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Elucidation of the Cell Division Mechanism and Characterization of Tubulins in the Bacterial Phylum *Verrucomicrobia*

Martin Pilhofer

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(Università di Pisa, Italy)

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meiner Familie

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ABBREVIATIONS

AMP	adenosine monophosphate
ATP	adenosine triphosphate
bklc	bacterial kinesin light chain
bp	basepair
BSA	bovine serum albumin
btub	bacterial tubulin
Ca	Candidatus
cDNA	complementary DNA
CODEHOP	consensus degenerate hybrid oligonucleotide primer
Da	dalton
dcw	division and cell wall
ddl	d-alanine d-alanine ligase
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen
°C	degree Celsius
DNA	deoxyribonucleic acid
DTT	dithiothreitol
FRAP	fluorescence recovery after photo bleaching
fts	filamentous temperature sensitive
GDP	guanosine diphosphate
GFP	green fluorescent protein
GTP	guanosine triphosphate
h	hour
His	histidine
IF	intermediate filament
IgG	immunoglobulin G
IMG	Integrated Microbial Genomes
IPTG	isopropyl-beta-D-thiogalactopyranosid
kb	kilobases
kDa	kilodaton
klc	kinesin light chain
kV	kilovolt

M	molar
µF	microfarad
µg	microgram
µm	micrometer
µM	micromolar
MT	microtubule
MTOC	microtubule organizing center
mg	milligram
ml	milliliter
min	minute
ng	nanogram
Ni	nickel
nm	nanometer
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
Pde	*Prosthecobacter debontii*
Pdj	*Prosthecobacter dejongeii*
rpm	rounds per minute
Pva	*Prosthecobacter vanneervenii*
PVC	*Planctomycetes-Verrucomicrobia-Chlamydiae*
PVDF	polyvinylidene fluoride
RNA	ribonucleic acid
rRNA	ribosomal RNA
RT	reverse transcription / real time
SDS	sodiumdodecylsulphate
TBS	tris buffered saline
TEM	transmission electron microscopy
TPR	tetratricopeptide repeat
V	volt
v/v	volume/volume
Vsp	*Verrucomicrobium spinosum*
w/v	weight/volume

ORIGINAL PUBLICATIONS

Some main results of this study and the corresponding discussion, conclusions and materials and methods are described in detail in the publications listed below. The original articles and the corresponding author contributions can be found in the section Appendix A-D. Also, the study contains unpublished data for which additional materials and methods are specified in section Appendix E.

The symbol ➲ at the beginning of a chapter designates that the chapter content was published as part of the indicated appendix / publication.

Appendix A **Pilhofer M**, Rosati G, Ludwig W, Schleifer KH, Petroni G.
Coexistence of tubulins and *ftsZ* in different *Prosthecobacter* species.
Molecular Biology and Evolution 2007 Jul;24(7):1439-42.

Appendix B **Pilhofer M**, Bauer AP, Schrallhammer M, Richter L, Ludwig W, Schleifer KH, Petroni G.
Characterization of bacterial operons consisting of two tubulins and a kinesin-like gene by the novel Two-Step Gene Walking method.
Nucleic Acids Research 2007 Nov;35(20):e135.

Appendix C **Pilhofer M**, Rappl K, Eckl C, Bauer AP, Ludwig W, Schleifer KH, Petroni G.
Characterization and evolution of cell division and cell wall synthesis genes in the bacterial phyla *Verrucomicrobia*, *Lentisphaerae*, *Chlamydiae* and *Planctomycetes* and phylogenetic comparison with rRNA genes.
Journal of Bacteriology 2008 doi:10.1128/JB.01797-07.

Appendix D **Pilhofer M**, Ludwig W, Schleifer KH, Petroni G.
Phylogenetic relationships of bacterial tubulins within the Tubulin/FtsZ superfamily: Implications on their evolutionary origin
Prepared for submission.

A GENERAL INTRODUCTION

A.1 The Cytoskeleton: Eukaryotes versus Bacteria

In the last 15 years, our view of the bacterial cytoskeleton has basically changed. Before 1990, the cytoskeleton was thought to have evolved only in Eukaryotes. The "tea-bag model", considering the bacterial cytoplasm unorganized and its constituents randomly distributed, is meanwhile outdated. Through advances in cellular imaging, it has become clear that the subcellular environments of bacterial cells are highly organized (reviewed in Gitai 2007).

The eukaryotic cytoskeleton includes both stable filamentous structures that are composed largely of intermediate filament proteins and dynamic structures such as tubulin-derived microtubular structures and actin filaments that can assemble, disassemble and redistribute rapidly within the cell in response to signals that regulate cellular functions such as cell cycle progression, intracellular organelle transport, motility, and cell shape. The common characteristics of these systems are their polymeric filamentous nature and their long-range order within the cell. Actin filaments determine the shape of the cell's surface and are necessary for whole cell locomotion. Microtubules determine the positions of membrane-enclosed organelles and direct intracellular transport. Intermediate filaments provide mechanical strength and resistance to shear stress (Alberts et al. 2002).

Also in *Bacteria*, cytoskeletal proteins play a major role in subcellular organization. Bacterial homologs of the three eukaryotic cytoskeletal families, actin, tubulin and intermediate filaments have been identified (Gitai 2007; Larsen et al. 2007; Pogliano 2008).

A.1.1 Homologs of tubulin: FtsZ, bacterial tubulins and TubZ

Microtubules are long, hollow cylinders made of the protein tubulin (Figure 1). The subunit of each protofilament is a tubulin heterodimer, formed from a very tightly linked pair of alpha- and beta-tubulin monomers. The GTP molecule in the alpha-tubulin monomer is so tightly bound that it can be considered as an integral part of the protein. The GTP molecule in the beta-tubulin subunit monomer, however, is less tightly bound and has an important role in filament dynamics (Alberts et al. 2002).

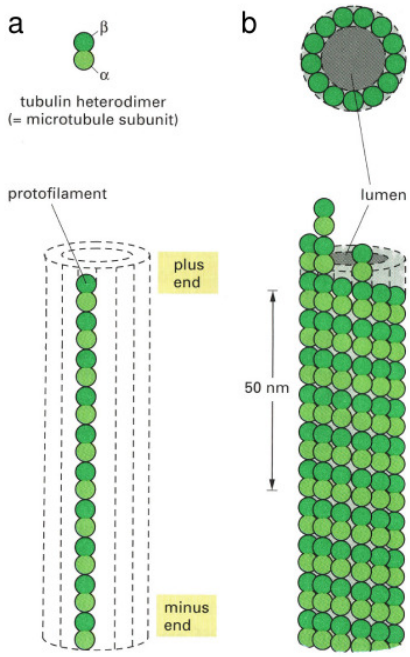

Figure 1. The structure of a eukaryotic microtubule and its subunit [Alberts et al. (2002), modified]

a | One tubulin subunit (alpha-beta heterodimer) and one protofilament are shown schematically. Each protofilament consists of many adjacent subunits with the same orientation. **b** | The microtubule is a stiff hollow tube formed by 13 protofilaments aligned in parallel.

Microtubules, which are frequently found in a star-like cytoplasmic array emanating from the center of an interphase cell, can quickly rearrange themselves to form a bipolar mitotic spindle during cell division. They have an important contribution in chromosome segregation. Microtubules can also form motile whips called cilia and flagella on the surface of the cell, or tightly aligned bundles that serve as tracks for the transport of materials down along neuronal axons (Alberts et al. 2002).

Besides alpha and beta tubulins, further subfamilies were identified in Eukaryotes. Another representative is gamma tubulin, which is permanently associated with basal bodies or, more general, with different microtubule-organizing centers (MTOCs) (Libusova and Draber 2006). Apart from the conventional tubulin genes, a number of new members of the tubulin family have been described: delta, epsilon, zeta, eta, theta, iota and kappa (Libusova and Draber 2006). Epsilon tubulins were shown to be important for basal body assembly and anchorage, delta tubulins for C-tubule assembly and eta tubulins for basal body development. For the others, the function remains to be elucidated (Libusova and Draber 2006).

In *Bacteria*, four groups of tubulin homologs have been identified: FtsZ (Erickson 1995), bacterial tubulins (BtubAB) (Jenkins et al. 2002), bacilli TubZ (Larsen et al. 2007) and

archaeal FtsZ-likes (Larsen et al. 2007). Except for the latter ones, all were shown to polymerize into protofilaments. But none of them has ever been observed to form microtubule-like structures. The bacterial tubulin homologs are briefly reviewed in the following sections (A.1.1.1 - A.1.1.3).

A.1.1.1 The bacterial cell division protein FtsZ

FtsZ is the most widely conserved bacterial cytoskeletal protein and is found in nearly all *Bacteria* and *Euryarchaea*, as well as in several eukaryotic organelles (Margolin 2005). It is required for cytokinesis (cf. A.2.1), by forming a ring at the plane of division (Bi and Lutkenhaus 1991). The secondary structure of FtsZ mirrors tubulin (Figure 2) and the protein displays *in vitro* similar dynamic properties (Lowe and Amos 1998; Nogales et al. 1998). Like tubulins, FtsZ polymerizes into linear protofilaments in a GTP-dependent fashion. The monomers within an FtsZ filament were shown to turn over very dynamically (Stricker et al. 2002). Although FtsZ is incapable to form microtubule-like structures, the combined structural and functional properties make it unlikely that FtsZ and tubulin proteins evolved twice (Erickson 1998); therefore, eukaryotic tubulin and bacterial FtsZ are considered to be homolog proteins.

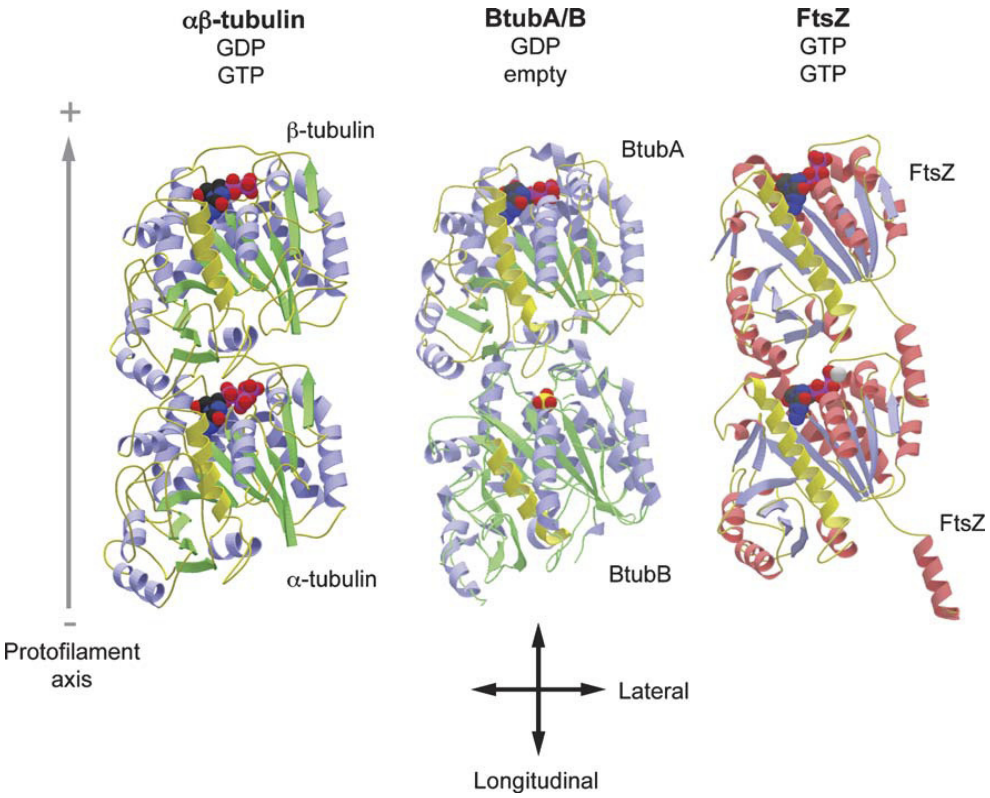

Figure 2. Structure comparison of tubulin homologs (Michie and Lowe 2006)

Structures of the α/β-tubulin heterodimer (Lowe et al. 2001), the BtubA/BtubB heterodimer (Schlieper et al. 2005) and the FtsZ dimer (Scheffers et al. 2000), showing the position of the nucleotide at the dimer interface, the conservation of fold, and the axis of protofilament extension (*up the page*). Lateral interactions between protofilaments could be formed at all or any of the interfaces perpendicular to the longitudinal axis of protofilament assembly.

A.1.1.2 Bacterial tubulins in *Prosthecobacter*

In 2002, Jenkins et al. described genes with higher similarities to eukaryotic tubulin than to FtsZ in the bacterial genus *Prosthecobacter*. Two adjacent loci, *btubA* and *btubB*, were detected in the genome sequence data of *Prosthecobacter dejongeii*; *btubAB* could be also detected in the remaining species of the genus. RT-PCR results indicated that *btubA* and *btubB* are transcribed in *P. dejongeii*.

BtubA and BtubB had their top Blast matches with eukaryotic alpha and beta tubulin, respectively and were shown to share 31-35% and 34-37% sequence identity with these proteins. Sequence identity with FtsZ was in the range of 8-11%, suggesting a specific relationship to eukaryotic tubulins. Although, using electron microscopy, no microtubule-like structures could be observed in thin sections of *P. dejongeii*. Also the Fingerprint sequence

analyses (Attwood et al. 2003) of *P. dejongeii* A and B tubulins suggested a relationship of BtubA to alpha tubulins and BtubB to beta tubulins (Jenkins et al. 2002).

Comparative sequence analysis of bacterial tubulins and representatives of eukaryotic tubulin subfamilies showed stable monophyletic clusters of BtubA and BtubB sequences, respectively. In contrast to Fingerprint and Blast analyses, relationships of the BtubA or BtubB cluster to eukaryotic subfamilies were rather instable. Also, no specific relationship to any eukaryotic lineage could be detected.

Whereas the comparative modeling results (*in silico*) of Jenkins and co-workers (2002) did not suggest the ability of BtubA and BtubB to polymerize, in 2005, two working groups could show that bacterial A and B tubulins are able to form homo- and heterodimers *in vitro* (Schlieper et al. 2005; Sontag et al. 2005). Moreover, these heterodimers were able to assemble into protofilament bundles even if they did not form microtubule-like structures. Immunofluorescence studies with antibodies against bacterial tubulin performed on recombinant *E. coli* expressing both BtubA and BtubB, showed that bacterial tubulins assemble into rods running the length of the cells, which sometimes seem to form a loose spiral at the membrane. No co-localization at Z ring like structures was observed. On the basis of these data, Sontag et al. (2005) speculated that bacterial tubulins could contribute to the peculiar morphology of *Prosthecobacter*, which possesses a prostheca at the older pole of the cell (A.3.1.2). However, direct studies on bacterial tubulin localization and function in *Prosthecobacter* were not performed.

The crystal structures of *Prosthecobacter* tubulins exhibit striking similarities to the eukaryotic counterpart (Figure 2) and clearly show that the polymerization mechanism is the same as that of eukaryotic tubulin (Nogales and Wang 2006; Schlieper et al. 2005). Despite of the strong similarities in structure between eukaryotic and *Prosthecobacter* tubulins, *Prosthecobacter* tubulins show divergent properties such as weak dimerization and chaperon-independent folding (Schlieper et al. 2005), which is more similar to FtsZ (Andreu et al. 2002). Both, Jenkins et al. (2002) and Schlieper et al. (2005) hypothesized that *btub*-genes could have been acquired by *Prosthecobacter* from a eukaryote.

A.1.1.3 TubZ in *Bacillus thuringiensis* and archaeal FtsZ-likes

Recently, a gene coding for another tubulin-like polymer (*tubZ*) has been identified on the virulence plasmid pBtoxis of *Bacillus thuringiensis* (Larsen et al. 2007). TubZ is also a member of the tubulin/FtsZ GTPase superfamily but is only distantly related to both FtsZ and tubulin. TubZ assembles dynamic, linear polymers that exhibit directional polymerization

with plus and minus ends, movement by treadmilling and a critical concentration for assembly. The *tubZ* gene is organized in an operon with *tubR*, which encodes a putative DNA-binding protein that regulates TubZ levels. TubZ was reported to be important for plasmid stability but up to now, it is not clear what specific role TubZ could play. At least four distinct types of very diverse TubZ-like sequences were found on different *Bacillus* plasmids, showing sequence identities below 25%.

Other sequences with similarities to both tubulin and FtsZ have been detected in some *Archaea*. They form a distinct group, cluster together on the phylogenetic tree and are referred to as FtsZ-like sequences. FtsZ-likes are not always encoded on plasmids and their function is up to now unknown (Larsen et al. 2007).

The sequence similarities within the GTP-binding and hydrolysis domains between TubZ, FtsZ-like, tubulin, and FtsZ suggest that they share a common ancestor. Larsen et al. (2007) hypothesize that TubZ defines a new prokaryotic cytoskeletal protein that may be an evolutionary link between the tubulin-based cytoskeletal proteins of Eukaryotes and the FtsZ-based cytokinetic ring of prokaryotes.

A.1.2 Homologs of actin: MreB and ParM

Actin filaments are two-stranded helical polymers of the protein actin. They appear as flexible structures and they are organized into a variety of linear bundles, two-dimensional networks and three-dimensional gels. Although the filaments are dispersed throughout the cell, they are most highly concentrated in the cortex, just beneath the plasma membrane. Thus, actin is the major protein which determines the eukaryotic cell shape (Alberts et al. 2002).

Bacterial actin homologs can be divided into two main classes, MreB/Mbl and ParM that differ in their sequences and polymerization dynamics. The MreB/Mbl class is always encoded in the bacterial chromosome and regulates a wide array of cellular functions. *parM* is always carried on extrachromosomal plasmids and is dedicated to the proper segregation of those plasmids. The cell division protein FtsA is also and ancestral homolog of actin (A.2.1).

