., 2012). Many oral bacteria can produce inhibitors of the host innate immune system (e.g., hydrogen peroxide or volatile fatty acids), change the pH or affect the reduction/oxidation potential of their environment (Marsh, 2000). For example, some of the *Streptococcus salivarius* can prevent the colonisation of the mouth by Lancefield Group A streptococci (Sanders & Sanders, 1982) by producing a bacteriocin, called salivaricin, which acts against exogenous organisms (Sanders & Sanders, 1982).

All in all, knowing that its imbalance can cause oral, as well as systematic diseases (e.g., cardiovascular disease, bacterial pneumonia, diabetes mellitus), homeostasis of the oral cavity microbiota is crucial to human health (X. Li et al., 2000). Therefore, deciphering the molecular mechanisms of oral cavity microbiota growth and division may enable the development of new drugs and therapies. With this overreaching goal in mind, my research study (and the next chapter) focus on the oral cavity symbiont *Alysiella filiformis* and *Simonsiella muelleri*, belonging to one of the most abundant phyla to populate the mouth (Proteobacteria) (Perera et al., 2016).

ALYSIELLA FILIFORMIS AND SIMONSIELLA MUELLERI, TWO ORAL CAVITY RESIDENTS

The non-pathogenic gammaproteobacteria *Alysiella filiformis* and *Simonsiella muelleri* (Hedlund & Tønjum, 2015) (family Neisseriaceae) colonize the oral cavity of many mammals, including humans (Hedlund & Kuhn, 2006). Successful cultivation of *S. muelleri* and *A. filiformis* is possible since the 60s. These aerobic chemoorganotrophs are attached to the epithelium of the palate forming multicellular chains. *A. filiformis* forms ribbon-like filaments of variable length that using fimbriae can glide on the oral mucosa (Figure 1A). *A. filiformis* cells are disk-shaped and, likely to secure their attachment to the host epithelia, bear fimbriae on one of their cell poles (Tønjum, 2015). *S. muelleri* eight-cells long watchband-like filaments are flat and curved. Each concave-shaped individual cell is oriented toward the surface of the host epithelial cells with its ventral side (Hedlund & Tønjum, 2015) (Figure 1B). Covering the ventral side of *S. muelleri*, 100 nm-long fimbriae are probably involved in the adhesion, as well as the gliding on the oral mucosa. Individual *S. muelleri* cells are 1.9–6.4 μm long and 0.5–1.3 μm wide (Hedlund & Tønjum, 2015; Tønjum, 2015). The percentage of healthy humans bearing *S. muelleri* may be up to 40% (Hedlund & Tønjum, 2015; Kuhn et al., 1974) and the percentage of animals (such as dogs and cats) up to 99% (Nyby et al., 1977).

The closed genome of *A. filiformis* and *S. muelleri* (<https://www.ncbi.nlm.nih.gov/sra>; accession numbers SRR292335 and SRR330055 for *S. muelleri*, and SRR3503032 for *A. filiformis*; T. Viehboeck, unpublished data) indicated that these monochromosomal genomes are around 2.4 Mbp in size, with a GC content of 46% and 41%, respectively. Moreover no plasmids were identified. Even though these oral cavity dwellers, based on genomics, possess similar division machinery as other aerobic chemoorganoheterotrophs, they are not able to synthesis B₁₂ vitamin. Nevertheless, they encode for the TonB-dependent vitamin B₁₂ transporter BtuB and vitamin B₁ transporter. Interestingly, the genome of these oral cavity symbionts encode free iron, as well as amino acid transporters (respectively, the iron uptake system EfeOUB and Met, Arg, and Glu amino acid transporters; T. Viehboeck, unpublished

data). *A. filiformis* and *S. muelleri* genomes also encode for proteins important for host lysozyme inhibition (PliC) (Callewaert et al., 2008) and resistance to the host neutrophils (Lst) (Smith et al., 1995). Another strategy for oral cavity symbionts to survive the host immune system is expression of IgA protease (Roussel-Jazédé et al., 2014) and EsiB (Pastorello et al., 2013) proteins.

All in all, the genomes of *A. filiformis* and *S. muelleri* suggest that they can take up and exploit organic compounds and essential nutrients found in the vertebrate saliva. Furthermore, they suggest that these oral cavity symbionts may tolerate the host immune system and produce antibiotic effectors to deter pathogens thereby acting as defensive symbionts.

Figure 1. Phase-contrast microscopy images of *Alysiella filiformis* and *Simonsiella muelleri*. (A) An *A. filiformis* filament (upper panel) and four single cells obtained following a 2 min-long sonication (bottom panel). (B) A *S. muelleri* filament (left panel) and single cells obtained following a 10 min-long sonication (right panel). Scale bar: 2 μ m.

Function Protein	Activity or category	<i>E.coli</i>	<i>A.filiformis</i>	<i>S.muelleri</i>	<i>Ca.T. oneisti</i>	<i>Ca.T. hypermnestrae</i>
Cell elongation	Cytoskeletal structure, ATPase, GTPase	MreB	+	+	+	+
Cell elongation	MreB-associated protein	MreD	+	+	+	+
Cell elongation	MreB-associated protein	MreC	+	+	+	+
Cell elongation	MreB-associated protein	RodA	+	+	+	+
Cell elongation	MreB-associated protein	RodZ	+	+	+	+
Cell elongation	MreB-associated protein	Pbp2	+	+	+	+
Cell division	Cytoskeletal structure, GTPase	FtsZ	+	+	+	+
Cell division	'Early' association with the Z ring	FtsA	+	+	+	+
Cell division	'Early' association with the Z ring	ZapA	+	+	+	+
Cell division	'Early' association with the Z ring	ZipA	+	+	+	+
Cell division	Late' association with the Z ring	FtsW	+	+	+	+
Cell division	Late' association with the Z ring	FtsL	-	-	+	+
PG synthesis	GTases and DD-TPases (class A PBPs)	Pbp1A	+	+	+	+
PG synthesis	GTases and DD-TPases (class A PBPs)	PBP-1B	-	-	+	+
PG synthesis	GTases and DD-TPases (class A PBPs)	PBP-1C	-	-	-	-
PG synthesis	DD-TPases (class B PBPs)	PBP-2	+	+	+	+
PG synthesis	DD-TPases (class B PBPs)	PBP-3	+	+	+	+
Regulation of PG synthesis	Activator of PG synthase	LpoA	-	-	+	+
Regulation of PG synthesis	Activator of PG synthase	LpoB	-	-	-	-

Table 1. List of Essential cell division proteins from *E. coli* and their presence (+) or absence (-) in *A.filiformis*, *S. muelleri*, *Ca. T. oneisti*, *Ca. T. hypermnestrae*.

AIM OF THIS STUDY

Sequencing, closure and annotation of the genomes of two gram-negative oral cavity dwellers, *A. filiformis* and *S. muelleri* (T. Viehboeck, unpublished data) revealed that they possess highly similar cell growth machinery as the model organism *E.coli*. However, seminal ultrastructural studies suggested that they divide by longitudinal division by default. My morphological and molecular study confirmed the previous ones and, given the culturability of *A. filiformis* and *S.muelleri*, it represents a first step in the understanding of longitudinal division, a non-canonical way of division only observed in nematode symbionts, so far. The planned establishment of longitudinally dividing oral cavity symbionts as genetic systems will allow disclosing the conserved molecular mechanisms underlying cell growth and division in bacteria.

More specifically in my master thesis, I (1) performed morphometric measurements of *A. filiformis* to understand if it grows throughout the cell cycle or only during septation, (2) localized its DNA, (3) visualized septation with different PG precursors to confirm longitudinal septation and (4) visualized the localisation of proteins (e.g., MreB, FtsZ) predicted to underly growth and/or septation.

METHODS

ALYSIELLA FILIFORMIS AND *SIMONSIELLA MUELLERI* STRAINS AND CULTIVATION

A. filiformis and *S. muelleri* were ordered from the “Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures” (DSM No.: 16848, DSM No.: 2579). *A. filiformis* strain was originally isolated from sheep saliva in Australia.

After we obtained colonies of *A. filiformis* and *S. muelleri*, they were inoculated on Peptone-Yeast (PY) agar plates (pH ~ 7.5, see Table 2 for medium composition). On every second day, we transferred the colonies onto new PY agar plates. To prevent oral cavity symbionts from drying out, we construct a humid chamber in which we stored our plates (see Figure 2). To grow the oral cavity symbionts in the liquid culture we used PY medium and T25 liquid flasks (Table 3). The optimal pH of the liquid medium was 7.5. After transferring a one-day-old colony from the PY agar plate into 50 ml PY medium in a baffled flask, it was placed at 37°C and incubated overnight without shaking.

After reaching exponential growth, in liquid, *A. filiformis* started to form clumps and stopped growing. To overcome clump formation in a liquid medium we tipped into-, instead of picking the colony, and instead of plastic liquid flasks, we used glass made Duran Erlenmeyer flask with four baffles on the bottom. These baffled flasks (Sigma Aldrich Z680966-4EA) provide a larger surface for the liquid (250 ml), as well as a gas exchange which could be important in oxygen intake. Using this method we succeeded in growing *A. filiformis* in liquid culture.

If not stated otherwise all experiments were held out at room temperature (RT).

FIXATION

Cells were fixed in methanol for 20 min at -20°C. For morphometric analysis and immunostainings, cells were rehydrated with 1x PBS for 10 min.

SONICATION

To be able to do morphometric analysis of single *A. filiformis* cells, we dissociated filaments into single cells by applying sonication for at minimum 30 and up to 180 seconds. As in the case of oral cavity symbiont *S. muelleri*, sonication was applied for 10 min (Figure 1; lower panel). After sonication of both organisms, samples were mounted on 1% agar slides and imaged by fluorescence microscopy.

LABELLING OF *ALYSIELLA FILIFORMIS* AND *SIMONSIELLA MUELLERI* CELL WALL AND STAINING OF DNA

Insertion of new PG was tracked by incubating *A. filiformis* and *S. muelleri* with EDA-DA (Liechti et al., 2014). One day old colonies were incubated in EDA-DA (diluted in DMSO from 100 mM stocks to 1mM working concentration) for 1 min, 2 min or 5 min (*A. filiformis*) and 15 min 30 min or 45 min (*S. muelleri*) after which they were fixed in 70% methanol for 1h at -20°C. Samples were washed with 0.1% PBS-T three times and then blocked in 3% BSA PBS-T for 30 min. The cell clicking reaction was carried out with Click-iT Cell Reaction Buffer Kit (Invitrogen). After 30 min incubation in the dark at RT, the samples were washed with PBS-T and DNA was labelled using Hoechst stain 33342 (1:1000 dilution in 1x PBS).

For in situ PG insertion other metabolic probes (Hsu et al., 2017) as HADA (7-hydroxycoumarin-3-carboxylic acid-D-alanine), BADA (BODIPY FL-D-alanine) and TADA (TAMRA-D-alanine) were used on *A. filiformis* cells. These prelabeled fluorescent D-amino acids (FDAA) were (stored at -20°C) to 1 mM working concentration. After one-hour incubation with each prelabeled precursor, ice-cold ethanol (70%) was added to *A. filiformis* colonies. One hour later, ethanol-fixed cells were collected by centrifugation (10 000 g for 5 min, 4°C) and washed twice and resuspended with 1x PBS. Samples were stored on ice before imaging. Cells were mounted on agar slides (1% agarose) before microscopic imaging.

Polyclonal anti-E. coli fimbriae antibody (Abcam, ab35292) 1:500 diluted in 5% PBSM and rabbit anti-MreB antibody (provided by collaborator T. den Blaauwen) (Den Blaauwen et al., 2003) previously diluted 1:4 in PBSM were also tested on Western blot. The protocol was performed using same protocol as in the previous section. Secondary anti-sheep or anti-rabbit antibody (Amersham Biosciences) were diluted 1:5000 in PBSM.

WESTERN BLOTS

Western blots have been performed with the commercially available polyclonal anti-rabbit FtsZ antibody (Agrisera) and the rabbit anti-FtsZ antibody (kindly provided by our collaborator T. den Blaauwen) (Vischer et al., 2015). Protein extracts from *A. filiformis* were loaded onto SDS–polyacrylamide gels (Invitrogen) and separated for 1h in 1x MOPS (3-(N-morpholino) propane sulfonic acid) buffer. The proteins were transferred from the gel to the nitrocellulose membrane (Amersham Biosciences). Blotting membrane was incubated for 45 min in PBS - 5% non-fat milk (PBSM) at RT. After the addition of rabbit polyclonal anti-FtsZ (1:200 diluted in 5% PBSM) membrane was incubated overnight at 4°C. After five washing steps with PBSM to remove primary antibodies, the membrane was incubated for 1h at RT with horseradish peroxidase-conjugated secondary anti-rabbit FtsZ antibody, (diluted in PBSM 1:5000). FtsZ protein was visualised with detection reagents (Sigma Aldrich) and films (Amersham Biosciences).

A22 INCUBATION OF *ALYSIELLA FILIFORMIS*

To track *A. filiformis* cell wall growth PY agar plates, with different A22 (Sigma-Aldrich) concentrations as well as a negative control (without A22, just DMSO), were prepared. Previously A22 (10 mg/ml) was dissolved in 1 ml of the DMSO and stored at 4°C. From the final concentration, 2 µg/ml, 20 µg/ml and 40 µg/ml of A22 drug were added to freshly prepared PY agar medium. *A. filiformis* were then transferred from glycerol stock to A22 PY prepared plates and stored overnight at 37°C incubator. As negative control, the same volume of DMSO was added as the volume of A22 dissolved in DMSO to each PY agar plate.

After 24 h three colonies from each plate were chosen and A22 was added to freshly prepared PY medium in the same concentration as in the agar plates (2 µg/ml, 20 µg/ml and 40 µg/ml). To each Eppendorf tube, now containing A22 PY medium, HADA, BADA and TADA prelabeled precursors were added to a final concentration of 1 mM (from 100 mM stock). After we chose three colonies from the plate, pre-cut blue pipette tips were pressed around them to avoid possible cell separation from agar, 50 µl of A22 PY medium were added and incubated for 1 h at 37°C.

After 1 h of incubation, probes were carefully pipetted off to not disrupt the colonies and ice-cold 70% ethanol was added. Cells were fixed by incubation on ice for next hour. Ethanol-fixed cells were collected by centrifugation (10,000 g for 5 min, 4°C) and then washed 2x and resuspended with 1x PBS (pH 7.4). Samples were stored on ice, in the dark until imaging.

MORPHOMETRIC AND FLUORESCENCE MEASUREMENTS

A. filiformis cells were immobilized on 1% agarose and imaged using a Nikon Eclipse 50i microscope equipped with either a DS-Fi1 camera (Nikon). Epifluorescence images were obtained using the NIS Elements F 3.22 software (Nikon) and then analysed using the program ImageJ in combination with plugins ObjectJ (Vischer et al., 2015) or MicrobeJ (Ducret et al., 2016). Cell outlines were automatically recognized, and errors removed manually. Cells were selected based on phase-contrast images. Based on cell width we were able to distinguish between dividing and non dividing cells. In overlays, DNA fluorescence is false coloured in red and incorporated clickable D-amino acid dipeptide Ethynyl-D-Ala-D-Ala (EDA-DA) in green. Sonicated cells used in polarized DNA plot as well as DNA density plot were flipped manually towards the DNA fluorescence signal. In the density plot, only the localization of the brightest pixel is indicated. Prelabeled precursors HADA and BADA were false coloured blue and green (respectively) and representative pictures were chosen based on contrast images. Image contrast was adjusted for better fluorescence signal visualisation.

Quantification of cells within filaments has been made by manual segmentation process (DNA-Fimbriae plot, PG precursors plots) in quantitative analyser MicrobeJ.

Illustrations were graphically edited with Inkscape (Bah, 2011).

Peptone from meat	7.5 g
Yeast extract	1.5 g
NaCl	2.5 g
K ₂ HPO ₄	1.25 g
D+ Glucose	1.25 g
Agar-agar	6 g
Distilled water	500 ml

Table 2. Ingredients for PY agar plates

Peptone from meat	7.5 g
Yeast extract	1.5 g
NaCl	2.5 g
K ₂ HPO ₄	1.25 g
D+ Glucose	1.25 g
Distilled water	500 ml

Table 3. Ingredients for PY medium

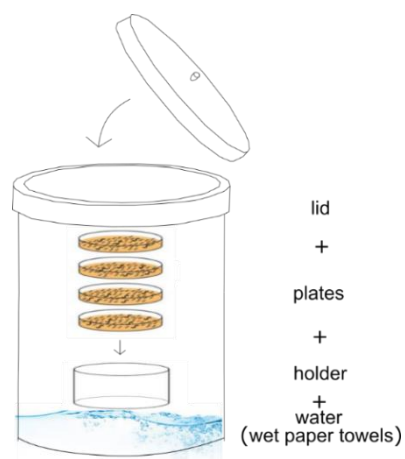


Figure 2. Humid chamber for *A. filiformis* and *S. muelleri* incubations.