MreB homologs of *Escherichia coli*, *Bacillus subtilis* and *Caulobacter crescentus* all form a helical structure that extends from pole to pole. When MreB is perturbed, cells lose their characteristic shapes and round up (Gitai et al. 2004), polar proteins become mislocalized (Gitai et al. 2004; Nilsen et al. 2005; Shih et al. 2005) and chromosome segregation is impaired (Gitai et al. 2005). MreB thus appears to function as a key integrator of spatial and temporal information in bacterial cells. The structure of MreB closely resembles that of actin despite their low level of sequence similarity (van den Ent et al. 2001). MreB self assembles

into linear filaments in the presence of ATP or GTP, which is thought to be hydrolyzed after monomer polymerization. The MreB filaments have been observed to bundle into a rigid structure (Esue et al. 2006).

ParM is the main representative of the second class of bacterial actin homologs. Together with ParR and the *parS* locus, the ParM system is the simplest and best characterized system for segregation of bacterial DNA. A ParM filament, bound to two copies of a plasmid, acts as a tension rod to push the plasmids apart – one to each of the two extreme cell poles (Moller-Jensen et al. 2003). Though the structure of ParM is similar to that of actin (Moller-Jensen et al. 2002), the kinetics of its polymerization are more similar to that of tubulin, as it exhibits dynamic instability (Garner et al. 2004).

The evolutionary relationships of the homologs MreB, ParM and actin are unclear.

A.1.3 Crescentin: A bacterial protein similar to eukaryotic Intermediate-Filaments

Intermediate filament (IF) proteins comprise the third major class of eukaryotic cytoskeletal proteins. Until the identification of the IF-like protein crescentin, IF proteins were thought to be unique to animal cells (reviewed in Shih and Rothfield 2006). In Eukaryotes they line the inner face of the nuclear envelope, forming a protective cage for the cells DNA. In the cytosol they are twisted into strong cables that can hold epithelial cell sheets together, help neuronal cells to extend long and robust axons, or allow us to form tough appendages such as hair and fingernails (Alberts et al. 2002).

Both crescentin and animal IFs have four characteristic coiled-coil domains and can self-assemble *in vitro* in the absence of nucleotides or other cofactors, in contrast to tubulin and actin homologs.

Crescentin was identified during a screen for cell shape mutants in *Caulobacter crescentus*. It is responsible for the shape of the comma-shaped organism, being present as an extended filamentous structure along the concave side of the cell. Interestingly crescentin and also MreB cytoskeletal structures were both shown to be required for production of a comma-shaped cell. MreB is important for the longitudinal mode of cell growth that leads to a rod shape, whereas the role of crescentin is to impart the curvature to the rod-shaped cell (Ausmees et al. 2003).

A.1.4 Additional families of bacterial cytoskeletal proteins

With the discovery of bacterial homologs for each of the eukaryotic cytoskeletal families, the list of bacterial cytoskeletal types was thought to be complete. It is now clear, however, that bacteria have additional cytoskeletal families without clear eukaryotic counterparts.

The ParA/MinD superfamily of Walker A ATPases was the first to be characterized and consists of the subgroups MinD and ParA (reviewed in Gitai 2007). MinD proteins regulate cell division by regulating both the localization and timing of FtsZ assembly (A.2.2). ParA acts as the alternative partitioning system, (type I partitioning system) to the actin homolog ParM (type II partitioning system).

There were also reports of several other filamentous intracellular structures in prokaryotic organisms. However, the responsible proteins mainly remain to be elucidated and formation of polymeric filaments *in vitro* was usually not demonstrated. Some examples of these structures are the fibrillar structures in *Spiroplasma*, cytoplasmic filaments in *Treponema*, intracellular filaments in *Myxococcus* and filamentous structures in *Mycoplasma* (reviewed in Shih and Rothfield 2006).

A.2 Bacterial Cell Division

A.2.1 Formation of the Z ring and septal protein recruitment

In most bacteria division occurs symmetrically, across the middle of the cell, between the partitioned nucleoids. Advances in understanding the molecular details of bacterial cell division began with the visualization of *E. coli* FtsZ (filamenting temperature sensitive Z) in a ring structure (the Z ring) at the leading edge of the septum of a dividing bacterium (Bi and Lutkenhaus 1991). The FtsZ protein is nearly universally conserved within *Bacteria* and *Euryarchaea*. Also, mitochondria in protists and chloroplasts in photosynthetic Eukaryotes possess FtsZ (Osteryoung 2001). Immunofluorescence and GFP fusion proteins have been used for visualization of FtsZ and other proteins which are recruited to the divisome (Errington et al. 2003).

A number of events must occur before cells are able to separate. A septal apparatus must be formed at the site of division and the concerted activity of its components must generate sufficient force to constrict the cytoplasmic membrane against the osmotic pressure of the cytoplasm. Along with the invagination of the cytoplasmic membrane, synthesis of new murein (peptidoglycan) must occur to accompany this invagination. In Gram-negative bacteria, invagination of the outer membrane must follow (Dajkovic and Lutkenhaus 2006).

It was proposed, that there is a nucleation site on the cytoplasmic membrane which becomes activated and from which the Z ring forms bidirectional (Addinall and Lutkenhaus 1996). There are indications that FtsZ polymers are organized into helices that coalesce into the Z ring at the appropriate time in the cell cycle (Dajkovic and Lutkenhaus 2006). Although the Z ring is observed as a static structure in fluorescence microscopy, it was shown to be a very dynamic structure using fluorescence recovery after photo bleaching (FRAP) in living cells. The subunits of the ring are constantly exchanging with the pool of cytoplasmic FtsZ (Anderson et al. 2004). It was suggested that the Z ring consists of multiple short filaments, because a ring composed of a continuous polymer would be unable to turn over as fast as determined by FRAP.

An essential requirement for the formation of the Z ring is the attachment of FtsZ to the membrane (Figure 3a). In *E. coli*, two proteins can do this: FtsA and ZipA, both of which bind to the conserved sequence present in the carboxyl tail of FtsZ that is not involved in assembly (Ma and Margolin 1999). ZipA is a membrane protein with an N-terminal

transmembrane domain, a flexible linker and a carboxyl domain (Ohashi et al. 2002). FtsA is an ancestral homolog of actin possessing a C-terminal amphiphatic helix responsible for interaction with the cytoplasmic membrane (Pichoff and Lutkenhaus 2005). The conservation of FtsA in bacteria and the analyses of *zipA* mutants suggested FtsA being the principal factor responsible for the association of FtsZ with the membrane in most bacteria.

Once assembled (involving FtsA and ZipA), the Z ring recruits several other membrane-associated cell division proteins. These proteins seem to be recruited in a linear order, with FtsA and ZipA required for all the others to arrive (Figure 3b).

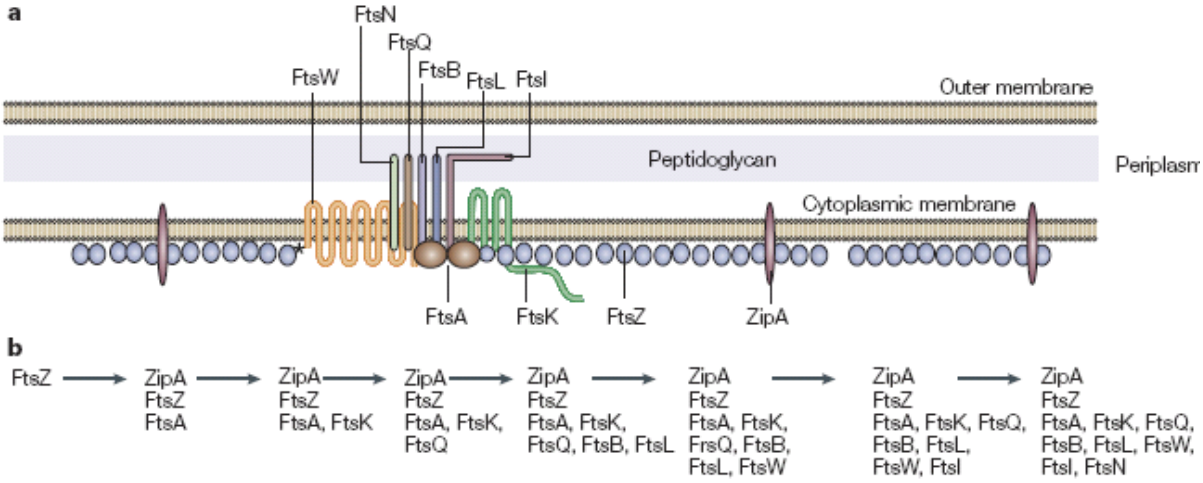

Figure 3. Model of the septum and order of protein recruitment (Margolin 2005)

a | A model of the *Escherichia coli* Z ring and its essential protein partners is shown in cross section. FtsZ is shown as a series of single protofilaments at the membrane, although the actual structure of FtsZ in the Z ring is unknown. Both ZipA and FtsA contact FtsZ as well as the membrane in *E. coli*. However, FtsZ contacts FtsW directly in *Mycobacterium tuberculosis* (asterisk), which lacks ZipA and FtsA. A single transmembrane subassembly associated with an FtsA dimer is shown, based on the low relative amounts of most of the integral membrane proteins that are essential for cell division. These membrane proteins include FtsQ, FtsB, FtsL, FtsI and FtsN, which are bitopic proteins that each have a single transmembrane and periplasmic domain, and FtsW and FtsK, which are polytopic proteins with multiple transmembrane and periplasmic domains. The network of protein–protein associations is implied by the proximity of the proteins in the diagram. Proteins implicated in stabilization of the ring structure are labelled below the cytoplasmic membrane lines, whereas proteins implicated in later functions in septum formation, such as septum synthesis, are labelled above the lines.

b | The dependency order of recruitment of essential cell-division proteins to the Z ring, as deduced from the requirement of a given protein for another's localization to the Z ring.

The mechanism by which the visible contraction of the Z ring at the leading edge of the inner membrane translates into the pinching of cell membranes and cytokinesis is not clear. One possibility is that the Z ring contracts passively ahead of invaginating septal wall growth, which, in turn, is promoted by proteins of the divisome such as FtsI, the septum-specific cell wall transpeptidase. Another hypothesis suggests that the rapid net loss of FtsZ monomers, which are tethered to the membrane by other proteins of the machine, excerts a pinching force on the membrane (Margolin 2005).

A.2.2 Spatial regulation of the Z ring

The position of the Z ring is determined by gradients of negative regulators of Z ring assembly, which have a minimum at midcell. A variety of negative regulators can be used by a bacterium and different bacteria use the same or different regulators (Lutkenhaus 2007). Two of these negative regulators, MinC and MipZ, promote the disassembly of FtsZ polymers. In *E. coli* a gradient of MinC is formed on the membrane by MinD and MinE, which oscillate from one cell pole to the other (Hu et al. 1999; Raskin and de Boer 1999). In its ATP-bound form, MinD associates with the membrane, activates and recruits MinC to the membrane (Hu et al. 2003). Thus, MinC is only an efficient inhibitor in regions where it is attached to the membrane by MinD. MinE imparts topological specificity to the MinCD inhibitor by inducing it to oscillate between the ends of the cell (Hu and Lutkenhaus 2001). Following, FtsZ assembly is inhibited most at the cell poles and least at midcell. This causes an FtsZ disassembly wave to oscillate from pole to pole (Thanedar and Margolin 2004).

A.2.3 Organization of cell division genes in the division and cell wall (*dcw*) gene cluster

Generally, *ftsZ* is located in a cluster of genes involved in cell division and in the synthesis of peptidoglycan precursors (Ayala et al. 1994; Vicente et al. 2004). A prominent feature of this division and cell wall (*dcw*) cluster is that it is conserved in many bacterial genomes over a broad taxonomic range (Nikolaichik and Donachie 2000; Tamames 2001; Vicente et al. 1998). Further, a strong relationship between the organization/conservation of the *dcw* cluster and the morphology of the cells was detected. In contrast to cocci, the majority of rod-shaped and filamentous bacteria were found to possess a conserved *dcw* cluster (Tamames 2001). The correlation suggests that cotranslational assembly of some *dcw* cluster gene products links gene order with cell morphology; this mechanism was referred to as genomic channeling

(Mingorance et al. 2004; Mingorance and Tamames 2004). As the last common ancestor of extant bacteria was suggested to have a rod-shape (Koch 2003), it was inferred that it also had a large *dcw* cluster (similar to that of *E. coli*) which has been maintained in some lineages and has been reduced in others (Nikolaichik and Donachie 2000). Some examples of *dcw* gene cluster organization are illustrated in Figure 4.

The *dcw* cluster comprises 16 genes in *E. coli* (Figure 4). The first two genes at the 5-prime terminus (*mraZW*) are coding for proteins with unknown functions (Carrion et al. 1999). The *mur* genes together with *mraY* and *ddl* are essential and involved in the synthesis of murein (peptidoglycan) precursors (van Heijenoort 1996). In addition, the *dcw* cluster comprises six other *fts* genes, which are essential for cell division; their products have been shown to localize at the division site during septation (Margolin 2004).

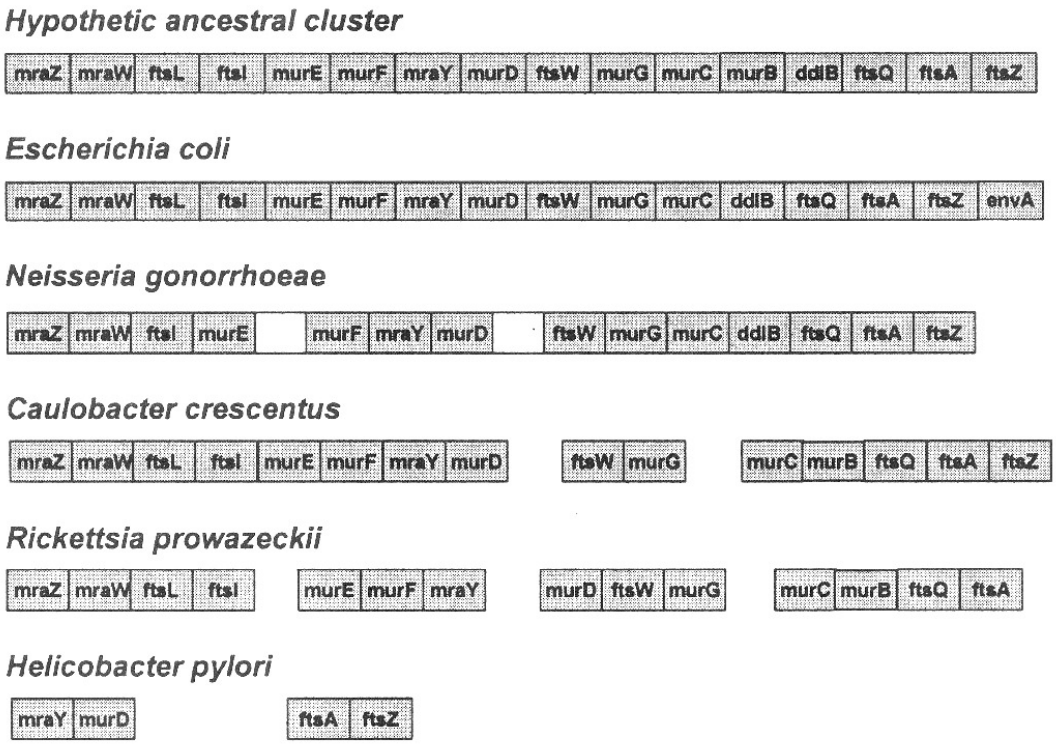

Figure 4. *dcw* gene cluster in different bacteria (Mingorance and Tamames 2004)

Schematic representation of the *dcw* cluster in several bacterial genomes. Adjacent boxes represent contiguous genes. White boxes correspond to genes that are not known to be related with cell division or cell wall synthesis. Groups of genes separated by spaces represent groups that are separated in the genome. Isolated genes are not represented but most are present in all the genomes.

A.3 The Bacterial Phylum *Verrucomicrobia*

A.3.1 Tubulins in *Verrucomicrobia*

Certainly, the most prominent peculiarity of the bacterial phylum *Verrucomicrobia* is the report of tubulins, a typical eukaryotic feature, in different representatives. Genes with sequence similarities to eukaryotic tubulin have been detected in the genus *Prosthecobacter* (Jenkins et al. 2002). Microtubule-like structures have been reported in Epixenosomes, bacterial symbionts of a ciliate (Rosati et al. 1993).

A.3.1.1 Microtubule-like structures in Epixenosomes

Epixenosomes are episymbionts of the ciliate *Euplotidium* (Ciliophora) and were investigated as symbionts of the species *E. arenarium* and *E. itoi* (Rosati 1999; Verni and Rosati 1990). In 2000, Petroni and co-workers resolved the relationship of Epixenosomes to representatives of the bacterial phylum *Verrucomicrobia* using comparative sequence analyses of 16S rRNA genes and *in situ* hybridization (Petroni et al. 2000).

Epixenosomes have a typical localization in a well defined cortical band on the host dorsal surface (Figure 5a). The life cycle of Epixenosomes consists of two main stages. During stage I cells are spherical, have a simple bacteria-like cell organization and are able to divide by direct binary fission (Figure 5b). During transformation of stage I to stage II, Epixenosomes gradually acquire a more complex structure (Rosati et al. 1993; Verni and Rosati 1990). Stage II Epixenosomes are larger and egg shaped and show the following intriguing features (Figure 5c): 1) an electrondense, dome-shaped zone under the cell membranes in the upper region of the cell body, containing DNA and basic proteins (like eukaryotic chromatin); 2) a basket built up of bundles of regularly arranged tubules, which are remarkably similar to eukaryotic microtubules concerning the ultrastructure and the sensitivity to nocodazole (interferes with the polymerization of MTs) and low temperature; 3) a sophisticated extrusive apparatus (Petroni et al. 2000). This apparatus appears as a ribbon rolled up around a central core and immersed in a complex protein matrix. The ribbon is ejected after activation of the adenylate cyclase-cAMP system by membrane receptors. During ejection, the extrusive apparatus unrolls from the inside and forms an approx. 40 µm long, hollow tube (Figure 5e). The head of the ribbon consists of the apical portion of the Epixenosome (Figure 5d), i.e. a portion of the cytoplasm along with the densely packed genomic material (Rosati et al. 1993). By this

ejection mechanism, Epixenosomes can defend their host from predators, such as e.g. *Litonotus* (Rosati et al. 1999).

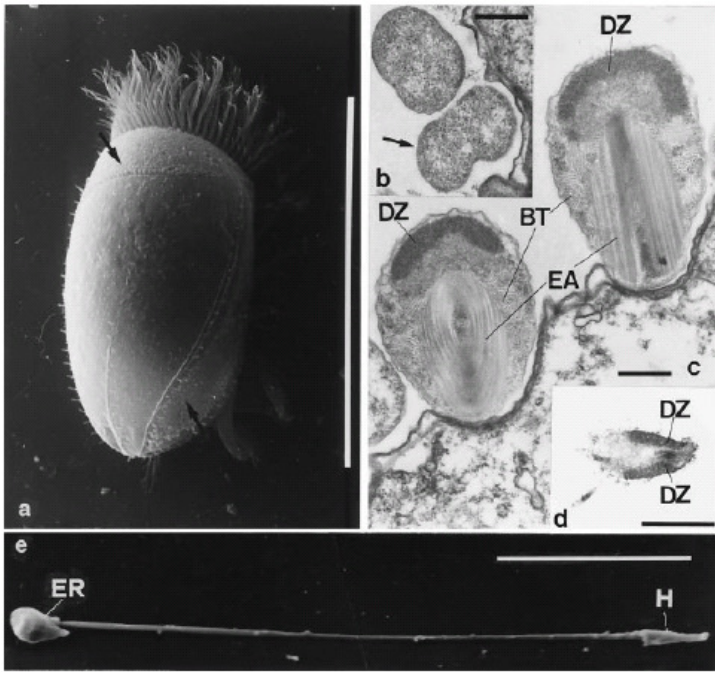

Figure 5. Epixenosomes and their host (Petroni et al. 2000)

a | Dorsal view of *Euplotidium itoi* at scanning electron microscope. Arrows indicate Epixenosomes in the cortical band. (Bar = 100 µm.) **b** | Sectioned stage I Epixenosomes. Arrow indicates the dividing one. **c** | Stage II Epixenosomes sectioned at different levels. DZ, Apical (dome-shaped) chromatin-like zone. EA, Extrusive apparatus. BT, Microtubule-like elements forming a "basket" around EA. **d** | Section through the head of an ejected epixenosome. (Bars = 1 µm.) **e** | The tube at the end of ejection. ER, Epixenosome remnant. H, Head. (Bar = 10 µm.)

A.3.1.2 Tubulin genes in the genus *Prosthecobacter*

The genus *Prosthecobacter* was named based on the morphology of its representatives, which produce prosthecae (Staley et al. 1976). Prosthecae are narrow extensions of the cell wall that contain cytoplasm (Staley 1968). They greatly increase the surface area of cells and thus it is proposed that these structures confer several advantages to aerobic, heterotrophic bacteria. These advantages include enhanced respiration and nutrient uptake (Schmidt 1971), as well as decreased sedimentation in aquatic environments (Poindexter 1978; Stove and Stanier 1962). In addition holdfasts on the tips of some prosthecae allow bacteria to attach to solid substrates in favorable local environments (Merker and Smit 1988).

The most widely known prosthecate bacteria are the caulobacters. In 1935, Henrici and Johnson reported that stalked bacteria were an important component of the bacterial periphyton of lakes. The authors had submerged glass slides in lakes. One of the more numerous forms they observed attached to the slides were unicellular organisms with a single polar stalk. They proposed the genus *Caulobacter* for these bacteria and noted that there were two morphologically distinct types. One was of vibrioid to rod-shaped stalked cells which, upon division, produced non-stalked daughter cells they surmised to be motile. The other type, referred to by them as the “fusiform type” because of the shape of the organism, was inferred to be immotile because the daughter cells were frequently attached to the glass slides at the time they separated from the mother cell (Figure 6) (Henrici and Johnson 1935).

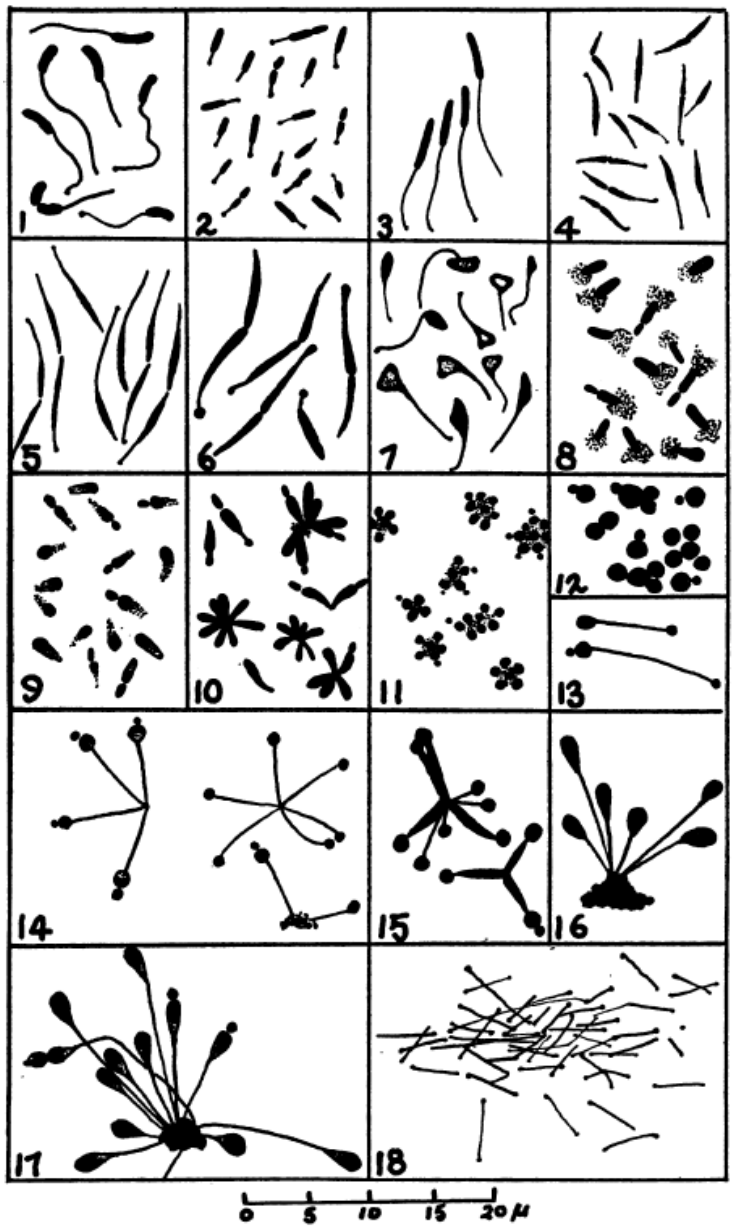

Figure 6. Stalked bacteria from Lake Alexander (Henrici and Johnson 1935).

Figure shows drawings of different types of stalked bacteria which were observed attached to glass slides, which were submerged in Lake Alexander. Panel 4-6 was referred to as the “fusiform type”, which was later placed into the genus *Prosthecobacter* (De Bont et al. 1970; Staley et al. 1976).

The first report of a pure culture of the "fusiform type" was published by DeBont et al. (1970), who obtained a strain from enrichments from a lake in Michigan. Subsequently three other strains were isolated and all were placed in a single genus and species, *Prosthecobacter fusiformis* (Staley et al. 1976). Whereas most well-known prosthecate, heterotrophic bacteria belong to the alpha-2 subgroup of the *Proteobacteria* (Stackebrandt et al. 1988), Hedlund et al. (1996) placed *Prosthecobacter* in the phylum *Verrucomicrobia* and later described the four current species of the genus, as there are *P. debontii* (Figure 7), *P. vanneervenii*, *P. fusiformis* and *P. dejongeii* (Hedlund et al. 1997).

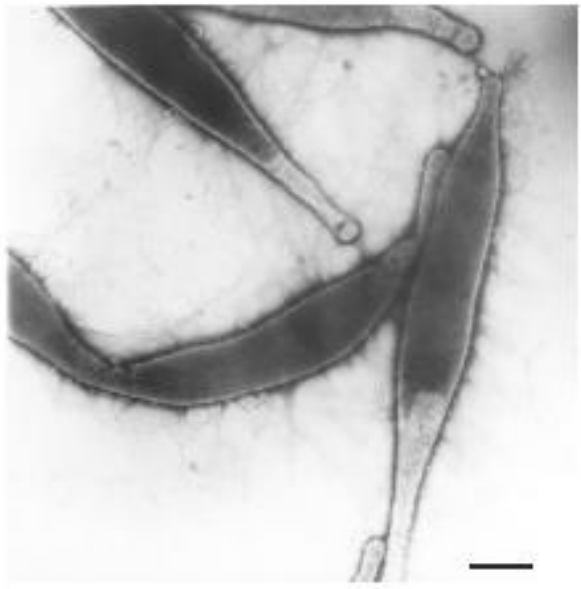

Figure 7. *Prosthecobacter debontii*
Electron micrograph image of *P. debontii* (negatively stained with phosphotungstic acid; 10,000x). Cells show a prostheca with a bulbous tip (holdfast) and numerous fimbriae. Bar represents 0.5 µm.

In 2002, Jenkins and co-workers reported genes with similarities to eukaryotic tubulin in the genus *Prosthecobacter*. Initially, two genes, referred to as *btubA* and *btubB*, were detected in the still incomplete genome sequence data (95%) of *Prosthecobacter dejongeii*. The authors found *btubA* and *btubB* as adjacent loci on the genome, like eukaryotic alpha and beta tubulin (Vaughan et al. 2000). *btubA* and *btubB* genes could be also detected in the remaining species of the genus *Prosthecobacter* which are *P. vanneervenii*, *P. debontii* and *P. fusiformis*.
Phylogenetic, structural and functional investigations of the *Prosthecobacter* tubulins performed by Jenkins et al. (2002), Sontag et al. (2005), and Schlieper et al. (2005) were outlined in section A.1.1.2.

A.3.2 Taxonomy, phylogeny and ecology of *Verrucomicrobia*

Cultivation-independent surveys have shown that members of the phylum *Verrucomicrobia* are very diverse (Freitag and Prosser 2003; Haukka et al. 2005; Hugenholtz et al. 1998; O'Farrell and Janssen 1999). However, only a few members of the phylum have yet been cultivated. Verrucomicrobial 16S rRNA sequences of members were retrieved from various soils (Hackl et al. 2004; He et al. 2006), confirming their importance in these systems. It has been estimated that *Verrucomicrobia* comprise up to 10% of the total bacteria in soil. Very recently, the first relevant biogeochemical reaction of *Verrucomicrobia* was reported. Three studies isolated extremely acidophilic *Verrucomicrobia* which oxidize methane (Dunfield et al. 2007; Islam et al. 2008; Pol et al. 2007).

Members of the *Verrucomicrobia* phylum were also recently detected in bogs (Dedysh et al. 2006; Juottonen et al. 2005) and in a mesocosm simulating the flooding of an unplanted paddy soil (Noll et al. 2005). 16S rRNA sequences affiliated with *Verrucomicrobia* were also obtained from aquatic systems, including drinking water (Martiny et al. 2005), lake mesocosms (Haukka et al. 2005; Haukka et al. 2006), lakes (Eiler and Bertilsson 2004; Lindstrom et al. 2005), marine sediments (Polymenakou et al. 2005) and even from hot springs (Islam et al. 2008; Kanokratana et al. 2004) and volcano mudpots (Pol et al. 2007). Furthermore, *Verrucomicrobia* thrive in manmade ecosystems such as acid rock drainage (Okabayashi et al. 2005) and leachate from a municipal solid waste landfill (Huang et al. 2005) and in various reactor systems (Chouari et al. 2005; Kimura et al. 2003).

Interestingly, several *Verrucomicrobia* live in association with Eukaryotes. For example, *Verrucomicrobia* were isolated from the gut of the sea cucumber *Stichopus japonicus* (Sakai et al. 2003a), from sponges (Scheuermayer et al. 2006; Yoon et al. 2007b), from termite guts (Shinzato et al. 2005; Stevenson et al. 2004) and from the human intestine (Derrien et al. 2004; Wang et al. 2005), where they contribute to mucine degradation. Furthermore, *Verrucomicrobia* live as obligate endosymbionts in ectoparasitic nematodes of the genus *Xiphinema*, which feed on fruit crops (Vandekerckhove et al. 2002). Other symbiotic *Verrucomicrobia*, the Epixenosomes, were described above (A.3.1.1).

The phylum *Verrucomicrobia* has been historically divided into five subdivisions (Figure 8) based on 16S rRNA gene sequences (Hugenholtz et al. 1998). Subdivision 1 includes the freshwater species *Verrucomicrobium spinosum* (Schlesner 1987), four freshwater species of the genus *Prosthecobacter* (Hedlund et al. 1996; Hedlund et al. 1997), the humanintestinal species *Akkermansia muciniphila* (Derrien et al. 2004) and three sponge associated *Rubritalea* species (Scheuermayer et al. 2006; Yoon et al. 2007b). Isolates belonging to subdivision 2

(*Chthoniobacter flavus*) and subdivision 3 (Ellin514, Ellin5102 amongst others) were obtained in cultivation attempts using soil liquid serial dilutions in Peter H. Janssen's group (Joseph et al. 2003; Sangwan et al. 2004; Sangwan et al. 2005). There are several identified species in subdivision 4, the soil-borne species *Opitutus terrae* (Chin et al. 2001; Chin and Janssen 2002; Janssen et al. 1997), *Alterococcus agarolyticus* (Shieh and Jean 1998), isolated from a hot spring and 'Fucophilus fucoidanolyticus' (Sakai et al. 2003b). Very recently, further five species were described belonging to the genera *Pelagicoccus* (Yoon et al. 2007c; Yoon et al. 2007e), *Coralomargerita* (Yoon et al. 2007d), *Cerasicoccus* (Yoon et al. 2007a) and *Puniceicoccus* (Choo et al. 2007). They were all isolated from marine samples, namely seagrass, hard corals, sediment and seawater respectively. Another representative of subdivision 4 are the microtubule-like structure producing Epixenosomes [A.3.1.2; (Petroni et al. 2000)]. Subdivision 5 is only represented by 16S rRNA sequences of yet-uncultivated strains.

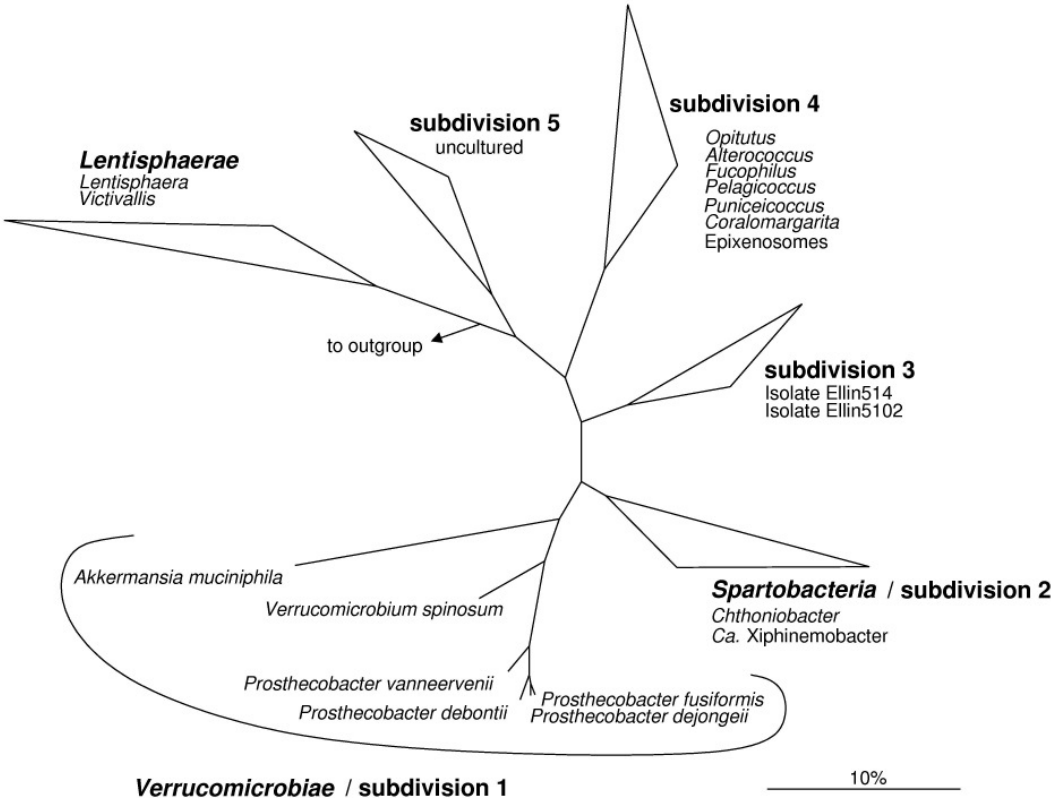

Figure 8. 16S rRNA tree showing the sister-phyla *Verrucomicrobia* and *Lentisphaerae*

Verrucomicrobia can be divided in five subdivisions with few cultured representatives. Selected genera with cultured or characterized members are assigned to each subdivision. Maximum likelihood tree was produced using an extended ARB database originally developed by Bauer (2005). Environmental sequences of subdivision 1 were removed after tree calculation. Bar, 10% estimated evolutionary distance.