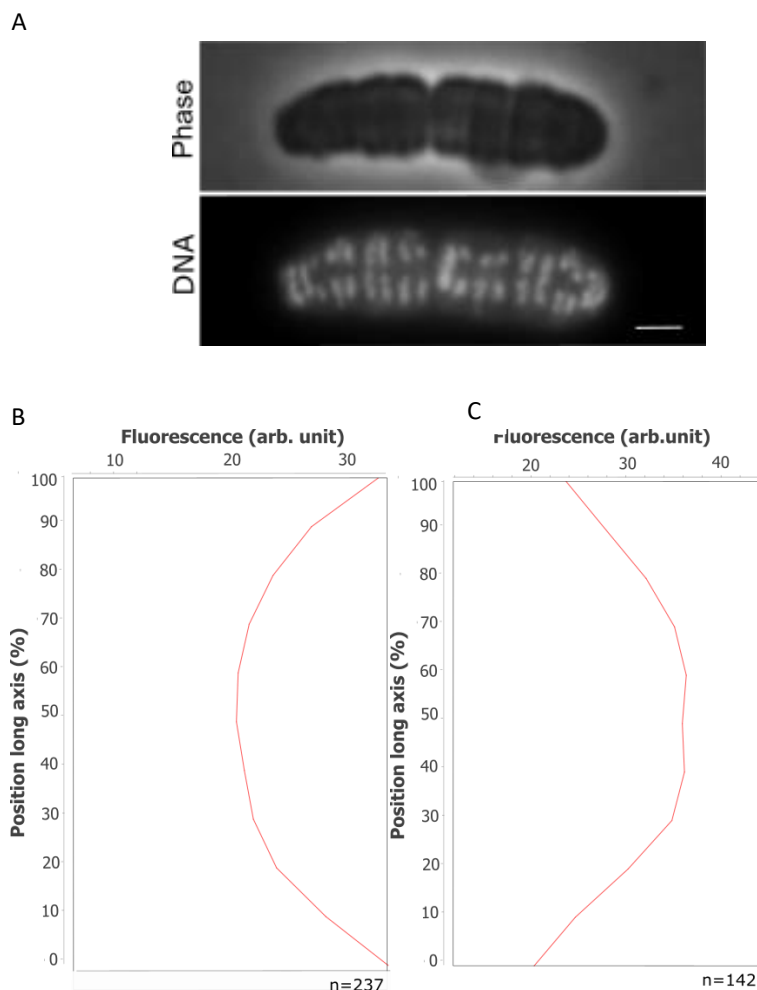
RESULTS

SIMONSIELLA MUELLERI

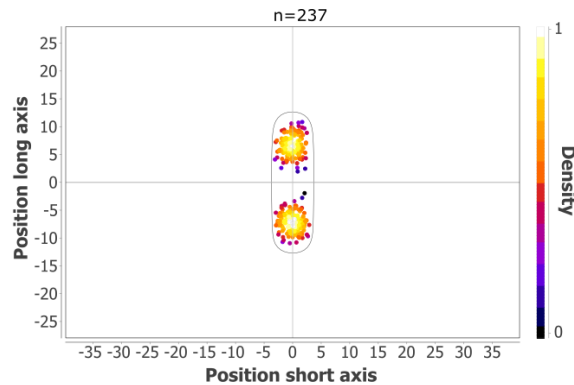
DNA LOCALISATION IN *SIMONSIELLA MUELLERI*

The quantification of 379 *S. muelleri* cells stained with the Hoechst 33342 revealed two distinct subcellular localisation patterns of the symbiont DNA (Figure 3): 237 cells were DNA-poor in their dorsalmost regions (Figure 3C, E), whereas 142 cells had their DNA disperse throughout the cell, but the poles (Figure 3B, D).

We conclude that *S. muelleri* DNA distribution along the cell long axis can be observed in two complementary patterns. Namely, cells either localized their DNA throughout except at their poles or in the proximal and distal thirds leaving the central (dorsalmost) third almost DNA-free.



D



E

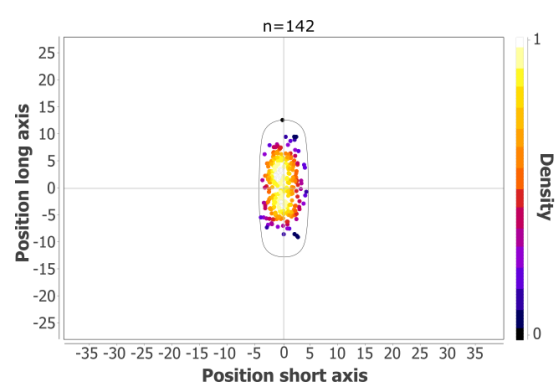


Figure 3. DNA localisation in the oral cavity symbiont *S. muelleri*. Representative *S. muelleri* filaments stained with Hoechst 33343. (A) The upper panel shows phase contrast image and the lower panel shows DNA fluorescence signal. Scale bar: 2 μ m.

(B, C) Total DNA fluorescence (red) expressed in arbitrary units, of 237 cells with DNA at the cell poles and 142 cells with disperse DNA localisation, plotted against the normalized cell length displayed in %.

(D, E) The position of each focus is plotted as fraction of the normalized cell width and length (%) of the cell that contained them. Full lines represent the long and the short cell axis, and midcell is defined as the point in which they intersect

LONGITUDINAL SEPTATION IN THE ORAL CAVITY SYMBIONT *SIMONSIELLA MUELLERI*

Previous ultrastructural analysis suggested that *S. muelleri* septates longitudinally (Hedlund & Tønjum, 2015). To confirm this, we incubated *S. muelleri* with the PG precursor EDA-DA for 60 min or 75 min. Following 60 min incubation, in the six central cells the signal appeared only medially and as a dot on each pole (Figure 4, bottom left panel). However, longer incubation times (75 min) resulted in new PG incorporation throughout the long axis except at midcell (Figure 4, bottom right panel). Concerning the terminal cells, they either displayed a focus of new PG incorporation at midcell (right terminal cell in the left panel of Figure 4), or throughout the long axis (right terminal cell in the right panel of Figure 4).

We conclude that *S. muelleri* central cells divide (and perhaps also widen) by medial incorporation of newly synthesized PG and that new PG incorporation starts at the poles and progresses toward midcell. As for the terminal cells, additional experiments are needed to assess whether they also divide longitudinally.

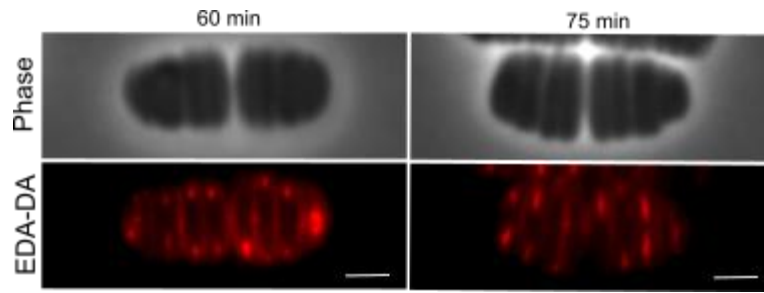


Figure 4. PG metabolic probe incorporation pattern in *S. muelleri*. Two representative filaments incubated for either 60 or 75 min in the presence of the PG precursor EDA-DA. Upper panels show the phase-contrast images and lower panels show the EDA-DA fluorescence signal (in red). Scale bar: 2 μm .

ALYSIELLA FILIFORMIS

WIDENING OF *ALYSIELLA FILIFORMIS* THROUGHOUT THE CELL CYCLE

To determine whether *A. filiformis* is widening and if it does so throughout the cell cycle, or only while it is dividing (as previously shown for other longitudinally dividing bacteria) (Leisch et al., 2012, 2016; Pende et al., 2018), we analysed dividing ($n = 174$) and non-dividing ($n = 134$) cells morphometrically. By doing so, we found that the average length of the non-dividing cell is $1.9 \pm 0.17 \mu\text{m}$ and the average width is $1 \pm 0.19 \mu\text{m}$. In the case of dividing cells, the average length is $2.06 \pm 0.21 \mu\text{m}$ and the average width is $1.7 \pm 0.24 \mu\text{m}$ (Table 3, Figure 5).

We concluded that *A. filiformis* width increases by 70%, whereas its cell length does not vary significantly throughout the cell cycle. Furthermore, a positive linear relationship between cell width and cell volume of both non-dividing and dividing cells ($R^2 = 0.6114$) indicates that *A. filiformis* widens throughout the cell cycle (Figure 5).

Table 3. Size measurements of *A. filiformis* (n=300)

	Non-dividing cells Length (μm)	Non-dividing cells Width (μm)	Dividing cells Length (μm)	Dividing cells Width (μm)
Minimum	1.52	0.59	1.47	1.25
Maximum	2.46	1.48	2.69	2.41
Average	1.96	1.02	2.08	1.69
SD	0.17	0.19	0.21	0.24

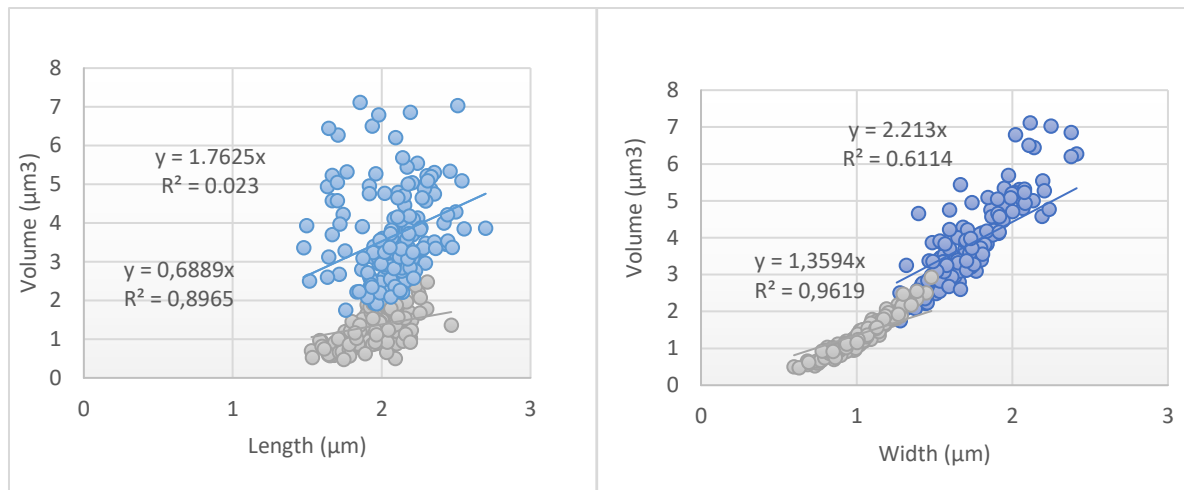


Figure 5. Morphometric analysis of *A. filiformis*. Cell volume of non-dividing (grey) or dividing (blue) *A. filiformis* cells plotted against cell length (left plot) or width (right plot) (total n=308).

ANTI-*E. COLI* FtsZ ANTIBODIES DO NOT DETECT *ALYSIELLA FILIFORMIS* FtsZ ON WESTERN BLOTS

The product of the *ftsZ* gene identified in the available *A. filiformis* (DSM 16848) genome (T.V., unpublished data) is 54% identical and 72% similar to *E. coli* K12 FtsZ and the sequence alignment of *E. coli* and *A. filiformis* FtsZ protein sequences is shown in Figure 6. To determine whether *A. filiformis* expresses FtsZ, we tested two rabbit polyclonal anti-*E. coli* FtsZ antibodies, one commercially available (Agrisera) and the second one kindly provided by T. den Blaauwen (Vischer et al., 2015) on western blots of *A. filiformis* protein extracts. Unfortunately, both antibodies failed to detect a protein running at the expected molecular weight (ca. 40 kDa; Figure 7).

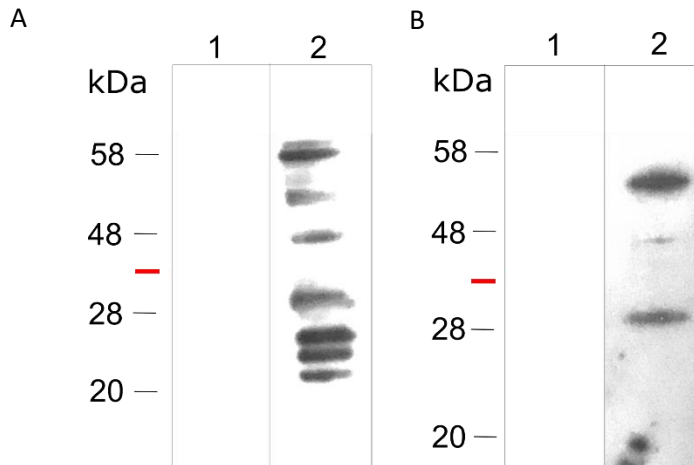
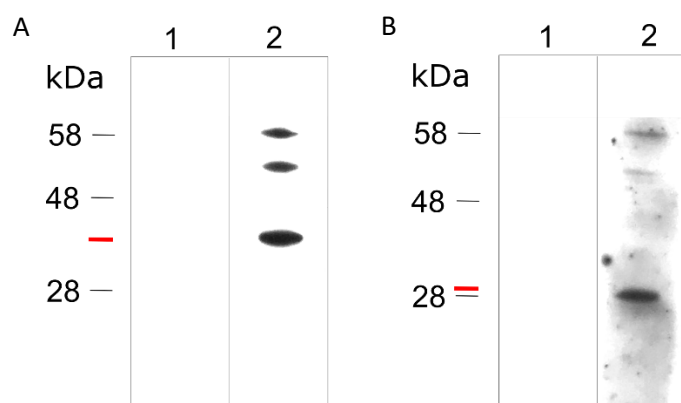


Figure 7. Western blots of *A. filiformis* protein extracts. Blots were probed with a commercially available rabbit anti-FtsZ (panel A, lane 2), with a previously published rabbit anti-FtsZ antibody³⁴ (panel B, lane 2) or without any primary antibody (lane 1 in both A and B). Red dashes indicate the expected MW of FtsZ (40 kDa).

ALYSIELLA FILIFORMIS MreB AND FIMBRIAL PROTEINS DETECTED ON WESTERN BLOTS

After failing to detect FtsZ protein in *A. filiformis*, we focused on the actin homolog MreB, important for cell preseptal growth in model Proteobacteria (see introduction). The product of the *mreB* gene identified in the available *A. filiformis* (DSM 16848) genome (T.V., unpublished data) is 61.2 % identical to *E. coli* K12 MreB and the sequence alignment of *E. coli* and *A. filiformis* MreB proteins is shown in Figure 8. We tested an anti-*E. coli*-MreB antibody kindly provided by our collaborator T. den Blaauwen; Den Blaauwen et al. 2003) on western blot and detected a band of the expected molecular weight of MreB (36 kDa; Figure 9A, lane 2). Additionally, we probed *A. filiformis* protein extracts with a commercially available sheep polyclonal antibody raised against *E. coli* K88 fimbrial protein AB/FaeG (<http://www.uniprot.org/uniprot/P02970>) to confirm that fimbriae localise to the host-attached pole only via immunostaining (Kaiser & Starzyk, 1973). Luckily, subjecting *A. filiformis* protein extracts to anti-*E. coli* pili antibody enabled the detection of a band running, approximately, at the expected molecular weight of 29 kDa (Figure 9B, lane 2).

In conclusion, *A. filiformis* appears to express both MreB and the fimbrial protein FaeG and we can use the available antibodies to analyse the expression patterns of these proteins. Importantly, we can use the fimbrial signal to distinguish the proximal from the distal pole.

[illegible]

DNA IN *ALYSIELLA FILIFORMIS* IS ASYMMETRICALLY LOCALIZED TO THE PROXIMAL CELL HALF

Analysis of the DNA localization pattern of 112 *A. filiformis* cells immunostained with an anti-*E. coli* fimbriae antibody (see previous section) showed DNA accumulation in the proximal cell moiety, the one in contact with the oral mucosa (Figure 10).

We conclude that the *A. filiformis* DNA is asymmetrically localized to the substrate-attached and, putatively, host-attached cell half.

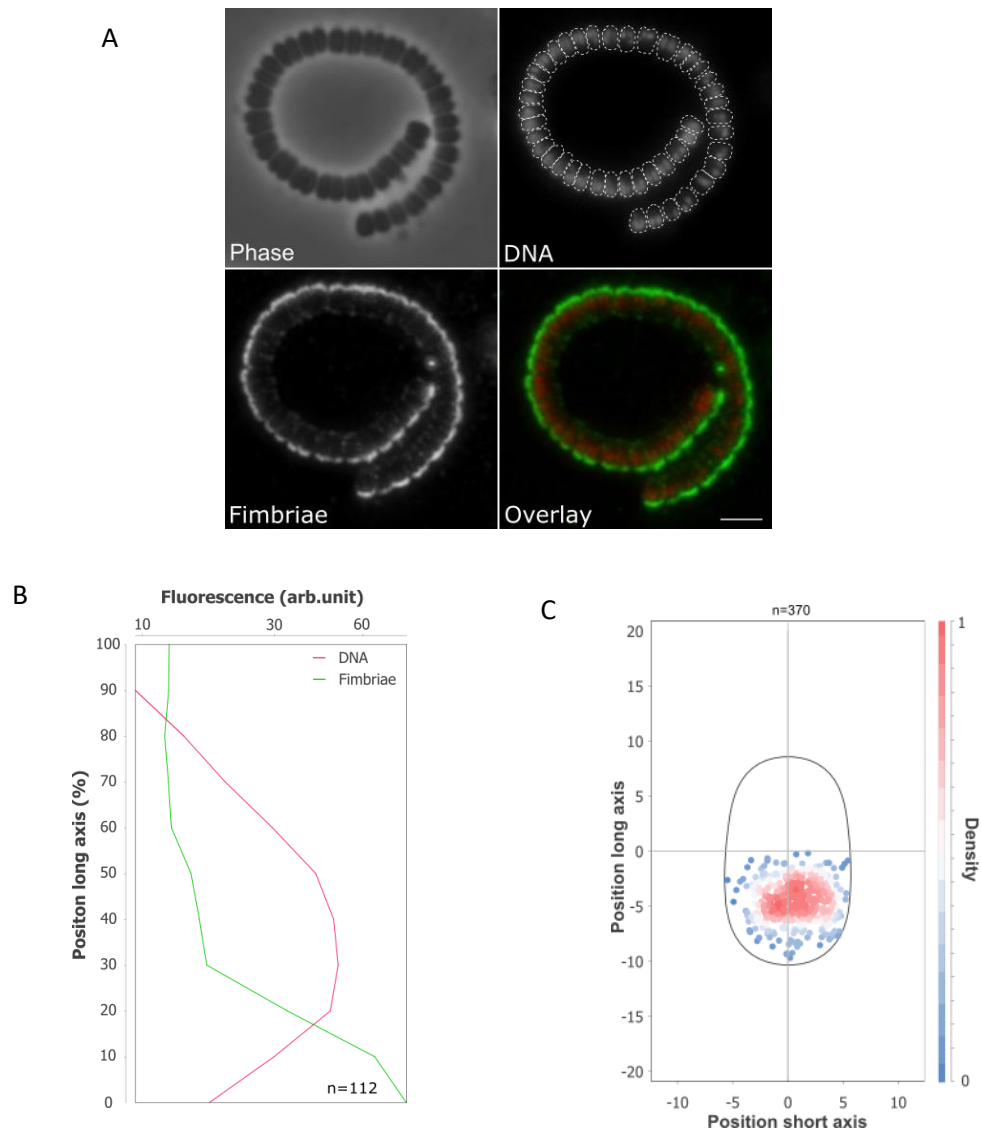


Figure 10. DNA asymmetric localization in *A. filiformis*. (A) Image of a representative *A. filiformis* filament in phase-contrast together with fluorescence signal images of (red) DNA and (green) fimbriae signals. Cell outline was deduced from the corresponding phase-contrast image (white dotted lines). Scale bar: 4 μ m. (B) Total fluorescence of DNA (red) and fimbriae (green) plotted against normalized cell length in % (n=112). (C) Subcellular localization of DNA fluorescence foci in *A. filiformis* (n= 370) cells displaying one fluorescence focus only. The focus represents the position of maximal DNA fluorescence intensity. The position of each focus is plotted against the normalized cell width and length (%) of the cell that contained them. The full lines represent the long and the short cell axis, and midcell is defined as the point in which they intersect.