In the past, there were several studies, addressing the phylogenetic relationships between *Verrucomicrobia* and other bacterial phyla, based on 16S rRNA, 23S rRNA or protein sequences (Cho et al. 2004; Griffiths and Gupta 2007; Hedlund et al. 1997; Jenkins and Fuerst 2001; Liesack et al. 1992; Strous et al. 2006; Teeling et al. 2004; Wagner and Horn 2006; Ward et al. 2000; Ward-Rainey et al. 1997; Weisburg et al. 1986).

The closest relatives of *Verrucomicrobia* were shown to be representatives of the phylum *Lentisphaerae* (Cho et al. 2004). The sister phylum comprises two cultivated species, *Lentisphaera araneosa* and *Vivtivallis vadensis*, isolated from seawater and a human faecal sample, respectively (Cho et al. 2004; Zoetendal et al. 2003). Studies about the relationships of *Verrucomicrobia* to other bacterial phyla were more controversial. Some studies detected the formation of a monophyletic cluster of *Verrucomicrobia* together with *Chlamydiae* and *Planctomycetes*, whereas other authors disagreed (Griffiths and Gupta 2007; Hugenholtz et al. 1998; Ward et al. 2000). Nevertheless, on the basis of 16S rRNA gene phylogeny, Wagner and Horn (2006) proposed a PVC (*Planctomycetes-Verrucomicrobia-Chlamydiae*) superphylum, comprising *Verrucomicrobia*, *Lentisphaerae*, *Chlamydiae*, *Planctomycetes* and two additional candidate phyla (Poribacteria and OP3), in which the sister phyla *Lentisphaerae* and *Verrucomicrobia* are more closely related to *Chlamydiae* than to *Planctomycetes*.

Interestingly, all genome projects on *Chlamydiae* and *Planctomycetes* representatives revealed that they are missing genes coding for the major bacterial cell division protein FtsZ (Glockner et al. 2003; Horn et al. 2004; Jenkins et al. 2002; Read et al. 2000; Strous et al. 2006). This is also the case for the unfinished genome data (95%) of *Prosthecobacter dejongeii* (Jenkins et al. 2002). Nothing is known about how cell division is performed in these organisms. In contrast to all other members of the PVC superphylum, the *Chlamydiae* have been studied extensively for decades. *Chlamydiae* are obligate intracellular bacteria characterized by a unique developmental cycle and, as such, are well-known pathogens of animals and humans. They were also found to be associated with amoebae, arthropods, crustaceans or fish (Collingro et al. 2005; Draghi et al. 2004; Kostanjsek et al. 2004).

In contrast to *Chlamydiae*, *Planctomycetes* have more diverse lifestyles, ranging from associations with Eukaryotes to free-living species in very different environments. Some peculiarities of *Planctomycetes* are the ability of anaerobic ammonium oxidation in anammox bacteria (Op den Camp et al. 2006) and a unique cell plan in all *Planctomycetes*, in which the cell cytoplasm is divided into compartments by one or more membranes, including a major cell compartment containing the nucleoid (Fuerst 2005).

A.4 Aims of This Study

The apparent absence of *ftsZ* in the incomplete genome sequence data (90 %) of *Prosthecobacter dejongeii* and the concomitant presence of bacterial tubulin genes suggested a vicarious takeover of the FtsZ function through the novel tubulins. Related to this hypothesis, the aim of this study was to answer the following questions:

- Are there bacterial tubulin genes in other *Verrucomicrobia* representatives?
- What is the major structural cell division protein in the genus *Prosthecobacter*?
- What is the major cell division protein in other subdivisions of *Verrucomicrobia*?
- Does 23S rRNA phylogeny support a PVC superphylum?
- How are cell division and cell wall synthesis genes organized in the genomes?
- How did the *dcw* cluster evolve in *Verrucomicrobia* and in its related phyla?
- How are bacterial tubulin genes organized in the genome?
- Do bacterial tubulin genes have a function in *Prosthecobacter*?
- Where are the products of bacterial tubulin genes localized in *Prosthecobacter* cells?
- What is the origin of bacterial tubulin genes?

To answer these questions, a variety of standard molecular biological and microbiological techniques was applied. However, for the characterization of the genomic environment of genomic sequences which were detected by PCR using primers targeting conserved protein domains, a novel, highly efficient gene walking technique had to be developed.

B RESULTS AND DISCUSSION

B.1 Development of the Two-Step Gene Walking Method

➲ Appendix B

A fundamental part of this study was the detection of protein coding genes by PCR with degenerate primers (Rose et al. 1998; Rose et al. 2003). Subsequent gene walking had to be applied to obtain full sequences and to elucidate the genomic organization of the characterized genes. For this reason, the straightforward Two-Step Gene Walking method was developed.

The method is based on randomly primed polymerase chain reaction (PCR); it is illustrated in Appendix B, Figure 1 (p. 123). The method presents a simple workflow, which comprises only two major steps - a Walking-PCR with a single specific outward pointing primer (step 1) and the direct sequencing of its product using a nested specific primer (step 2). In the first 30 cycles of the Walking-PCR (Appendix B, Table 1, p. 123), the primer binds at stringent conditions and singlestranded DNA is specifically amplified. One subsequent cycle at low annealing temperature allows unspecific binding of the primer at different sites on the singlestranded DNA as reverse primer. Thus, doublestranded DNA with the primer sequence incorporated at each 5-prime end is produced. Subsequently, the doublestranded DNA is exponentially amplified in thirty cycles at stringent conditions. The resulting PCR product is a heterogeneous mixture of specific fragments differing in length, what is due to different drop off sites of the polymerase and due to different sites of primer binding during the unspecific cycle. The PCR product is sequenced directly by using a specific nested primer.

Using Two-Step Gene Walking, over 85% of the performed attempts were successful. In the other cases it was possible to keep on walking replacing either the PCR primer, the sequencing primer or both of them. The method was successfully applied to DNA extractions of various pure cultures (*Verrucomicrobia*, *Gammaproteobacteria*) and to two different complex non-pure culture systems. One was the epixenosome system (Rosati 1999), including Epixenosomes, the host organism *Euplotidium*, as well as several free-living bacteria that are present in the host culture and some of the algae used as food source for the host. The second non-pure culture sample was the *Caedibacter caryophilus* system (Schmidt et al. 1988), including *C. caryophilus*, the host *Paramecium caudatum*, food bacteria and a diverse community of free-living bacteria.

In comparison to other gene walking techniques, Two-Step Gene Walking has striking advantages, which are detailed below. Two-Step Gene Walking is the first gene walking technique, which was shown to work in complex non-pure culture samples. The method was

always processed using the standardized protocol without any optimization. The required minimal amount of DNA (50 ng) makes it also feasible to perform gene walking in organisms for which a limited amount of biomass is available, such as slowly growing or even uncultivable organisms. The two-step workflow can be processed within one day. The derived sequence reads show very high quality up to 900 bp (Appendix B, Figure 2, p. 124).

Two-Step Gene Walking overcomes the major disadvantages of other gene walking techniques. Inverse PCR (Huang 1994; Kohda and Taira 2000; Ochman et al. 1988; Triglia et al. 1988) and ligation-mediated PCR (Diatchenko et al. 1996; Drancourt et al. 2001; Kilstrup and Kristiansen 2000; Lin et al. 2005; Nthangeni et al. 2005; Rishi et al. 2004; Rosenthal and Jones 1990; Shyamala and Ames 1989; Siebert et al. 1995; Yuanxin et al. 2003) require time-consuming and labor-intensive enzymatic modifications of the target DNA, a relatively high amount of target DNA (several µg) and other DNA modifying enzymes in addition to DNA polymerase. The advantages of Two-Step Gene Walking compared to other randomly primed PCR methods (Addinall and Holland 2002; Antal et al. 2004; Caetano-Anolles 1993; Dominguez and Lopez-Larrea 1994; Gould et al. 2004; Hermann et al. 2000; Karlyshev et al. 2000; Liu and Whittier 1995; Malo et al. 1994; Min and Powell 1998; Mishra et al. 2002; Parker et al. 1991; Parks et al. 1991; Spector et al. 1999; Tan et al. 2005; Trueba and Johnson 1996) are the independence of (i) modified oligonucleotides, (ii) special walking primers, (iii) successive PCRs, (iv) cloning steps, (v) procedures to screen for specificity or to enrich specific fragments and (vi) other enzymatic modifications than the DNA polymerase. Some methods (Karlyshev et al. 2000; Spector et al. 1999) are restricted and optimized to only one walking step starting always from the same known sequence (e.g. an inserted transposon). In contrast, the newly developed method theoretically enables to completely characterize one DNA molecule using successive gene walking steps and primer walking on Walking-PCR products. Potential applications of Two-Step Gene Walking are (i) rapidly obtaining full gene sequences, finding the corresponding promoters and regulatory elements, (ii) characterization of complete operons starting from a small known DNA fragment, (iii) characterization of primer binding sequences, (iv) identification of transposon integration sites in known or unknown genomes during functional studies, and (v) gap closure in genome sequencing projects. The latter one could be also performed in a high-throughput scale due to the simple and standardized protocol of Two-Step Gene Walking.

B.2 23S rRNA Phylogeny of *Verrucomicrobia*: Inter- and Intraphylum Relationships

➲ Appendix C

In the past, there were several studies, addressing the phylogenetic relationships between *Planctomycetes*, *Chlamydiae* and *Verrucomicrobia* based on 16S rRNA (Cho et al. 2004; Hedlund et al. 1997; Liesack et al. 1992; Wagner and Horn 2006; Weisburg et al. 1986), 23S rRNA (Ward et al. 2000) or protein sequences (Griffiths and Gupta 2007; Jenkins and Fuerst 2001; Strous et al. 2006; Teeling et al. 2004; Ward-Rainey et al. 1997). In most of the cases a relationship of *Chlamydiae* and *Verrucomicrobia* could be detected, whereas the formation of a monophyletic cluster of all three lineages was controversial.

Despite some studies disagree (Griffiths and Gupta 2007; Hugenholtz et al. 1998; Ward et al. 2000), Wagner and Horn (2006) proposed the establishment of a new superphylum on the basis of 16S rRNA phylogeny. The PVC (*Planctomycetes-Verrucomicrobia-Chlamydiae*) superphylum comprises *Verrucomicrobia*, *Chlamydiae*, *Planctomycetes*, *Lentisphaerae* and two additional candidate phyla (Poribacteria and OP3).

The present study tested the hypothesis of the PVC superphylum using comparative sequence analyses of 23S rRNA genes. The 23S rRNA sequences of the following species were determined: *Prosthecobacter debontii*, *Prosthecobacter dejongeii*, *Prosthecobacter vanneervenii*, *Akkermansia muciniphila*, *Chthoniobacter flavus*, isolate Ellin514, and *Opitutus terrae*. Thus, all subdivisions with cultivable representatives were covered. For tree calculations all available 23S rRNA sequences of *Verrucomicrobia*, *Lentisphaerae*, *Chlamydiae*, *Planctomycetes* and selected species of the major bacterial groups, some *Archaea* and some 28S rRNA sequences of *Eukarya* were included. Trees were calculated using different treeing methods in combination with different minimum similarity filters.

All trees showed a stable monophyly of all included members of the PVC superphylum (Appendix C, Figure 4A, p.139). The study by Ward *et al.* (2000) also using 23S rRNA as a phylogenetic marker could not detect a significant relationship of the three phyla. However, their dataset was rather limited, *Planctomycetes* were represented by 13 sequences (16 representatives in this study), *Chlamydiae* by one sequence (26 representatives in this study), *Verrucomicrobia* by three sequences (11 representatives in this study) and *Lentisphaerae* were completely lacking (2 representatives in this study). The much more comprehensive phylogenetic analyses in the present study showed a very good agreement between trees derived from 23S and 16S rRNAs. Therefore, these analyses support the suggestion of a PVC

superphylum, in which the phyla are branching in the order *Planctomycetes*, *Chlamydiae*, *Lentisphaerae* and *Verrucomicrobia* (Wagner and Horn 2006).

The treeing pattern within *Verrucomicrobia* was also stable. All subdivisions with cultivable representatives (1-4) were recovered monophyletically and match the 16S rRNA phylogeny (Appendix C, Figure 4A, p.139).

B.3 Detection of Tubulin Genes in *Verrucomicrobia*

➲ Appendix A / D

The absence of *ftsZ* in the unfinished genome sequence of *P. dejongeii* [95% coverage (Jenkins et al. 2002; Staley et al. 2005)], the detection of tubulin genes in *Prosthecobacter* (Jenkins et al. 2002) and the report of microtubule-like structures in Epixenosomes (Petroni et al. 2000) suggested the possibility of a vicarious takeover of the FtsZ function through the bacterial tubulins in *Verrucomicrobia*.

To evaluate this hypothesis, the following verrucomicrobial strains were screened for tubulin-like genes using PCR: *Verrucomicrobium spinosum* and *Akkermansia muciniphila* (both subdivision 1), *Chthoniobacter flavus* (subdivision 2), isolate Ellin514 (subdivision 3), *Opitutus terrae*, *Opitutus* sp. strain VeGlc2 and Epixenosomes (all subdivision 4). Attempts to detect tubulin genes were performed using PCR with a high number of different combinations of primers, designed according to different strategies [consensus primers, degenerate primers, CODEHOP (Rose et al. 1998; Rose et al. 2003)]. For a complete list of primers see Appendix A, Supplementary Material (p. 112). Using some combinations of these primers in PCR, it was possible to amplify simultaneously eukaryotic alpha and beta tubulin as well as *Prosthecobacter* tubulins. However, no tubulin genes could be identified in any of the investigated verrucomicrobial strains.

Because of host and culture derived contaminations, a subtractive PCR approach using subtractive hybridization in microplates (Zwirglmaier et al. 2001) was performed on the Epixenosome-host system. Alpha, beta and gamma tubulin genes from the ciliate host (M. Hartmann and G. Petroni, unpublished data) were used as subtractor-DNA. In no case, genes similar to *Prosthecobacter* tubulins were found.

The absence of tubulin genes in *Verrucomicrobia* (except *Prosthecobacter*) was further supported by a later BLAST analysis of unfinished genome project data which became very recently available for *Verrucomicrobium spinosum*, *Akkermansia muciniphila* (both subdivision 1), isolate TAV2 (subdivision 4) and isolate DG1235 (subdivision 4).

B.4 Cell Division in the PVC Superphylum

B.4.1 Coexistence of tubulins and *ftsZ* in *Prosthecobacter*

➲ Appendix A

The restriction of bacterial tubulin genes just to members of the genus *Prosthecobacter* suggested them not being the major cell division protein in *Verrucomicrobia*. Despite the apparent absence of *ftsZ* in the *Prosthecobacter dejongeii* genome sequence data (Jenkins et al. 2002; Staley et al. 2005), it was possible to detect a sequence coding for FtsZ in that organism using PCR and Consensus Degenerate Hybrid Oligonucleotide Primers [CODEHOP; (Rose et al. 1998; Rose et al. 2003)]. Moreover, *ftsZ* was also identified in *Prosthecobacter debontii* and *Prosthecobacter vanneervenii*. The retrieved sequences were used to detect an open reading frame with protein sequence similarities to FtsZ also in the incomplete sequence data of the *Verrucomicrobium spinosum* (closest cultivable relative of *Prosthecobacter*) genome project.

Vaughan et al. (2004) analyzed publicly available FtsZ sequences and defined criteria for functional FtsZ. *Prosthecobacter* and *Verrucomicrobium* FtsZs exhibit most of these typical FtsZ features and some peculiar characteristics. Like typical bacterial FtsZ, they can be divided into four domains (N-terminus, core, spacer, and C-terminus). Six out of six characteristic motifs of FtsZ were identified by PRINTS fingerprint scan (Attwood et al. 2003) (Appendix A, Table 2, p.102), whereas the *E*-values were rather low in comparison to *E. coli* FtsZ. The tubulin signature motif [S/A/G]GGTG[S/A/T]G is always present and perfectly conserved (Appendix A, Supplementary Figure S1, p. 106). Amino acids which contact guanosine diphosphate (Lowe and Amos 1998; Nogales et al. 1998) are conserved or conservatively exchanged with few exceptions (Appendix A, Supplementary Figure S2, p. 107-108).

FtsZ residues that have been demonstrated to be involved in protein interactions with FtsA (Haney et al. 2001) or which have been shown to be important for the protein conformation (Mosyak et al. 2000) are located in a nonapeptide in the C-terminal domain of FtsZ. This feature is considered typical of a functionally active FtsZ (Vaughan et al. 2004) and is also present and conserved in *Verrucomicrobia* (Appendix A; Supplementary Figure S3, p. 109).

Phylogenetic sequence analyses of the FtsZ core domains were performed using an ARB database, comprising *Prosthecobacter* and *Verrucomicrobium* FtsZs and representatives of

the other major bacterial and archaeal groups. The calculated trees showed a steady monophyly of verrucomicrobial FtsZs (Appendix A, Figure 1, p. 103), thus supporting the existence of specific evolutionary constraints for these genes. The branch length of verrucomicrobial FtsZs was rather long, compared to the other major bacterial groups. In most cases the phylogenetic information retained by FtsZ was not sufficient to resolve relationships above the phylum level, as it was also shown in earlier studies (Faguy and Doolittle 1998; Gilson and Beech 2001).

Using Two-Step Gene Walking (Appendix B), the genomic environment of *P. debontii* and *P. vanneervenii* FtsZ was additionally investigated. It shows upstream the presence of an open reading frame similar to the bacterial cell division gene *ftsA*.

In comparison to other bacterial FtsZs, the fingerprintscan, the amino acid sequence alignment and comparative sequence analyses detected sequence peculiarities and a higher divergence of *Prosthecobacter* FtsZs. Nevertheless, the following properties indicate that the identified *ftsZ* genes are functionally active: 1) all characteristics typical of functional FtsZ are present; 2) verrucomicrobial FtsZs are evolutionary constrained and 3) another typical bacterial cell division gene (*ftsA*) is present in *Prosthecobacter*. The simultaneous presence of apparently functional FtsZ in *Prosthecobacter* spp. together with the restricted occurrence of tubulin genes in the genus *Prosthecobacter* but not in other *Verrucomicrobia* indicates that FtsZ and not tubulin could be the major protein involved in cell division in *Verrucomicrobia*. However, the phylum *Verrucomicrobia* (Hedlund et al. 1997; Hugenholtz et al. 1998) comprises five subdivisions consisting of very heterogeneous, free-living and symbiotic species with only very few cultivable representatives. Therefore, a further aim of this study was to elucidate the cell division mechanism in all verrucomicrobial subdivisions with cultivable representatives to set up a hypothesis for the complete phylum.

B.4.2 FtsZ based cell division mechanism in *Verrucomicrobia* and *Lentisphaerae*

➲ Appendix C

CODEHOP primers were used to detect *ftsZ* in *Prosthecobacter*. Attempts using a high number of different combinations of these primers in PCR failed to detect *ftsZ* in other *Verrucomicrobia*. Therefore, CODEHOP primers were designed to target conserved domains of the D-Ala:D-Ala Ligase gene (*ddl*), which is usually located upstream of the *ftsQAZ* cluster. Despite the overall higher sequence divergence of the *ddl* gene in comparison to *ftsZ*, highly conserved amino acid motifs could be identified which were suitable for primer design. Genes with similarities to *ddl* could be sequenced in strains of all subdivisions with cultivable representatives: Prosthecobacters (subdivision 1), *Akkermansia muciniphila* (subidivision 1), *Chthoniobacter flavus* (subdivision 2), isolate Ellin514 (subdivision 3) and *Opitutus terrae* (subdivision 4). For these organisms, either specific primers targeting *ddl* and *ftsZ* were used in PCR to specifically amplify and subsequently sequence the intervening region comprising *ftsQA*, or Two-Step Gene Walking was applied to characterize the genomic environment of the *ddl* or *ftsZ* gene, respectively.

Very recently, preliminary genome sequence data of other *Verrucomicrobia* and *Lentisphaerae* (closest relatives of *Verrucomicrobia*) became available: isolate DG1235 (*Verrucomicrobia*, subdivision 4), isolate TAV2 (*Verrucomicrobia*, subdivision 4), *Lentisphaera araneosa* (*Lentisphaerae*) and *Victivallis vadensis* (*Lentisphaerae*). Therefore, the Integrated Microbial Genomes (IMG) System (Markowitz et al. 2006) and BLASTP (Altschul et al. 1997) were used to screen for the presence of *ftsZ*, *ddl* and other *dcw* cluster genes in these organisms.

In summary, sequences with similarities to *ddl* and *ftsZ*, respectively, could be detected in all verrucomicrobial subdivisions with cultivable representatives. With the exception of *Prosthecobacter vanneervenii* and *Akkermansia muciniphila*, the intervening region of *ddl-ftsZ* (generally *ftsQA*) was shown to be conserved in *Verrucomicrobia* (Appendix C, Figure 1, p. 136). *ddl*, *ftsQ*, *ftsA* and *ftsZ* were also detected in *Lentisphaera araneosa*, but the single genes are organized isolated at different locations on the genome. In the incomplete *Victivallis vadensis* (*Lentisphaerae*) genome sequence data, only the *ddl* gene could be identified (Appendix C, Figure 1, p. 136).

The detected sequence similarities were verified by searching for conserved domains of the Pfam library (Bateman et al. 2004). In general, verrucomicrobial Ddl, FtsQ, FtsA and FtsZ

sequences showed good matches to the respective Pfam domains (Appendix C, Table 1, p. 138). However, the *L. araneosa* FtsZ was different because it did not match the tubulin/FtsZ-family-GTPase Pfam domain, nor did it the FtsZ PROSITE pattern, BLOCKS database or PRINTS motif. However, the sequence was initially detected using BLASTP and shows similarities to the conserved domain FtsZ [cd02191; (Marchler-Bauer et al. 2005)] and to FtsZ from *P. debontii* with *E*-values of $5e^{-06}$ and $6e^{-04}$, respectively.

All FtsZ sequences were additionally searched for the tubulin signature motif [S/A/G]GGTG[S/A/T]G, which is present and perfectly conserved in all investigated verrucomicrobial FtsZs. Again, the putative *L. araneosa* FtsZ homolog does not show the tubulin signature motif. A similar observation was made during the analysis of amino acid positions which contact GDP (Lowe and Amos 1998; Nogales et al. 1998). All important positions are conserved among *Verrucomicrobia*, even if the new FtsZ sequences of subdivision 2 – 4 are added. *L. araneosa* FtsZ shows no conservation in the majority of these amino acid residues.

The transcription of *ddl*, *ftsQ*, *ftsA* and *ftsZ* was verified in *P. dejongeii* and *P. vanneervenii* using reverse transcription of mRNA. Total RNA was isolated from *Prosthecobacter* cultures and was subsequently incubated with DNAse. Total RNA was reversely transcribed using random hexamers. The cDNA was then used as template in PCR with gene specific primers. Specific amplification from cDNA was detected for all investigated genes in both species [Appendix C, Figure 3, p. 137; Eckl C. (2008)].

FtsZ and Ddl sequences were used for phylogenetic tree reconstructions. The FtsZ ARB database (B.4.1) was extended with the newly obtained verrucomicrobial and *Lentisphaera* sequences. Attempts to calculate global trees could recover a relationship of *L. araneosa* FtsZ and verrucomicrobial FtsZs in most of the cases. All calculated trees showed stable tree topologies, with stable monophyletic groups representing the different subdivisions. In all trees, the branching pattern of the FtsZ sequences of the different verrucomicrobial subdivisions (1, 2, 3 and 4) is consistent with the pattern obtained in rRNA phylogeny [Appendix C, Figure 4A, p. 139; (Cho et al. 2004; Hugenholtz et al. 1998; Sangwan et al. 2004; Wagner and Horn 2006)]. One representative maximum likelihood tree is shown in Appendix C, Figure 4B (p. 139).

A Ddl ARB database was established by importing the Ddl protein sequences obtained in this study and Ddl sequences of other bacterial groups from the EMBL nucleotide database. Initial attempts to calculate global trees using different methods and filters showed that in most of the cases the major bacterial groups could be recovered, whereas the relationships between

the major bacterial groups were unstable, as is also the case for FtsZ phylogeny (B.4.1). Notably, all global Ddl trees showed a stable relationship of "*Candidatus* Protochlamydia amoebophila" (*Chlamydiae*) Ddl with *Physcomitrella patens* (Viridiplantae) Ddl (nuclear encoded), indicating an inter-kingdom horizontal gene transfer event.

All calculated trees showed stable monophyletic groups representing the two sister-phyla *Verrucomicrobia* and *Lentisphaerae*. Within the *Verrucomicrobia*, each subdivision (1, 2, 3 and 4) was recovered monophyletically. One representative maximum likelihood tree is shown in Appendix C, Figure 4C (p. 139). The branching pattern matches the pattern obtained using comparative sequence analyses of 16S rRNA genes (Cho et al. 2004; Hugenholtz et al. 1998; Sangwan et al. 2004; Wagner and Horn 2006), 23S rRNA genes (AppendixC, Figure 4A, p. 139) and FtsZs (Appendix C, Figure 4B, p. 139).

This consistency suggests the absence of horizontal gene transfer influence on FtsZ and Ddl evolution in *Verrucomicrobia* and strongly indicates that the genes are conserved due to functional constraints. This applies also to the isolated genes of the non-conserved *dcw* clusters (in terms of gene order) of *Prosthecobacter vanneervenii* and *A. muciniphila* (Appendix C, Figure 1, p. 136).

The constant presence of *ftsQAZ* in *Verrucomicrobia*, the phylogenetic conservation of FtsZ, the fulfilment of all criteria of functional FtsZ within the gene sequence and the proof of transcription of *ftsQAZ* in actively growing cultures provide a strong indication of functionality and suggest an FtsZ-based cell division mechanism in all members of the phylum *Verrucomicrobia*.

Concerning the phylum *Lentisphaerae*, represented by the cultivated species *Victivallis vadensis* and *Lentisphaera araneosa*, the situation is less clear. Considering that the genome sequence of *V. vadensis* is still unfinished, the present inability to detect *ftsQAZ*-like sequences in the genome data is not significant. Indeed, its closest relative *L. araneosa* possesses *ftsQ*, *ftsA* and *ftsZ*. Concerning *L. araneosa* FtsZ, similarities to *Prosthecobacter* FtsZ and to the conserved domain FtsZ could be clearly detected, whereas the criteria of functional FtsZ (Vaughan et al. 2004) are not fulfilled at all. *L. araneosa* FtsQA have matches to the respective Pfam motifs with high E-values (Table 1), which could be an indication of functionality. Up to now, no bacteria could be identified having a non-FtsZ-based cell division mechanism and possessing at the same time *ftsA* (the first protein recruited to the Z ring and directly interacting with FtsZ). In summary, the present data suggests that *Lentisphaerae* also perform FtsZ-based cell division like *Verrucomicrobia* and the majority of other bacteria. However, the failure to meet the criteria of functionality could indicate a

modified mechanism or even that the gene is (at least in cell division) non-functional. Future studies on *L. araneosa* FtsZ could verify whether the fundamental criteria of functional FtsZ (Vaughan et al. 2004) have to be revised.

B.4.3 Evolution of the *dcw* gene cluster in the PVC superphylum

➲ Appendix C

The identification of *ftsZ* genes in *Verrucomicrobia* has further evolutionary implications, as the related phyla *Chlamydiae* and *Planctomycetes* are lacking *ftsZ* (Glockner et al. 2003; Horn et al. 2004; Read et al. 2000; Strous et al. 2006). Cell division and cell wall synthesis genes are generally organized in the highly conserved *dcw* gene cluster [A.2.3; (Nikolaichik and Donachie 2000; Tamames et al. 2001; Vicente et al. 2006)]. Some of these genes were also detected during genome projects in *Chlamydiae* and *Planctomycetes*. Thus, the question came up if the *dcw* cluster is also conserved in the PVC superphylum and how the last common ancestor of the included phyla performed cell division. A thorough screening of published and unpublished genome project data should help to answer this question.

The analyses of the available sequence data demonstrated that at least in one verrucomicrobial organism (DG1235) the *dcw* cluster is nearly perfectly conserved in comparison to the ancient type of cluster, which comprises 16 genes [Appendix C, Figure 2, p. 137; (Tamames et al. 2001)]. The only differences to the ancient type are: 1) the replacement of *ftsL* through a hypothetical protein; 2) the insertion of *lysM* between *murD* and *ftsW* and 3) the *murC-murB* fusion (Appendix C, Figure 2, p. 137). At least in five other cases (all investigated subdivision 1 organisms), the gene order in the cluster shows variations (Appendix C, Figure 1, p. 136).

A comparison of the *dcw* cluster from several bacterial genomes showed that there is a relationship between the organization of the gene cluster and the shape of the cells, so that the cluster tends to be conserved in rod-shaped and filamentous bacteria, and tends to be disrupted in bacteria with other shapes (Tamames et al. 2001). This observation is supported in this study, as none of the *Verrucomicrobia/Lentisphaerae* with dispersed *dcw* cluster genes forms classical cylindrical rods with hemispherically capped ends.

Ongoing and finished genome projects of the phyla *Lentisphaerae*, *Chlamydiae* and *Planctomycetes* were also investigated for the presence of *dcw* cluster genes. With the exception of *ftsL*, *L. araneosa* was found to possess also all genes of the proposed ancient *dcw* cluster; however the genes generally located in the 3'-terminal region of the cluster are

dispersed in the genome (Appendix C, Figure 2, p. 137). In comparison to the ancestral *dcw* cluster type, *Chlamydiae* show the gene order conservation of many *dcw* genes. Some are absent but the remaining genes are arranged in two isolated clusters, which are remarkably more similar to the DG1235 cluster than to the ancestral cluster (Appendix C, Figure 2). In representatives of *Planctomycetes*, the *dcw* gene content and order conservation varies highly between the different representatives. 12 out of 16 genes are conserved in the *Planctomycetes* representative "*Candidatus* Kuenenia stuttgartiensis" (Appendix C, Figure 2, p. 137). They are organized in a cluster which is very similar to that predicted for the ancestral type. In contrast, *Rhodopirellula baltica* and *Blastopirellula marina* genome data present only some isolated *mur* genes and none of the *fts* genes (*ftsQ* in *B. marina*). *Planctomyces maris* possesses most of the *dcw* genes but they are clustered at different locations in the genome (Appendix C, Figure 2, p. 137).

The high similarity of *dcw* clusters within *Chlamydiae* could be derived from the adaptation to their similar intracellular life styles and to the consequent functional constraints. Although up to now the existence of peptidoglycan in *Chlamydiae* has not been proven, the genes coding for a nearly complete pathway for peptidoglycan synthesis were suggested to be functional [reviewed in (McCoy and Maurelli 2006)]. In contrast to *Chlamydiae*, *Planctomycetes* show a high degree of variability concerning the *dcw* cluster which could be related to the very different life styles of its representatives.

The hypothesis that the last common ancestor of extant bacteria harbored a conserved *dcw* cluster and that some lineages lost this gene order during evolution (Nikolaichik and Donachie 2000) is strongly supported by the observations obtained in this study. The high degree of gene content and gene order conservation especially in DG1235 (*Verrucomicrobia*), in *Chlamydiae* and in the *Planctomycetes* representative "*Candidatus* Kuenenia stuttgartiensis", strongly indicates also that the last common ancestor of the PVC superphylum possessed a conserved *dcw* cluster.

Combined with the phylogenetic analyses (B.2), these data clearly support the view that many representatives of *Planctomycetes*, *Chlamydiae* and *Lentisphaerae* independently lost some of the *dcw* genes or the gene order within the *dcw* cluster. After the separate branchings of *Chlamydiae* and *Planctomycetes* from their last common ancestor with *Verrucomicrobia*, their cell division mechanisms most probably shifted independently from an FtsZ-based mechanism to a non-FtsZ-based mechanism. The major cell division gene *ftsZ* was completely lost or diverged so far from the original *ftsZ* that a relationship is no longer recognizable. FtsZ could have been replaced by two different division mechanisms

(concerning the structural protein of the division ring) in the different phyla and does not therefore necessarily have to be homologous. Another possibility is that there was an independent shift to the same division mechanism in *Chlamydiae* and *Planctomycetes*. If this was the case, it seems probable that the mechanism could have already been present in the last common ancestor of *Planctomycetes*/*Chlamydiae*/*Lentisphaerae*/*Verrucomicrobia*. To our best knowledge, up to now two *Ureaplasma* species are the only examples in bacteria besides *Chlamydiae* and *Planctomycetes* which were identified to lack *ftsZ*. Thus, a special predisposition of the last common ancestor of the PVC superphylum to evolve an FtsZ independent type of cell division can be speculated.

L. araneosa FtsZ shows the highest similarities to verrucomicrobial FtsZs. At the same time, the sequence divergence in comparison to other bacterial FtsZs is higher than that of verrucomicrobial FtsZ. Thus, from an evolutionary perspective, *L. araneosa* could be seen as an organism on the way to developing an FtsZ-independent cell division mechanism.

B.5 Characterization of Bacterial Tubulins

To get hints about the functionality of the bacterial tubulin genes in *Prosthecobacter*, their genomic organization (B.5.1), transcription (B.5.2), expression (B.5.3) and localization within the cell (B.5.4) were investigated. The results are discussed in the last section (B.5.5) of this chapter.

B.5.1 Genomic organization of bacterial tubulin genes

➲ Appendix A/B

Jenkins et al. (2002) published the complete nucleotide sequences coding for two bacterial tubulin genes, *btubA* and *btubB*, in *P. dejongeii* as well as the partial sequences for these genes in *P. vanneervenii* and *P. debontii*. In this study, degenerate primers have been developed, with which it was possible to amplify simultaneously bacterial A and B as well as eukaryotic alpha and beta tubulin genes (B.3). Using these primers, the presence of one A tubulin and one B tubulin gene was confirmed in *P. dejongeii* and *P. vanneervenii*. In addition, two further A and B tubulin genes could be detected and completely sequenced in *P. debontii*. This finding was also confirmed by Southern blotting and hybridization experiments. *P. debontii btubA* and *btubB* partial sequences characterized by Jenkins et al. (2002) do not exist as adjacent loci, but each of them is adjacent to the newly identified *btub*-genes. Therefore, the bacterial tubulin genes in *P. debontii* were renamed. Henceforth, *P. debontii btubA* (Jenkins et al. 2002) will be referred to as *btubA2* and *P. debontii btubB* (Jenkins et al. 2002) will be referred to as *btubB1* (Appendix A, Table 1, p. 102).