PROXIMAL LOCALISATION OF MreB IN *ALYSIELLA FILIFORMIS*.

After testing the anti-MreB and the anti-fimbriae antibodies on western blots, we used them to immunostain fixed *A. filiformis* cells. Representative filaments of *A. filiformis* (Figure 11A), as well as total MreB, fimbrial and DNA fluorescence plotted against the normalised cell length expressed in % (n = 36; Figure 11) indicated stronger MreB signal in the proximal cell third.

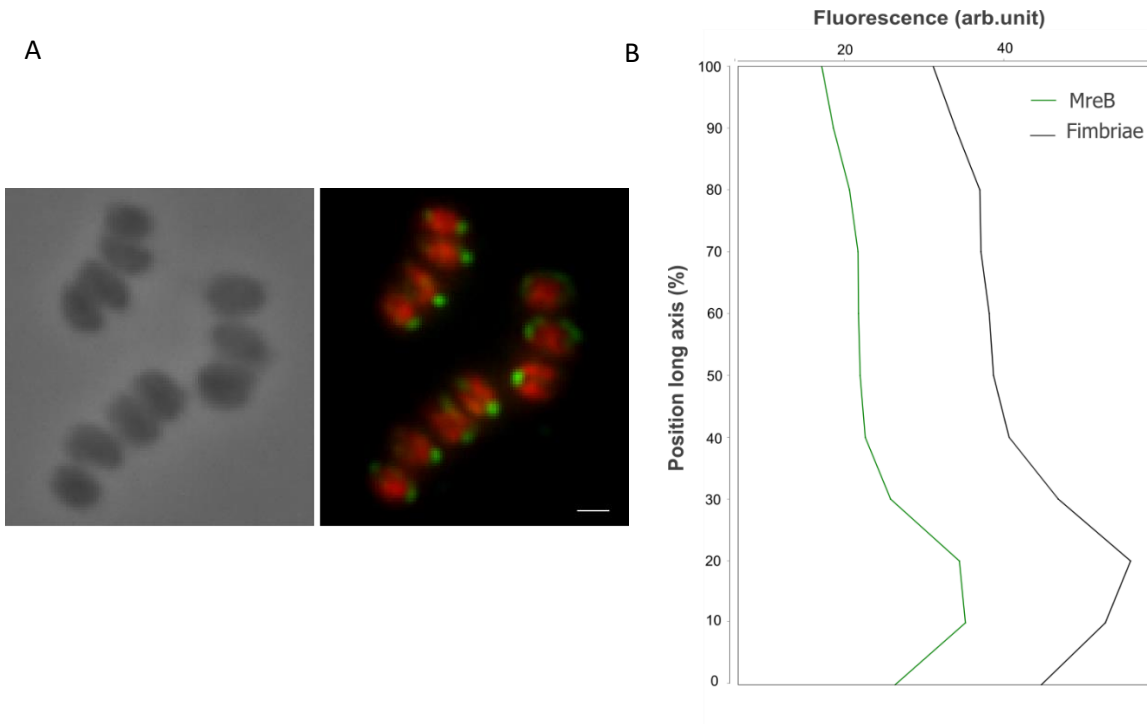


Figure 11. Proximal localisation of the MreB protein in *A. filiformis*. (A) Representative *A. filiformis* filaments stained with an anti-*E. coli* MreB antibody (green) and with Hoechst 33342 (red). (B) Total MreB (green), fimbrial (black) fluorescence of 36 cells (6 filaments) expressed in arbitrary units (arb. unit) plotted against the normalized cell long axis expressed in %. Scale bar: 1 μ m.

Given that no available anti-*E. coli* FtsZ antibody could detect *A. filiformis* FtsZ, we sought to confirm longitudinal fission by visualizing *A. filiformis* septation by incubating live cells with the cell wall precursor EDA-DA (D-amino acid dipeptide ethynyl-D-alanyl-D-alanine) (Liechti et al., 2014) (Figure 12A). In a filament subjected to a 2 min-long incubation, a focus of EDA-DA fluorescence was visible at one cell pole only, likely representing the leading edge of the invaginating septum. In a filament incubated for 5 min in the presence of EDA-DA, one fluorescence focus was visible at midcell, likely representing the leading edge of the septum moving from the invaginated pole towards the not yet invaginated one. As for the weaker, medial signal observed between the invaginated pole and the EDA-DA fluorescence focus it likely corresponds to the newly built septum. In a filament subjected to a 15 min-long incubation, one fluorescence focus is visible at the end of the almost complete new septum in the five leftmost cells, whereas the rightmost three appeared to have already started the second round of longitudinal septation before completing the first round (Figure 12; B, C).

Analysis of newly inserted PG expression pattern by employing HADA or BADA (Hsu et al., 2017), two cell wall precursors that do not need to be clicked to a fluorophore upon *A. filiformis* fixation, as they are already covalently bound to it prior incorporation, resembled what observed with EDA-DA (Figure 12) and allowed us to observe up to 16 cells undergoing the same septation stage indicating a remarkable degree of within filament-synchronicity (Figure 13; A, B).

We conclude that *A. filiformis* septation is longitudinal and asynchronous and that only one leading edge moves from one pole to the opposite one until it reaches it and septation is complete. We will hereafter refer to this new division mode as zipper-like to distinguish it to *Ca. T. hypermnestrae* division where both leading edges proceed toward one another, albeit the proximal one originates before the distal one.