Although no sequence data were released, Jenkins et al. (2002) described the existence of a third gene, located downstream of bacterial B tubulin in *P. dejongeii*, that was referred to as kinesin light chain homologue. In order to draw conclusions about the origin and a possible function of bacterial tubulins, it was fundamental to investigate the genomic environment of *btubAB* using Two-Step Gene Walking (B.1) in *P. dejongeii*, *P. vanneervenii* and *P. debontii*. Unexpectedly, all *Prosthecobacter btubB* genes are followed by an open reading frame that shows some similarity to kinesin light chain. According to the original designation (Jenkins et al. 2002), this third gene will be referred to as *bklc* for bacterial kinesin light chain. The presence of *bklc* genes downstream of all bacterial B tubulin genes so far characterized clearly suggests that the bacterial kinesin light chain is an essential part of a functional unit, likely an

operon, represented by one bacterial A tubulin, one bacterial B tubulin and one bacterial kinesin light chain (Appendix B, Figure 4, p. 125). Henceforth, this cluster of genes will be referred to as bacterial tubulin operon (*btub*-operon). Also gene expression features (e.g. promoters, ribosomal binding sites and terminators) support that these three genes are part of a single typical bacterial operon.

Genes showing low similarities to eukaryotic kinesin light chains have been described in *Bacteria* since 1997 [*Plectonema boryanum* (Celerin et al. 1997)], but nothing is known about their function. All *Prosthecobacter* bacterial kinesin light chain sequences show very low similarity to eukaryotic and bacterial kinesin light chain sequences detected using BLASTP (Table 1). Fingerprint analysis (Attwood et al. 2003) of verrucomicrobial kinesin light chain sequences could recover only two or three out of the six typical kinesin light chain motifs (Table 1). A tandem repeat of 4 respectively 5 tetratricopeptide repeat (TPR) domains could be detected in all *Prosthecobacter* Bklc sequences (Table 1). TPR typically contains 34 amino acids and is found in both bacteria and Eukaryotes; it is involved in many functions including protein-protein interactions (Blatch and Lassle 1999). Based on its genomic organization within the *btub*-operon and the presence of TPR domains, a functional relationship of *bklc* to the bacterial tubulin genes can be supposed.

Table 1. BLASTP E-values and kinesin light chain (klc) fingerprints of eukaryotic, bacterial and verrucomicrobial kinesin light chains.

			BLASTP[a]			PRINTS	
Organism	**Domain**	**Protein**	E-value to Bacterial Klc	E-value to Eukaryotic Klc	E-value to TPR domain	No. of detected Klc motifs	P-value for Klc fingerprint
Drosophila melanogaster	EUK	Klc	$2\ e^{-31}$	0.0	$8\ e^{-39}$	6 of 6	$7.3\ e^{-77}$
Plectonema boryanum	BAC	Klc	$5\ e^{-60}$	$1\ e^{-43}$	$2\ e^{-101}$	5 of 6	$9.3\ e^{-21}$
Prosthecobacter debontii	BAC	Bklc2	$3\ e^{-11}$	$8\ e^{-08}$	$1\ e^{-14}$	3 of 6	$3.6\ e^{-7}$
Prosthecobacter debontii	BAC	Bklc1	$2\ e^{-13}$	$2\ e^{-08}$	$3\ e^{-16}$	3 of 6	$4.4\ e^{-7}$
Prosthecobacter vanneervenii	BAC	Bklc	$3\ e^{-14}$	$5\ e^{-08}$	$2\ e^{-16}$	2 of 6	$3.7\ e^{-6}$
Verrucomicrobium spinosum[b]	BAC		$1\ e^{-04}$	0.001	$2\ e^{-07}$	2 of 6	$5.6\ e^{-6}$

Note. a, values refer to the highest similarity score to sequences belonging to the specified group; b, open reading frame of *V. spinosum* sequence was detected on contig 526 of genome sequence; BAC, *Bacteria*; EUK, *Eukarya*; klc, kinesin light chain; P-value, probability-value; TPR, tetratricopeptide repeat

The analysis of the genomic environment of the *btub*-operon showed that the genes upstream of bacterial A tubulins always appear functionally related to those downstream of the bacterial kinesin light chain gene. None of the bordering genes indicated a functional relationship to

cell cycle or cytoskeleton. In all three cases the *btub*-operon appears as an insert interrupting functionally related, but always different, genes (Appendix B, Figure 4, p. 125). This supports the hypothesis that *Prosthecobacter* acquired the *btub*-genes by horizontal gene transfer.

B.5.2 Transcriptional analyses of *btub*-operon genes

The transcription of *btubA* and *btubB* had been already verified in *P. dejongeii* (Jenkins et al. 2002). The results of transcriptional analyses of the *btub*-operon genes in a very recent study by Christina Eckl (2008; diploma thesis, Technical University Munich) are shortly summarized in this section.

The transcription of all *btubA* and *btubB* genes in the species *P. dejongeii*, *P. vanneervenii* and *P. debontii* could be demonstrated by using reverse transcription of mRNA and specific PCR amplification of the genes of interest from the cDNA. Transcription was detected independently from the growing stage (lag-, log-, stationary-phase) of the cultures used for total RNA isolation. For the first time, also the transcription of the *bklc* gene was proven (for all investigated *btub*-operons).

Subsequently, quantitative PCR was used in three different approaches to determine the levels of transcription of the *btub*-genes. One set of experiments focused on the comparison of the transcription level of the *btub*-operon in *P. dejongeii* at different growth stages using relative quantification. The results indicated that the transcription of the *btub*-operon is up-regulated in the stationary-phase in comparison to the log-phase.

Another set of experiments focused on the comparison of transcription levels of the different *btub*-operon genes (*btubA*, *btubB*, *bklc*) and also *ftsZ* in *P. vanneervenii* (using absolute quantification). The transcription levels of *btubB* and *ftsZ* were approx. 2-fold higher in comparison to *bklc* and ten-fold higher in comparison to *btubA*. This finding together with promoter analysis could indicate that the *btub*-operon might not be a typical bacterial operon but rather a gene cluster.

Finally, the comparison of the transcription levels of both *btub*-operons in *P. debontii* indicated that the *btub1*-operon had an approx. ten-fold higher transcription level than the *btub2*-operon.

B.5.3 Expression of *btub*-operon genes

B.5.3.1 Heterologous expression and antibody production

In order to allow the analysis of the expression of the *btub*-operon genes and the investigation of the localization of their gene products, the genes were heterologously expressed in *Escherichia coli*. For Materials and Methods see Appendix E.

The PCR-amplified fragments were cloned into the expression vector pHIS17, transformed in *E. coli* TOP10 and the inserts were resequenced. After transformation into the expression strains *E. coli* C41 or *E. coli* BL21, the expression of the inserts could be induced using IPTG. Via primer-design it was possible to produce recombinant proteins having a tag consisting of six histidine residues at the C-terminus. The following constructs were produced.

pHIS17-*btubA*(Pdj)
pHIS17-*btubA*(Pdj)-6xHis
pHIS17-*btubB*(Pdj)
pHIS17-*btubB*(Pdj)-6xHis
pHIS17-*bklc*(Pdj)
pHIS17-*bklc*(Pdj)-6xHis
pHIS17-*btubA-btubB-bklc*(Pdj)
pHIS17-*btubA-btubB-bklc*(Pva)

After induction the expression of the respective inserts was verified using SDS-PAGE. Compared to the control, a high intensity band in the expected molecular weight range was detected for all single gene constructs (Figure 9, lane 4-8). Recombinant *E. coli* strains, coexpressing BtubA and BtubB without the wild type spacer sequence between the two genes have been investigated by Sontag et al. (2005). It was suggested that BtubA was several-fold more abundant than BtubB. This is also supported by the heterologous expression of the complete *btub*-operon (*btubA-btubB-bklc* including also the wildtype spacers between the single genes) of *P. dejongeii* and *P. vanneervenii* in this study. The analysis of these constructs clearly demonstrated the expression of BtubA and BtubB; the amount of BtubA always seemed to be higher than that of BtubB (Figure 9, lane 2 and 3). A band representing overexpressed Bklc was not or only marginally visible (Figure 9, lane 2 and 3).

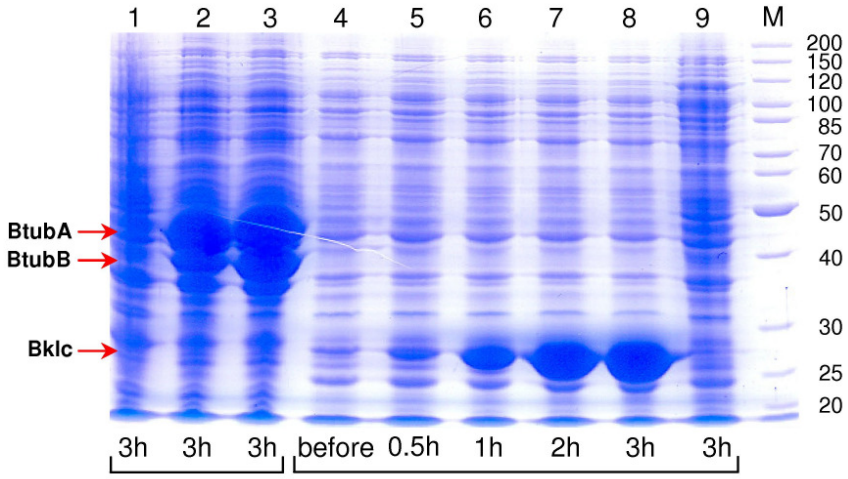

Figure 9. SDS gel showing cultures overexpressing different *btub*-operon genes
Crude cell extracts were loaded on a SDS gel and stained with Coomassie Blue. Lane 1, *E. coli* C41 control; Lane 2, *E. coli* C41/pHIS17-*btubA*-*btubB*-*bklc*(Pva); Lane 3, *E. coli* C41/pHIS17-*btubA*-*btubB*-*bklc*(Pdj); Lane 4-8, *E. coli* BL21/pHIS17-*bklc*(Pdj)-6xHis; Lane 9, *E. coli* BL21 control; M, Marker, numbers indicate molecular weight in kDa. Sampling was performed at the indicated periods of time after induction with IPTG. Red arrows indicate molecular weight range of BtubA, BtubB and Bklc.

Lanes 1-3: In comparison to the control (Lane 1), expression of BtubA and BtubB is clearly visible in both clones, overexpressing the complete *btub*-operons (*btubA*-*btubB*-*bklc*). The amount of BtubA seems to be higher than that of BtubB. The expression of Bklc is only marginally visible.
Lane 4-9: In comparison to the control (Lane 9), a gradual increase of expression of Bklc-6xHIS can be observed with increasing time periods after induction.

An induced *E. coli* C41/pHIS17-*bklc*(Pdj)-6xHis culture was used for protein purification of Bklc. The purification had to be performed at denaturing conditions, as the protein was almost exclusively expressed into inclusion bodies, which were dissolved using 8 M urea. Purification was based on the binding of the 6xHis-tag of the recombinant protein to a silica-based resin precharged with Ni^{2+}-ions. The protein was concentrated by precipitation using ammonium sulfate and redissolved it in a buffer containing 8 M urea. 3000 µg (4.4 mg/ml) of protein were sent to Sigma-Genosys (UK) for polyclonal antibody production (New Zealand White Rabbits, 77 days protocol). Specific antibodies directed against Bklc were purified from the supplied serum by incubation with recombinant protein blotted onto a PVDF membrane, different washings and elution at low pH.

B.5.3.2 Expression analyses of *btub*-operon genes

Expression of *btub*-operon genes in *Prosthecobacter* strains and in the different expression clones was investigated by Western blotting of SDS gels to a PVDF membrane and hybridization with specific antibodies. For Materials and Methods see Appendix E. Antibodies directed against *P. dejongeii* BtubA (anti-BtubA) and BtubB (anti-BtubB) were kindly provided by G. Petroni, University of Pisa. The polyclonal antibodies against *P. dejongeii* Bklc (anti-Bklc) were produced during this study (B.5.3.1).

For the verification of antibody specificities and the determination of specific hybridization conditions, the antibodies were used for the detection of the respective proteins in *E. coli* strains expressing either the single *btub*-operon genes (*btubA*, *btubB* or *bklc*) or the complete *btub*-operons (*btubAB-bklc*) of *P. dejongeii* and *P. vanneervenii*. Subsequently, the antibodies were used for the expression analysis of the *btub*-operon genes in *P. dejongeii*, *P. debontii* and *P. vanneervenii* cultures (Figure 10).

For *btubA*, *btubB* and *bklc*, expression could be detected in *Prosthecobacter* cultures independently from the growing stage of the cultures (Figure 10 C,F,I,J). The analysis of the hybridization patterns obtained during heterologous expression showed that the anti-BtubA antibody is able to hybridize with BtubA of all analyzed *Prosthecobacter* species. This is also true for the anti-BtubB antibody and BtubB. No cross-reaction was detected between anti-BtubA antibody and BtubB or between anti-BtubB antibody and BtubA. The anti-Bklc antibody binds only to *P. dejongeii* Bklc but not to Bklc of other prosthecobacters (Figure 10), probably based on the higher sequence divergence between Bklc proteins in comparison to BtubA and BtubB.

The hybridization of *P. debontii* samples with anti-BtubA antibody consistently showed a second signal in the range of 120 kDa, which was also observed in *P. dejongeii* samples at lower primary antibody dilutions (Figure 10 C). This observation could be based on a protein having a higher molecular weight and a protein domain which is very similar to BtubA. Another, more probable possibility would be the hybridization of the antibody with a tightly bound homo- or heteropolymer consisting of either several BtubA proteins or BtubA and other proteins.

A similar observation was made during hybridizations using the Anti-Bklc antibody. In addition to the expected signal three additional bands, all indicating higher molecular weights, were consistently detected in *P. dejongeii* samples. The intensity of the expected signal was only marginally higher than the additional signal in the range of 40 kDa. Also after the addition of high concentrations of dithiothreitol (DTT; used to prevent formation of intra- and

intermolecular disulfide bonds between cysteine residues of proteins) and the application of a 30 min heat denaturation step, the additional signals were still present (Figure 10 J). This suggests either the hybridization of the antibody to proteins having a domain which is very similar to Bklc, or a very tight binding (which overcomes the denaturing conditions) of Bklc to one or more other proteins. Raising the primary antibody dilution factor from 1/10,000 (Figure 10, Panel J) to 1/15,000 (Figure 10, Panel I) results in the single expected signal but with low intensity. It seems that the additional signals in hybridizations with the anti-Bklc antibody do not match the additional signal observed in anti-BtubA antibody hybridization.

During hybridizations with anti-BtubB antibody these additional signals could be detected only marginally at rather high primary antibody concentrations and due to resolution limits of SDS-PAGE it was difficulty to resolve whether they match the additional signals obtained using anti-BtubA and anti-Bklc antibodies.

Using the different antibodies at specific conditions, differences in signal intensities were detected. Anti-BtubA and anti-BtubB hybridizations were always roughly comparable. Hybridizations using anti-Bklc antibody showed comparable signal intensities only in combination with the recombinant *E. coli* strains expressing the single gene (*bklc*) construct. If complete operon expressing *E. coli* strains or *Prosthecobacter* samples were hybridized, the amount of sample applied in SDS-PAGE had to be increased (in comparison to anti-BtubA and anti-BtubB hybridizations) to obtain a signal. This indicates a lower expression level of Bklc in the wildtype operons and is supported by SDS-PAGE analyses (Figure 9).

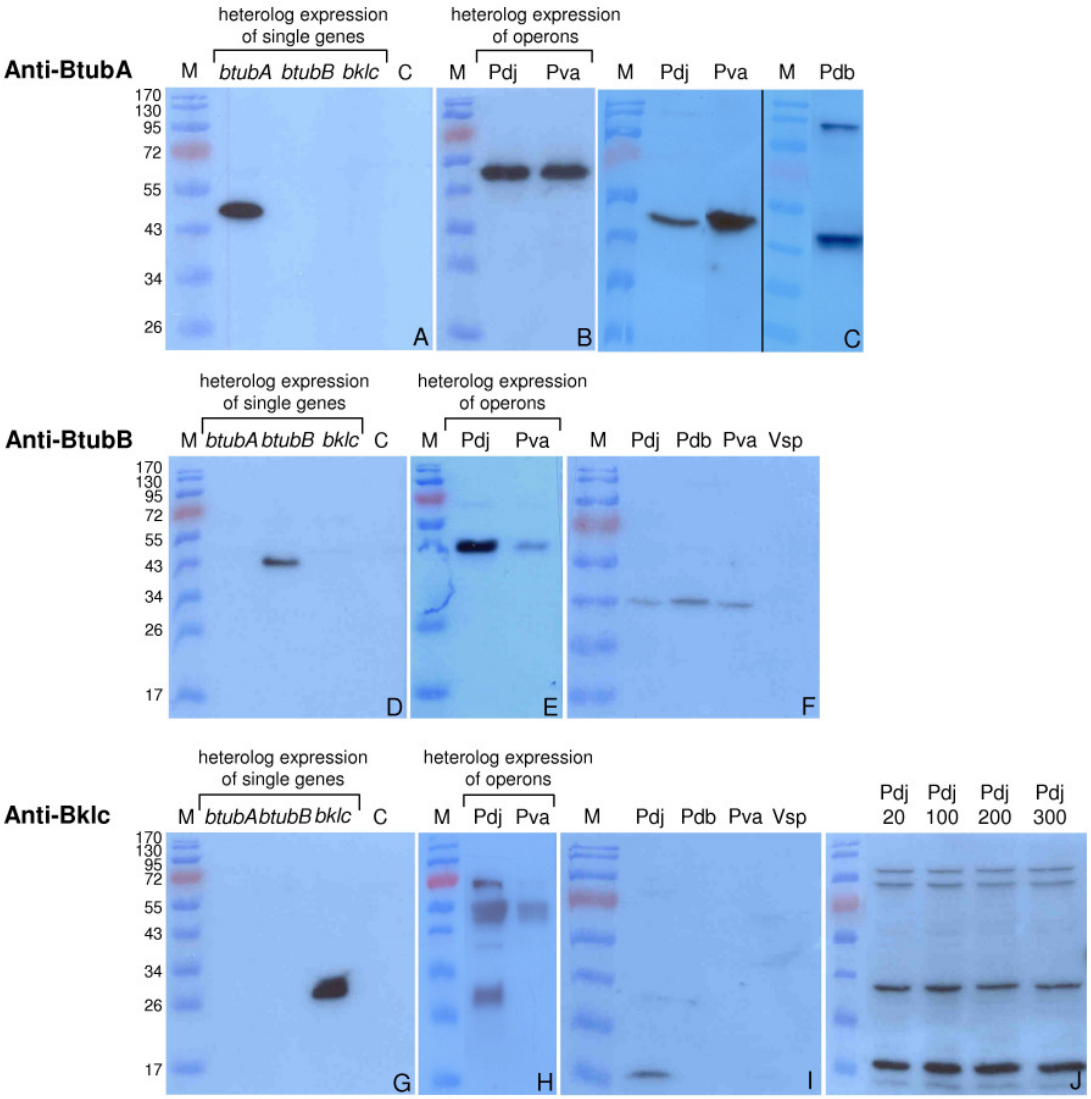

Figure 10. Western hybridizations using antibodies specific for *btub*-operon gene products

Crude cell extracts were loaded on SDS gels and subsequently blotted onto PVDF membranes. Loaded samples were *E. coli* C41/pHIS17 strains overexpressing the indicated single genes (without 6xHis-tag) of the *btub*-operon and *E. coli* C41 (C) as control [Panel A,D,G]; *E. coli* C41/pHIS17 strains overexpressing the complete *btub*-operon (*btubAB-bklc*) of *P. dedongeii* (Pdj) and *P. vanneervenii* (Pva) [Panel B,E,H]; and the *Prosthecobacter* strains *P. dejongeii* (Pdj), *P. vanneervenii* (Pva), *P. debontii* (Pdb) and *V. spinosum* (Vsp) as control [Panel C,F,I,J]. For Panel J samples were loaded with differing DTT concentrations indicated in mM (discussed in the text). Membranes were hybridized using specific antibodies for BtubA (51 kDa) [A-C], BtubB (46 kDa) [D-E], Bklc (29 kDa) [Panel G-J]. Primary antibody dilutions were 1:70,000 [Panel A-C], 1:60,000 [Panel D-F], 1:18,000 [Panel G], 1:15,000 [Panel H,I], 1:10,000 [Panel J]. M, Marker; numbers indicate molecular weight in kDa.