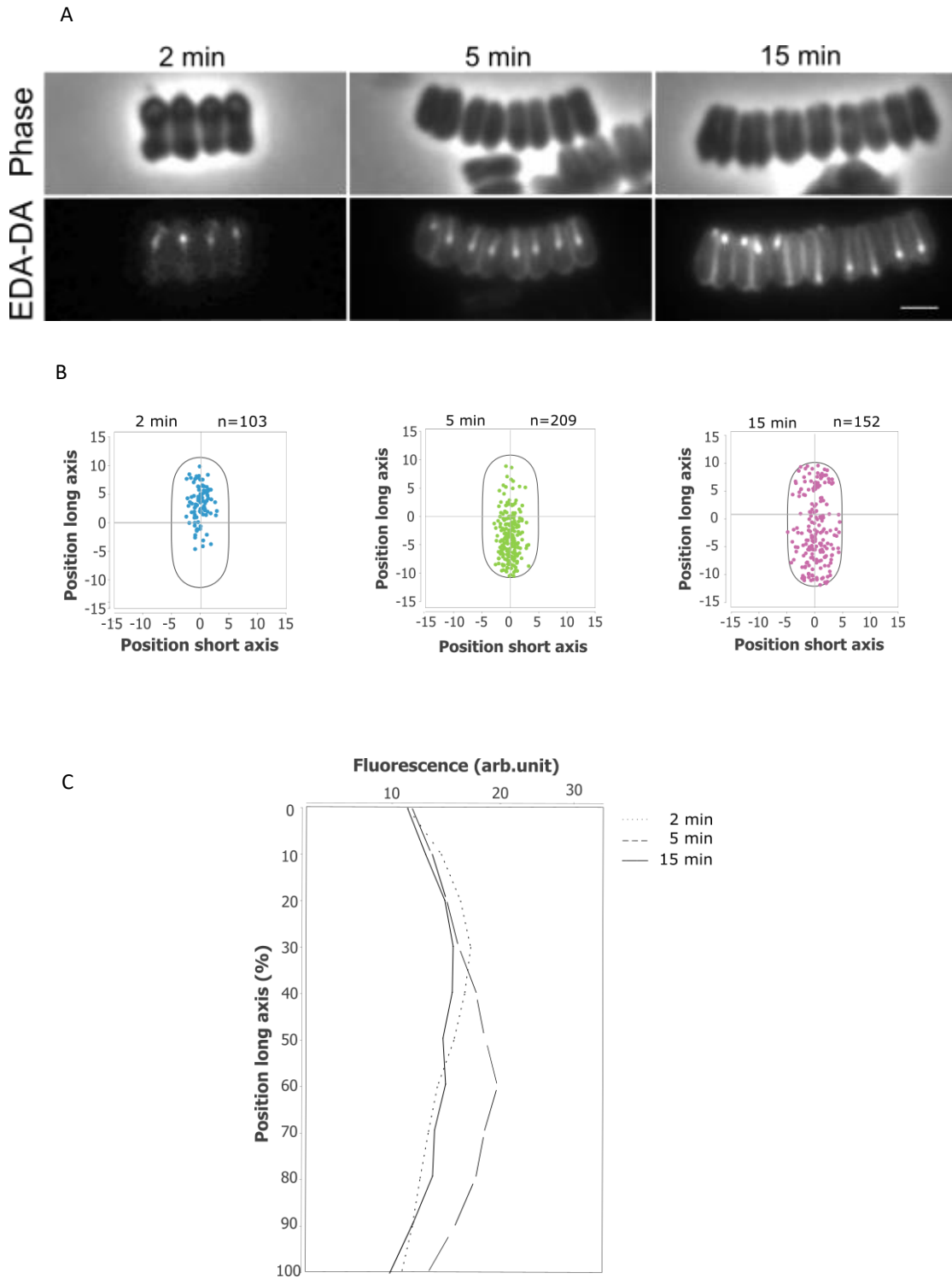


Figure 12. Pattern of new PG incorporation in *A. filiformis*. (A) Three representative filaments each incubated for either 2, 5 or 15 min in the presence of the PG precursor EDA-DA. Upper panels show the phase-contrast images and lower panels show the EDA-DA fluorescence signal. Scale bar: 2 μ m. (B) EDA-DA fluorescence foci position with respect to the normalized long cell axis in % (y-axis) and the normalized short cell axis in % (x-axis). The position of each focus is plotted against the normalized width and length (%) of the cell that contained them. Full lines represent the long and the short cell axis, and midcell is defined as the point in which they intersect. (C) Total EDA-DA fluorescence emitted by cells incubated for 2, 5 or 15 min in the presence of EDA-DA (n= 103; 209; 152, respectively; x-axis) plotted against the normalized long cell axis expressed in % (y-axis); 0% corresponds to the distal pole and 100% to the proximal (host-attached pole).

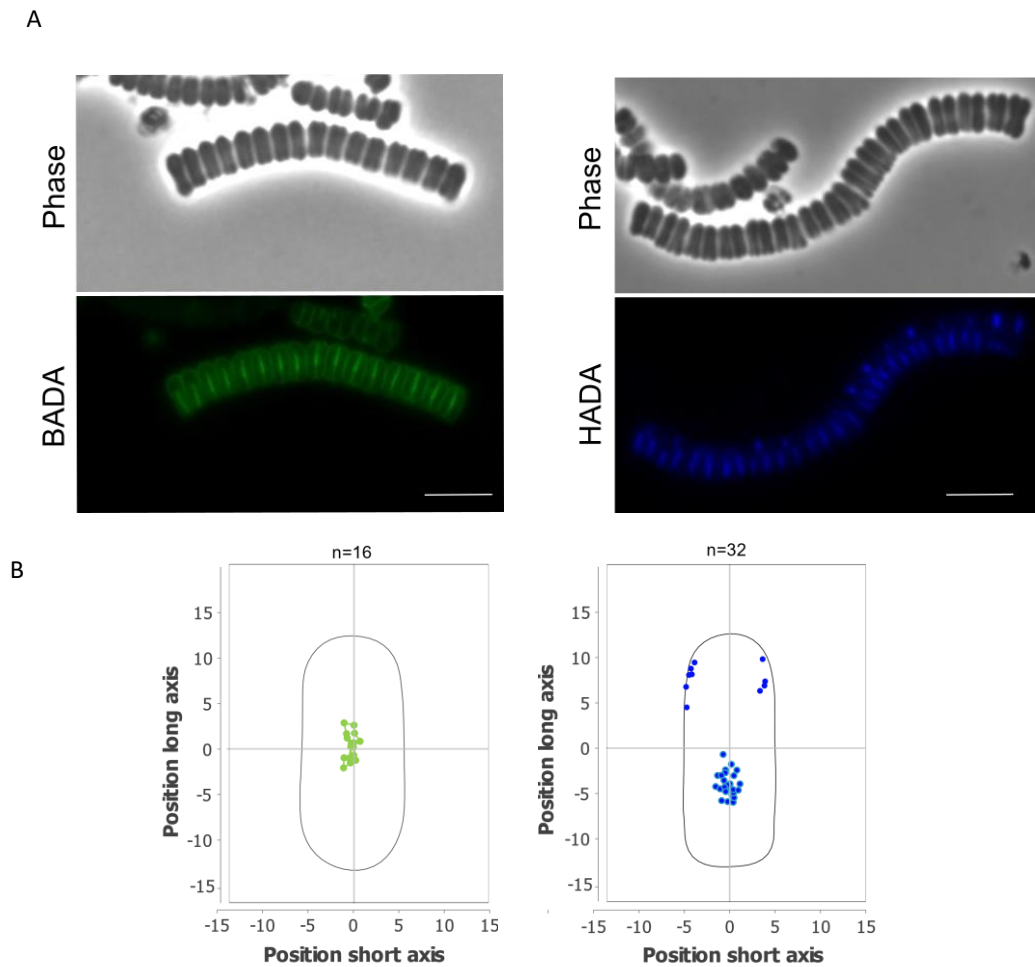


Figure 13. Within filament synchronicity in PG incorporation. A) Phase contrast (top) and epifluorescence (bottom) images of representative filaments after 1 h incubation with PG precursor HADA (7-hydroxycoumarin-3-carboxylic acid-D-alanine in blue) or BADA (BODIPY FL-D-alanine in green). Scale bar: 6 μ m. B) EDA-DA fluorescence emitted by each BADA- or HADA-stained (right and left plot, respectively; the number of cells per filament (n) is indicated on top of each plot).

A22-MEDIATED DEPOLYMERISATION OF MreB BLOCKS *ALYSIELLA FILIFORMIS* WIDENING

To understand the role of MreB in *A. filiformis* growth and division, cells were treated (or not treated) with the MreB-depolymerizing drug A22 (S-3,4-dichlorobenzyl-isothioureia) (Iwai et al., 2002) and incubated with the fluorescently labelled PG precursors TADA (TAMRA-D-alanine) or BADA. Morphometric analysis showed that cells not treated with A22 widened throughout the cell cycle as expected (Figure 14A, right plot). However, we could not observe any positive correlation between cell area and cell width in cells treated with A22 (40 μ g/ml) indicating that MreB depolymerization severely affected cell widening (Figure 14B, right plot).

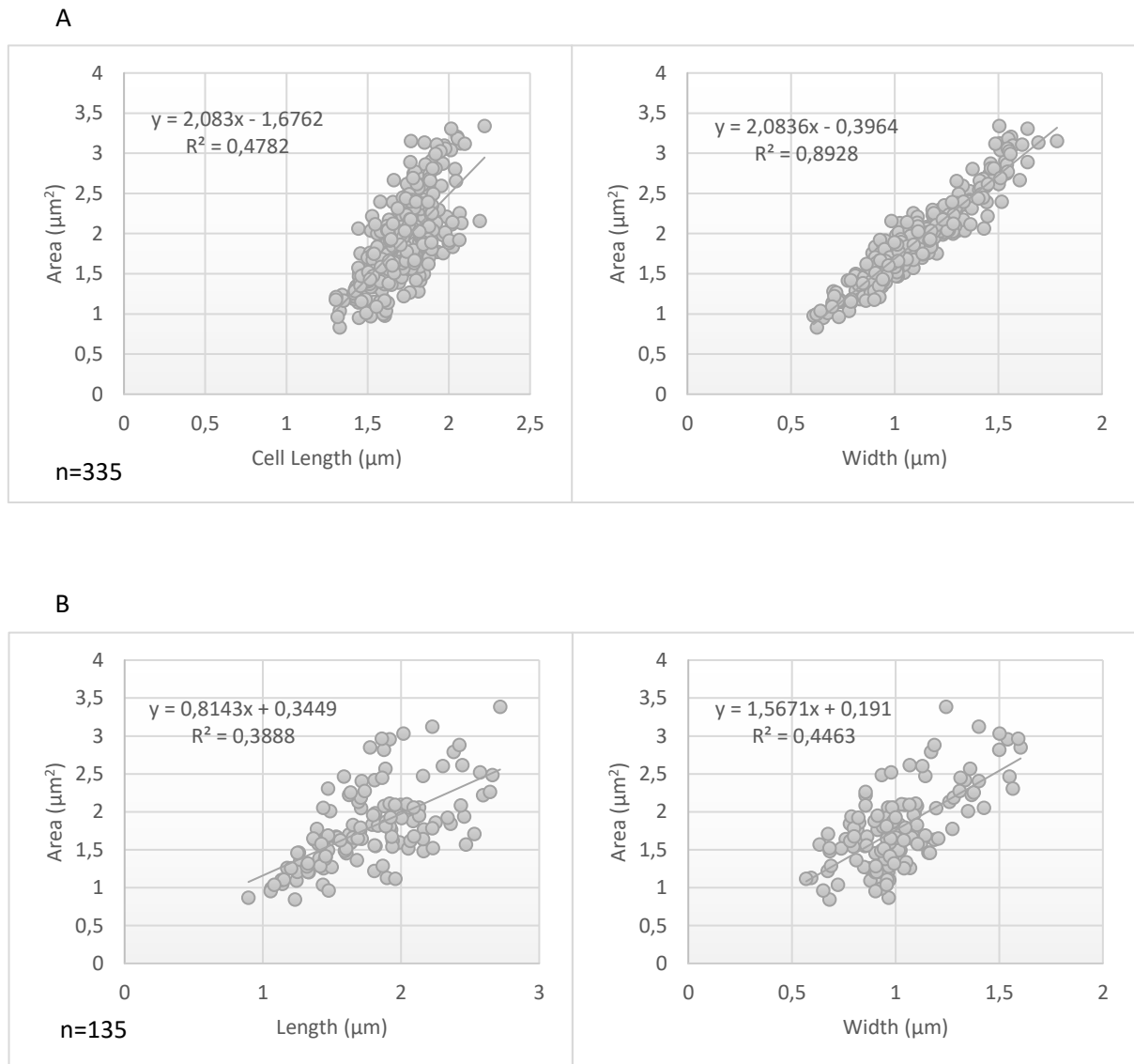


Figure 14. Morphometric analysis of *A. filiformis* cells treated and untreated with the MreB-depolymerizing drug A22. (A) Area of 335 untreated *A. filiformis* cells plotted versus cell length or cell width (left and right plot, respectively) and (B) area of 135 A22-treated (40 $\mu\text{g/ml}$) cells plotted against either cell length or cell width (left and right plot, respectively).

Consistently with the morphometric analysis, treatment of *A. filiformis* with A22 (20 or 40 $\mu\text{g/ml}$) seemed to decrease PG incorporation as shown by BADA and TADA weaker fluorescence in these cells (representative cells in Figure 15).

Based on the morphometric analysis of A22-treated and untreated *A. filiformis* cells, we conclude that, in these bacteria actin homolog MreB is required for cell widening but not required for rod-shape maintenance. Moreover, based on a preliminary analysis of PG

incorporation pattern in A22-treated and untreated *A. filiformis*, we hypothesize that MreB is required for the correct orientation of the septation plan.

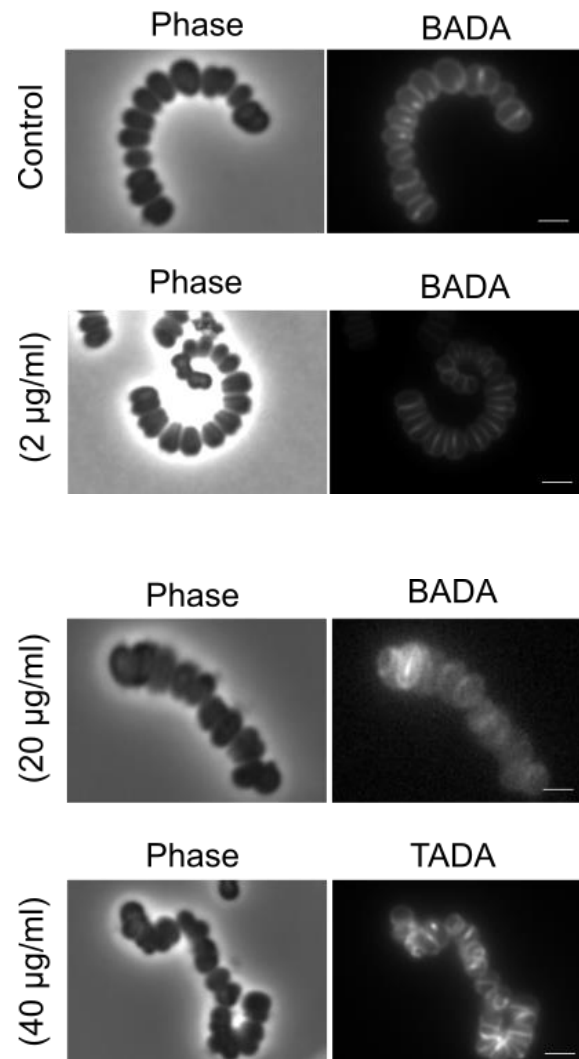


Figure 15. A22 affects new PG insertion in *A. filiformis*. Epifluorescence images of four representative filaments after 1 h incubation in the absence or in the presence of the MreB-depolymerising drug A22 (from top to bottom: control, 2 µg/ml, 20 µg/ml, 40 µg/ml) followed by 1 h incubation with BADA or TADA. Scale bar: 2 µm

DISCUSSION

Although the discovery of *S. muelleri* (SCHMID 1922) and *A. filiformis* (SIMONS 1922) dates back to almost a century, up to this one, there have been no molecular studies performed on oral cavity symbionts. However, their cultivability provides the unprecedented chance to understand the molecular mechanism of growth and division in animal symbionts, as well as bacterial multicellularity. We therefore investigated the growth mode of our two protagonists with emphasis on *A. filiformis* as it bears resemblance to longitudinally dividing symbionts by its rod shape and adhesion with only one cell pole to the mucosal squamous epithelium of the mouth (Kaiser & Starzyk, 1973).

Morphometric measurements showed that *A. filiformis* cells widen not only during septation, as observed in other longitudinally dividing bacteria (Pende et al., 2018), but throughout the cell cycle. Therefore, *A. filiformis* growth mode resembles that of model bacteria like *E. coli* and *B. subtilis* (Liechti et al., 2014), which grow both predivisionally and septally, i.e. both before and during septation. Furthermore, incubations with fluorescently labelled PG precursors suggest that septation is longitudinal in both *S. muelleri* and *A. filiformis*. More precisely, we determined that *S. muelleri* PG incorporation starts at the cell poles and progresses toward midcell. This simultaneous PG incorporation from both poles is also observed in *Ca. T. oneisti*. As for *A. filiformis*, its longitudinal septation differs from the asynchronous invagination of the cell poles reported in the nematode symbiont *Ca. T. hypermnestrae*: here, invagination starts at the proximal, fimbriae-rich pole and later the distal pole starts to constrict (Leisch et al., 2016), whereas in *A. filiformis* septation is zipper-like, i.e., it proceeds from one pole (the distal one) to the other pole (the proximal one). Even though asynchronous cell growth has been found in other gram-negative bacteria, such as *Proteus mirabilis* and *Caulobacter crescentus* (Yao et al., 2017), the host orientation of the PG synthesis machinery is unique in *Ca. T. hypermnestrae* (Pende et al., 2018) and *A. filiformis* (this work).

Not only is *S. muelleri* cell morphology unique, but it also varies within the same filament. Indeed, the terminal cells of a *S. muelleri* filament differ from the central ones, specifically, they are shorter and more round. We hypothesise that the two observed morphologies represent two different cell types with different functions. As for *S. muelleri* DNA localization, our analysis revealed that before cell cytokinesis DNA will accumulate either at the cell poles or at the midcell. DNA localization patterns are also observed in *C. crescentus* where chromosome organization and dynamics changes during cell cycle and DNA replication (Jensen & Shapiro, 2003).

Ultrastructural studies suggested that the oral cavity dweller *A. filiformis* adhere to the host palate (Kaiser & Starzyk, 1973) and after immunostaining of *A. filiformis* with an anti-*E. coli* pili antibody we could observe a proximal localization of its fimbriae. The proximal fimbriae might mediate gliding of the ribbon-like chains of *A. filiformis*. Noteworthy, in the case of *S. muelleri*, it is known that the ventral side is covered with fimbriae and both poles are attached to the host epithelial cells moulding rod shape into concave (Hedlund & Kuhn, 2006). Genes encoding for T4 pili likely responsible for attachment, as well as gliding motility were identified in the *A. filiformis* genome - *pilBCD*, *pilONM* and *pilTU* (T. Viehboeck, unpublished data). Similarly to the oral cavity symbiont *A. filiformis*, *Ca. T. oneisti* and *Ca. T. hypermnestreae* cells are attached via one of their poles to the cuticle of their nematode hosts, *Laxus oneistus* and *Robbea hypermnestra*, respectively (Leisch et al., 2012, 2016). They might have evolved the longitudinal cell division to ensure host attachment throughout the generations. Adhesins, as fimbriae, can promote bacterial attachment to host tissues, but can also function as virulence factors (for example, in uropathogenic *E. coli*) (Godaly et al., 2000). While bacterial adhesion via fimbriae helps bacteria to invade host cells, it also endangers them by contributing to the recognition and their clearance by the host innate immunity. Indeed, in today's medicine the ability of antibiotic to affect bacterial adherence, for example by inhibiting the expression of fimbriae, may have therapeutical use (Shibl, 1985).

Genome sequencing revealed that the gene encoding for the key division protein FtsZ is 50% and 58% identical to the *E. coli* FtsZ gene in *S. muelleri* and *A. filiformis*, respectively. Nevertheless, two different anti-*E. coli* FtsZ antibodies failed to detect their FtsZ proteins on western blots. Therefore, we do not know, at the moment, whether FtsZ co-localizes with- and may therefore mediate the insertion of new PG in the oral cavity symbionts as shown for *Ca. T. oneisti* and *T. hypermnestreae* (Pende et al., 2018). Although the *ftsZ* gene was identified in the *A. filiformis* genome, unsuccessful detection of the cell division protein FtsZ on western blot raises the possibility that this bacterium does not need the tubulin homologue to divide, as it is the case for members of *Planctomycetes*, *Ureaplasma urealyticum* (Bernander & Ettema, 2010) and in *Chlamydia* (Ranjit et al., 2020). Genome analyses of *Chlamydia* revealed lack of not only the *ftsZ* gene but also of almost all known FtsZ-associated cell division proteins; FtsA, FtsB, FtsP, FtsE, FtsX, ZipA and ZapA/B/C/D (Jacquier et al., 2015). Absence of FtsZ activity might have led chlamydial MreB and RodZ proteins to evolve the ability to mediate the insertion of septal PG (Ranjit et al., 2020).