Expression could be detected for all *btub*-operon genes directly in *Prosthecobacter* cultures. The anti-BtubA antibody is able to hybridize with BtubA of *P. dejongeii*, *P. vanneervenii* and *P. debontii*. This is also true for the anti-BtubB antibody and BtubB. There is no cross-reaction between anti-BtubA antibody and BtubB or between anti-BtubB antibody and BtubA. The anti-Bklc antibody binds only to *P. dejongeii* Bklc and not to Bklc of other prosthecobacters.

Due to difficulties in growing *Prosthecobacter* cultures, to date, the antibodies provided by H. Erickson (anti-BtubA, anti-BtubB) have only been used for expression analysis of recombinant *E. coli* strains, coexpressing BtubA and BtubB (Sontag et al. 2005). For the first time, this study could demonstrate the expression of BtubA, BtubB and also Bklc directly in exponentially growing *Prosthecobacter* cultures as well as in stationary-phase cultures. This strongly supports that these genes have a function in *Prosthecobacter*.

B.5.4 Localization of *btub*-operon gene products

As the expression of all *btub*-operon genes was demonstrated for prosthecobacters and recombinant *E. coli* strains, the analyses of the localization of the gene products should provide further hints about their function. For materials and methods see Appendix E.

B.5.4.1 Immunofluorescence staining

Fixed and permeabilized cells were spotted onto glass slides; the slides were blocked and hybridized using the primary antibody (anti-BtubA, anti-BtubB or anti-Bklc). After different washing steps, the samples were incubated with the secondary, fluorochrome-conjugated antibody (anti-rabbit-IgG). After different washing steps and embedding into anti-fading agent, the samples were analyzed using a confocal laser scanning microscope. The concentration and specificity of the primary antibody (anti-BtubA, anti-BtubB or anti-Bklc) were determined using recombinant *E. coli* strains expressing the different single gene constructs (B.5.3.1) or lacking the expression plasmid. The specificity of the secondary antibody was checked by a hybridization omitting the primary antibody.

Thus, for each of the antibodies, hybridizations could be performed at specific conditions showing signals only with the positive control. The respective cells (> 90%) showed whole cell fluorescence and a special signal accumulation around the inclusion bodies (anti-BtubA results shown in Figure 11). Inclusion bodies were constantly detectable in all strains expressing the single gene constructs.

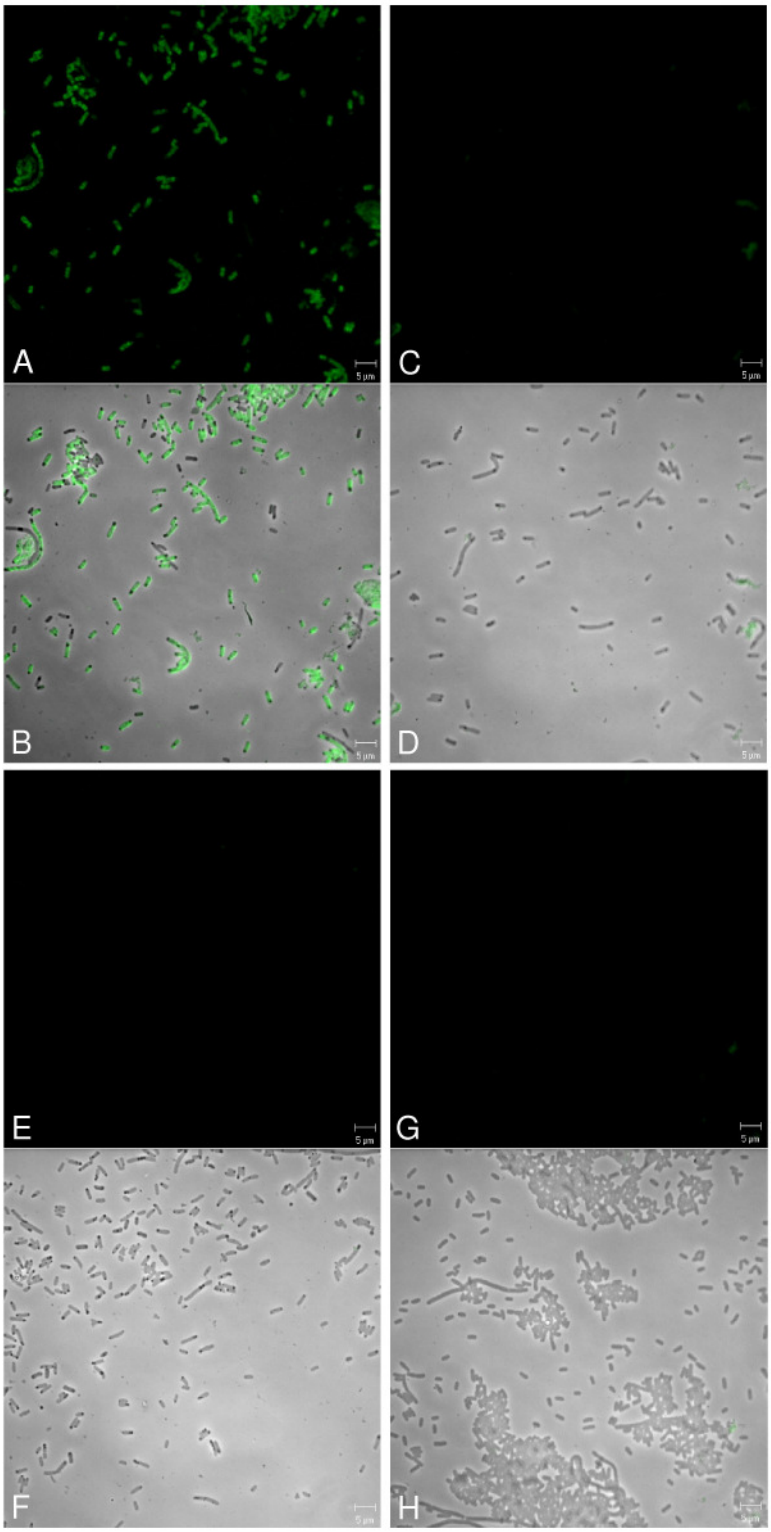

Figure 11. Determination of specificity for immunofluorescence staining

Fixed *E. coli* strains were hybridized using anti-BtubA (1:10,000) as primary antibody and FITC-labelled anti-rabbit-IgG (1:30). Fluorescence images (A,C,E,G) and merged fluorescence/phase contrast images (B,D,F,H) are shown. Strains were recombinant *E. coli* C41 expressing BtubA (A,B), BtubB (C,D), Bklc (E,F) and *E. coli* C41 without plasmid (G,H).

Signals were specifically detected in the BtubA (A,B) expressing strain (90% of the cells) and not in the controls (C,D,E,F,G,H). Only plasmid harbouring strains produced inclusion bodies.

The antibodies were also used with recombinant *E. coli* strains expressing the complete *btub*-operon from *P. dejongeii* or *P. vanneervenii* (B.5.3). In sum, there was no significant difference between the results of both strains; the observations described in this section will therefore apply for both strains.

Examination in the phase contrast already showed differences to all other strains. Firstly, the strains expressing the complete operons did only produce a low amount of inclusion bodies in contrast to the strains expressing the single gene constructs. Secondly and more strikingly, the cell morphology of a portion of the cells (approx. 10-20%) was different to the remaining cells in the culture, different to the control without plasmid and different to the strains expressing single gene constructs. These cells seemed to have a smaller diameter and were often observed to be curved. During immunostaining using anti-BtubA and anti-BtubB antibodies, only approx. 10-20% of the cells showed a signal. Interestingly, in most of the cases, the cells showing a signal matched exactly the cells having the peculiar morphology (Figure 12). The fact that only a subpopulation in the cultures showed a signal may be explained by the big size of the insert of the plasmid. It can be assumed that only the cells showing a signal do express the insert (*btub*-operon). The possibility that the different morphology is based on a different impact of the lysozyme permeabilization treatment on different cells was excluded. Omitting the lysozyme treatment of glutaraldehyde/paraformaldehyde or formaldehyde fixed *E. coli* cells resulted in the same type of signals and morphology after hybridization.

Only very faint signals without a specific localization were detected using the anti-Bklc antibody in combination with the complete *btub*-operon clones. This result is supported by SDS-PAGE and Western hybridization results (B.5.3), which indicated that Bklc is expressed in a much lower amount in comparison to the tubulin genes.

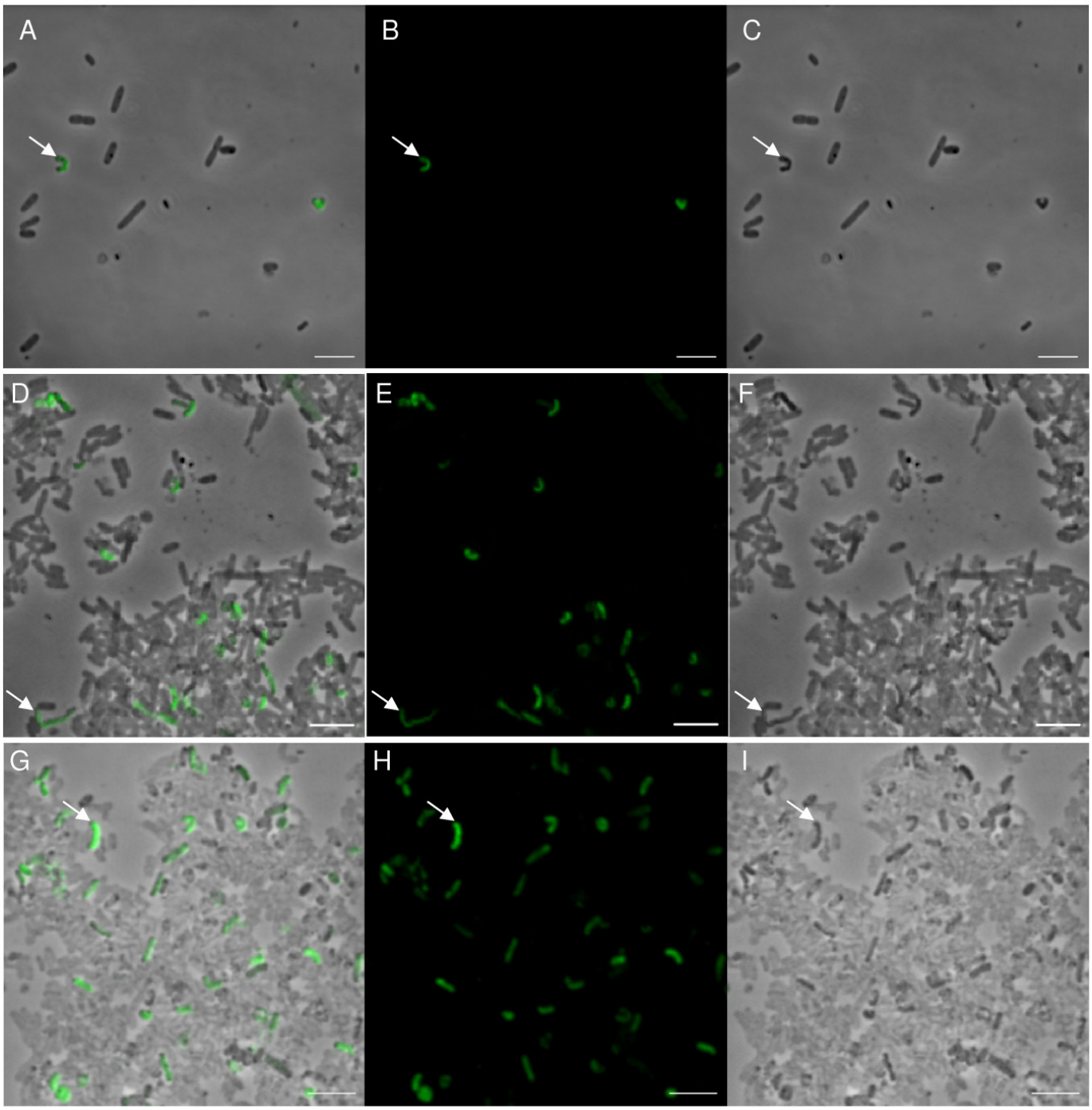

Figure 12. Immunofluorescence staining of recombinant *E. coli* expressing *btubA-btubB-bklc*
Fixed *E. coli* pHIS17-*btubA-btubB-bklc*(Pdj) was hybridized using anti-BtubA (A-F) or anti-BtubB (G-I) as primary antibodies (both 1:18,000) and Alexa488-labelled anti-rabbit-IgG (1:100) as secondary antibody. Merged fluorescence/phase contrast images (A,D,G), fluorescence images (B,E,H) and phase contrast images (C,F,I) are shown. Bar, 5 µm.

Results using anti-BtubA (A-F) and anti-BtubB (G-I) antibodies were comparable. Approx. 10-20% of the cells showed a signal. At the same time these cells were predominantly curved and appeared to have a smaller diameter than the remaining cells and the controls (see arrows for some examples).

A closer examination of the signal localization within recombinant *E. coli* cells expressing the complete *btub*-operon showed that in most of the cells, the signal had a curved rod shape, slightly thinner than the cell appeared in the phase contrast. Often, a buckling of the rod at both ends was observed. In some cells the signal appeared also rod-like but thinner. It stretched across the length of the cells (Figure 13A), similar to the type of signal which Sontag et al. (2005) described for recombinant *E. coli* expressing only fused *btubA* and *btubB*. In some cells, a thin rod-like structure could be even detected in the phase contrast, stretched across several dividing cells (Figure 13B).

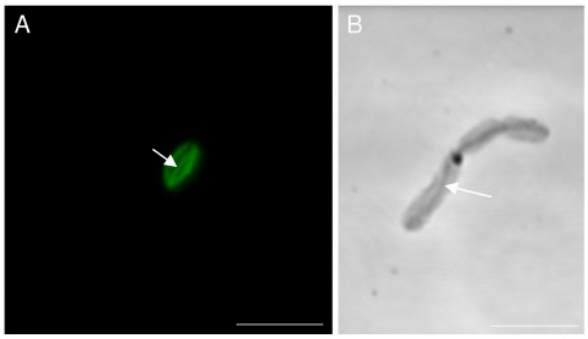

Figure 13. Intracellular rod-like structure in recombinant *E. coli* expressing *btubA-btubB-bklc*
E. coli cells expressing *btubA-btubB-bklc* are shown. Intracellular rod-like structures were detected in (A) immunofluorescence staining (anti-BtubA) and in some cells even in (B) phase contrast. Bar, 5 µm.

Immunofluorescence staining was also performed using *Prosthecobacter* cells. However, using antibody concentrations which were shown to be specific, no signal could be detected. Continuously for prosthecobacters and *Verrucomicrobium spinosum* (negative control), cell wall signals were observed when the dilution factor was decreased below the specific/unspecific threshold (determined in recombinant *E. coli*), probably based on unspecific binding of the antibodies to the cells. Previous experiments with *Prosthecobacter* cells in fluorescence *in situ* hybridizations (using digoxygenin labeled nucleic acid probes) indicated the need for rigid fixation and permeabilization procedures prior to hybridization to render the target accessible for the antibodies (M. Pilhofer, unpublished). However, if such procedures were applied, the poor preservation of the cell morphology made it impossible to detect specific signals. Thus, the inability to localize *btub*-operon gene products in *Prosthecobacter* using immunofluorescence is probably not based on a low expression level but rather on experimental limitations.