Concerning the role of the actin-homolog MreB in *A. filiformis*, we could show its expression on western blots of *A. filiformis* protein extracts. Moreover, immunostaining with an anti-MreB antibody showed an increased MreB signal in the proximal cell third, suggesting that it might direct new PG insertion. In two rods known for their longitudinal division, *Ca. T. oneisti* and

Ca. T. hypermnestrae, MreB localizes in areas of both low and high curvature (Pende et al., 2018). However, in model rods, including *E. coli*, the MreB protein is excluded from membrane regions of high curvature and appears to be the major coordinator for lateral PG synthesis and polymerisation (Nguyen et al., 2015).

To characterize and better understand the role of MreB during cell division in the oral cavity symbiont *A. filiformis*, we employed the MreB-depolymerizing drug A22. Upon incubation with 40 µg/ml A22, we observed a decrease in PG incorporation, in accordance to what previously published for a number of bacteria (Liechti et al., 2016; Hsu et al., 2017; Pende et al., 2018). Moreover, according to morphometric measurements, *A. filiformis* remained rod-shaped. This result is in contrast with what reported for *E. coli*, which became coccoid when treated with A22 (Gitai et al., 2005) but resembled what observed for nematode ectosymbionts which remained rod-shaped (Pende et al., 2018). Finally, upon incubation with 40 µg/ml A22, we observed misplacement of the septum, an unprecedented phenotype that has never been observed up to this study. All in all, we conclude that, in the oral cavity symbiont, MreB plays an important role not just for symbiont widening, but for cell division as well. Nevertheless, we need to take into account possible cyto- and genotoxicity effects of A22. It has been shown that a concentration of A22 from 32 µg/mL and above has cytotoxic effect (Bonez et al., 2016). Therefore, to confirm that the aberrations observed following A22 treatment are specifically due to MreB depolymerization, alternative inhibitors, such as MP265 (Takacs et al., 2010), should be tested.

Interestingly, not just the PG-synthesis but also its DNA appears to be host-polarized in *A. filiformis*. Namely, it appears to be restricted to the proximal cell half. Moreover, it was recently shown that the chromosome of *Ca. T. oneisti* displays a longitudinal chromosome configuration in which the origin of replication (*ori*) (together with *ftsQAZ* operon and two *parS* elements) locates to the proximal cell pole and terminus locates distally (Weber et al., 2019).

Longitudinal septation and multicellularity could represent adaptations to the oral cavity habitat of the filament-forming *Neisseriaceae* *Simonsiella muelleri* and *A. filiformis*.

As mentioned in the introduction, the genomes of the oral cavity symbionts *S. muelleri* and *A. filiformis* encode for secreted proteins that may enable the host to survive the vertebrate immune system, such as the host lysozyme inhibitor PlcC (Callewaert et al., 2008; Smith et al., 1995) or a protein that makes the lipooligosaccharide resistant to the attack of neutrophils (Lst) (Smith et al., 1995). We hypothesized that one of the reasons the oral cavity symbiont might have evolved longitudinal fission and filamentation is because they depend from host-derived essential nutrients. Genome annotation revealed that they are not able to synthesise vitamin

B12 but they have genes for the TonB dependent-vitamin B12 transporter BtuB and other vitamin B1 transporters Glu, Arg and Met (T. Viehboeck, unpublished data).

Extending bacterial cell biology to nonmodels and, more specifically, understanding the cell biological adaptations of *A. filiformis* and *S. muelleri* to the vertebrate oral cavity, promise to disclose fundamentals of cell growth and division.

CONCLUSIONS AND PERSPECTIVES

In this research study, we sought to shed light on the molecular mechanisms of cell growth and division in the oral cavity symbionts *A. filiformis* and *S. muelleri*, with special focus on the former. Namely, we examined two essential macromolecules, PG and DNA, as well as the FtsZ and MreB, homologues of the eukaryotic protein tubulin and actin, respectively.

We observed host polarized fimbriae localization in *A. filiformis* and its DNA appears to be restricted to the proximal cell half, a brand new finding. Based on immunostaining which indicated zipper-like longitudinal septation in *A. filiformis*, as well as DNA localization in the host-attached cell half, we drew a model of cell growth in *A. filiformis* (Figure 16, model kindly provided by Philipp M. Weber). Differently from what is observed in longitudinally dividing *Ca. Thiosymbion*, our morphometric analysis for *A. filiformis* revealed not just septal but also predivisional widening. Immunostaining of *A. filiformis*'s MreB revealed its septal, mainly proximal localization. Moreover, A22-mediated depolymerisation of MreB blocked *A. filiformis* widening but also led to septum misplacement, suggesting that the bacterial actin homolog is required not only for disperse but for septal growth, too.

In conclusion, our preliminary study of oral cavity symbiont growth, as well as division brings to light that several aspects of these processes differ from what has been learned from model systems. Ultimately, we believe that to identify the fundamentals of cell growth and division we must extend our research to nonmodels. Understanding the adaptations to the symbiotic lifestyle, might also have therapeutical relevance as oral cavity symbionts might promote oral health.

Continuation of *A. filiformis* and *S. muelleri* cell biological studies will help in understanding basic principles of bacterial cell shape maintenance, cell polarity and molecular organization.

New PG incorporation pattern analysis as well as MreB localization pattern analysis should be refined in *A. filiformis*. The resolution of microscopic images could be increased by applying super resolution microscopy techniques, such as 3D-SIM. Further efforts should lie in establishing techniques for expression of fluorescently labelled proteins (such as FtsZ, MreB and MinCDE), as well as deletion of canonical cell division or cell growth genes (the above mentioned and additionally of *ftsZ*, *ftsI*, *ftsK*, *mreB*). Finally, induction of random mutations would allow us to discover yet unknown genes involved in bacterial growth, division and/or multicellularity.

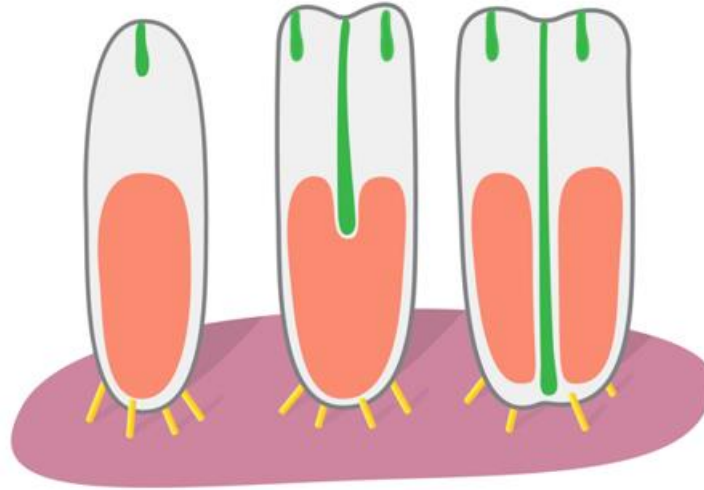


Figure 16. Model of cell growth in oral cavity symbiont *A. filiformis*. Schematic view of new PG incorporation (green) together with the DNA localization pattern (red). Attachment of bacteria to epithelial cells is mediated by fimbriae (yellow). Cells are arranged from the youngest to the oldest from left to right. Kindly provided by P.M. Weber.

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ABSTRACT GERMAN

Alsyella filiformis und *Simonsiella muelleri* gehören zu der Familie *Neisseriaceae*. Das sind Gammaproteobakterien, die in der Mundhöhle der Wirbeltiere gefunden wurden. Eine frühere (1922) Ultrastrukturalstudie von *A. filiformis* und *S. muelleri* hat gezeigt, dass ihre Ketten mittels Fimbrien an die Wirtsepithelzellen gebunden sind. Bis zu dieser Studie sind ihre Fortpflanzungsstrategien jedoch unerforscht geblieben. Hier haben wir durch morphometrische Analyse gezeigt, dass eine Verbreiterung von *A. filiformis* nicht nur während der Teilung sondern auch vor der Division auftritt. Außerdem haben wir beim Auftragen eines markierten Zellwandvorläufers bestätigt, dass die Septierung sowohl bei *S.muelleri* als auch bei *A.filiformis* in Längsrichtung und im Fall von *A.filiformis* asynchron ist, was bedeutet, dass ein Zellpol vor dem anderen septiert. Obwohl Western Blots mit Anti-*E.coli* FtsZ Antikörpern konnten dieses wichtige Zellteilungsprotein nicht nachweisen, die septale und proximale Anreicherung vom Aktinhomologen MreB in *A. filiformis* suggeriert, dass dies die Insertion neuer PGs nicht nur während Teilung, sondern auch während des Septumwachstums steuern könnte. Schließlich haben wir eine asymmetrische Verteilung der DNA in der polaren Zellhälfte von *A.filiformis*. Die Tatsache, dass die Symbionten der Mundhöhle kultivierbar sind, hat enormes Potenzial ein genetisch nachvollziehbares System zur Untersuchung der bakteriellen Längsspaltung als Anpassung an den symbiotischen Lebensstil zu etablieren. Da ihre Häufigkeit für die Mundgesundheit der Menschen sehr wichtig ist, könnte ein besseres Verständnis von *A.filiformis* und *S.muelleri* eine biomedizinische Relevanz haben.