B.5.4.2 Transmission electron microscopy

Transmission electron microscopy (TEM) was performed in order to detect protofilament-like structures in recombinant *E. coli* cells and in *Prosthecobacter*. Cells were fixed using osmium tetroxyde and glutaraldehyde, dehydrated in graded ethanols, embedded in epoxy resin and cut into thin-sections. Sections were counterstained using uranyl acetate and lead citrate.

E. coli cells expressing the *btub*-operon of *P. dejongeii* or of *P. vanneervenii* showed fundamental differences to the control without plasmid insert. Electron-dense, rod-like structures were observed in many cells (Figure 14 A-D). At higher magnifications these structures seemed to consist of protofilament bundles. The rod-like structures were running through the length of the cell and often even through the division site of dividing cells. Thus, these results support the observations made during immunofluorescence staining and phase contrast microscopy (B.5.4.1). Another peculiar feature was a region with low electron-density, which made up a big part of the cell. This region seemed to consist of a kind of meshwork and surrounded the rod-like structure. In some cases also inclusion bodies were detected. Granular cytoplasm was observed just below the membrane and in regions where no rod was visible. In contrast, the control showed an even distribution of granular cytoplasm throughout the cell.

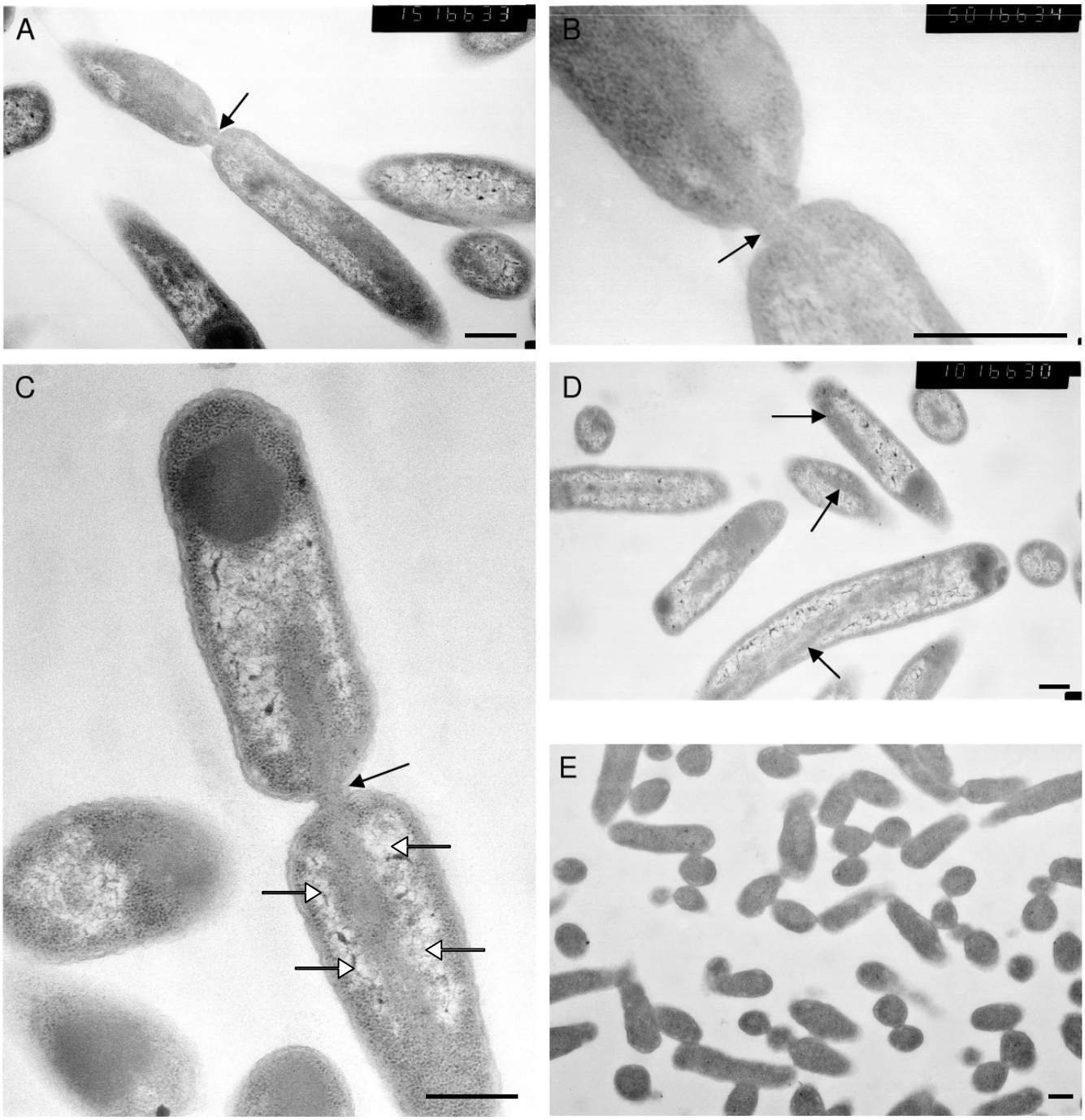

Figure 14. TEM micrographs of recombinant *E. coli* cells expressing the *btub*-operon
(A-D) *E. coli* expressing *btubA-btubB-bklc*; (E) *E. coli* C41 control. Cells fixed with glutaraldehyde/OsO_4 and stained with uranyl acetate/lead citrate. Bar, 0.5 µm.

Electron-dense, rod-like structures were observed in *E. coli* expressing the *btub*-operon (A-D; see black arrows). These structures seemed to consist of protofilaments and often stretched across the division site of dividing cells. Another specific feature was the low electron-density region consisting of a kind of meshwork, surrounding the rod-like structure (white arrows). Cells of the control (E) were lacking these structures.

Also *Prosthecobacter debontii* and *P. dejongeii* cells were subjected to transmission electron microscopical analyses. In contrast to a previous study (Jenkins et al. 2002), it was possible to detect electron-dense protofilament-like structures within both *Prosthecobacter* species. The protofilaments were often arranged in a rod-like bundle located in the center of the cells (Figure 15), resembling the rod-like structures in recombinant *E. coli* cells (Figure 14). In some cases, the protofilament structures appeared like a meshwork (Figure 15 C). Thin bundles of protofilaments could be detected in some cells also just below the membrane (Figure 15 A). Other features of the micrographs were proximal low electron-density regions and round, very electron-dense structures in the rod-like region. The latter ones could be possibly explained by metal accumulations within the cell.

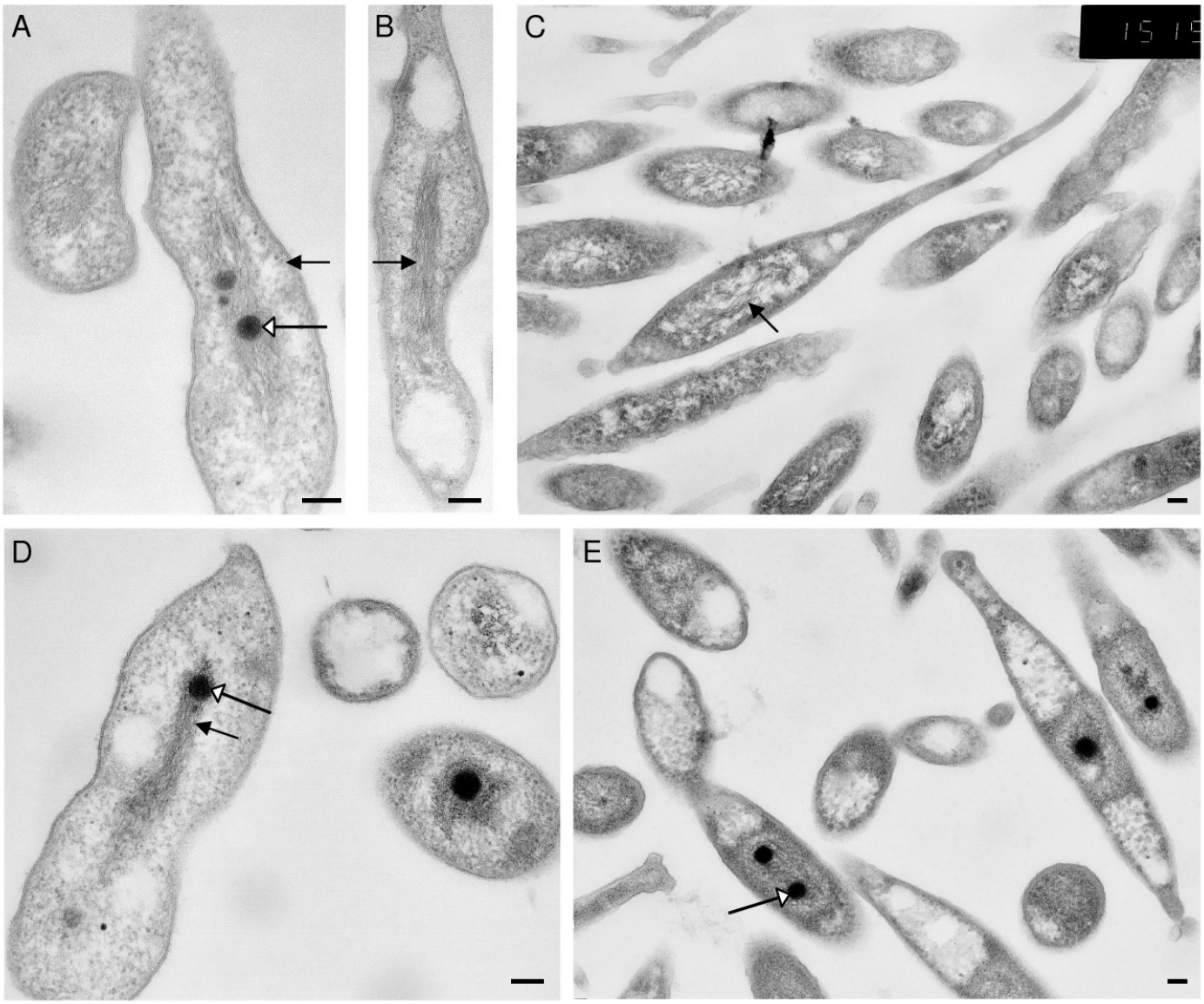

Figure 15. TEM micrographs of *P. debontii*

P. debontii cells were fixed with glutaraldehyde/OsO_4 and stained with uranyl acetate/lead citrate. Bar, 0.125 µm.

Protofilament-like structures could be identified, which were often arranged in a bundle in the center of the cell (B,D; black arrows) or sometimes just below the membrane (A; black arrow). In some cases the protofilaments appeared as a meshwork (C; black arrow). The very electron-dense round structures (white arrows) may be based on metal accumulations.

B.5.4.3 Electronmicroscopic immunocytochemistry

To verify the localization of the bacterial tubulins, the specific antibodies were used in combination with thin sections of *Prosthecobacter* samples and TEM.

Prosthecobacter cells were fixed in glutaraldehyde, dehydrated using graded ethanols, embedded in LR White resin and cut into thin sections. The sections were placed onto Ni-grids, blocked and incubated with the primary antibody (anti-BtubA or anti-BtubB). After washing steps, the grids were incubated with the secondary antibody (10 nm gold-conjugated anti-rabbit-IgG antibody) and washed again. The sections were counterstained using uranyl acetate and lead citrate and were examined in a transmission electron microscope. The specificity of the secondary antibody was checked by a hybridization omitting the primary antibody. The concentration of the primary antibodies was increased by factor 10 in comparison to immunofluorescence staining due to the lower sensitivity of the immunogold staining procedure.

The analysis of the sections revealed a poor preservation of the cell's ultrastructure probably due to LR White resin which was used for embedding. LR White resin was used because it enables a better permeabilization of the antibodies in the resin in comparison to epoxy resins. Therefore, gold-signals could be detected but in most of the cases it was difficulty to assign them unambiguously to a specific structure within the cell (Figure 16). The results were comparable using anti-BtubA or anti-BtubB antibodies. Often, clusters of several gold particles (10 nm) were observed, indicating polymeric structures of the bacterial tubulins. In longitudinal sections, the gold particles were predominantly observed in the region of the rod-like structure or just below the cytoplasmic membrane (Figure 16 C, D). A clustering of the particles was especially detected in cross sections. In most of the cases, these clusters were absent from the very low electron-density regions and localized again often just below the cytoplasmic membrane or more central (Figure 16 A, B).

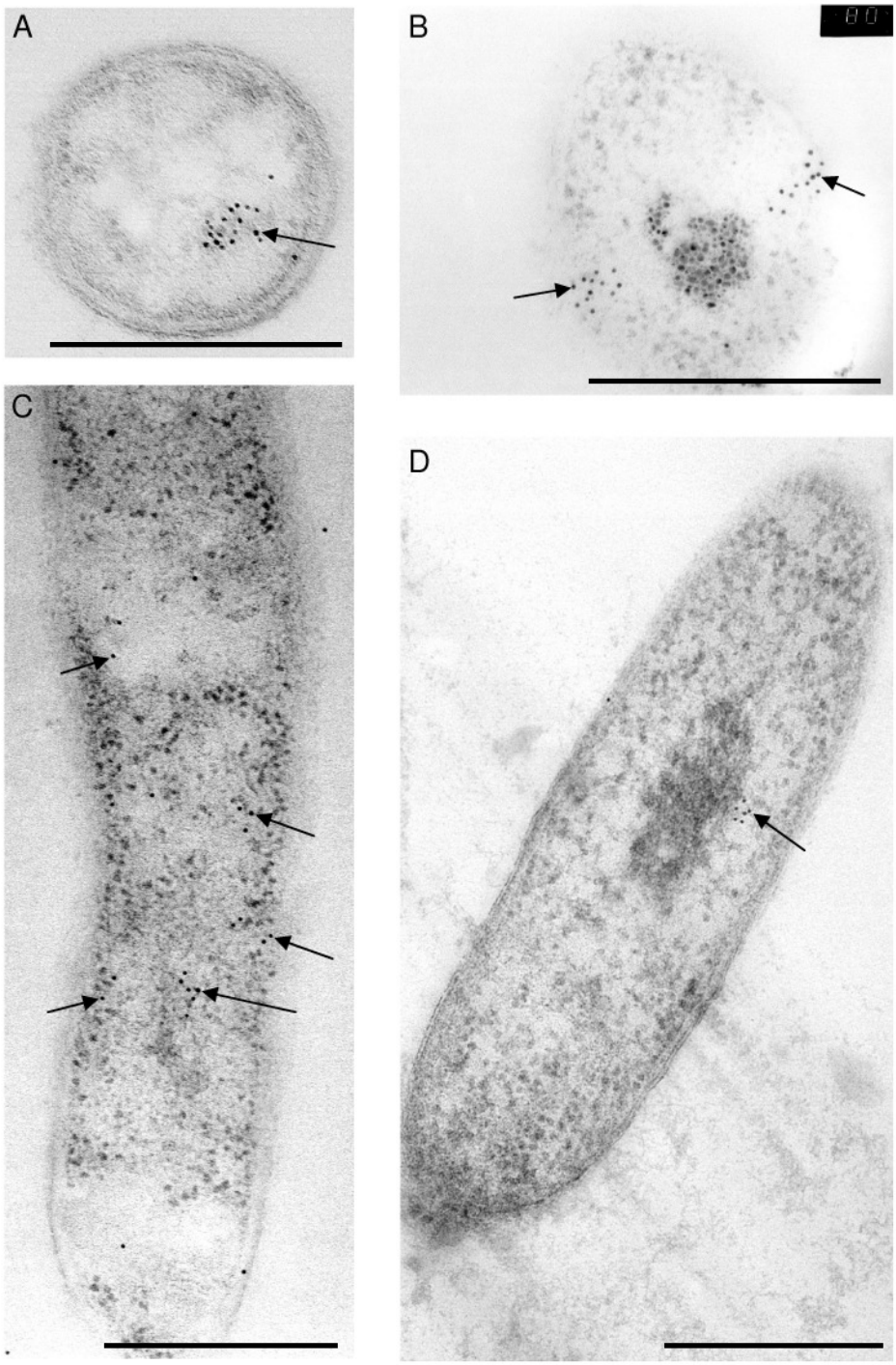

Figure 16. Immunogoldstaining of *Prosthecobacter* cells

Electron micrograph of *P. dejongeii* hybridized with anti-BtubA and anti-rabbit-IgG-gold. Bar, 0.5 µm.

Based on the used resin (LR White; optimized for permeabilization in immunogoldstaining), the ultrastructure preservation of the cells was imperfect. Often, clusters of gold particles (10 nm) were observed (arrows). In cross sections (A,B) gold clusters were predominantly absent from the very low electron-density regions but localized just below the membrane or more central. Longitudinal sections (C,D) also showed localization just below the membrane or in the region of the rod-like structure.

B.5.5 Conclusions about the functionality of bacterial tubulins

The analysis of the genomic organization of the *btubAB* genes revealed that the *bklc* gene constitutes an integral part of the *btub*-operons (B.5.1). Therefore, for the first time *bklc* was included in functional investigations of bacterial tubulin genes in *Prosthecobacter* in this study. The hypothesis that the peculiar *btub*-operon genes are functional in *Prosthecobacter* was strongly supported by the major results.

The transcriptional analyses showed that all *btub*-operon genes are transcribed in different growing stages. They even indicated that the *btub*-operon genes could have a special function during the aging process of the cells (B.5.2). Expression analyses revealed for the first time that bacterial tubulin genes and *bklc* are really expressed and translated into proteins in *Prosthecobacter* – even in different growing stages (B.5.3.2).

Previously, it was shown that bacterial B tubulin itself is able to form homopolymers (resulting in ring structures) and that bacterial A and B tubulins can form heteropolymers *in vitro*. The heteropolymers formed protofilaments which aggregated to bundles. These *in vitro* studies and crystal structure analyses suggested that bacterial A and B tubulins may alternate also *in vivo* along protofilaments (Schlieper et al. 2005; Sontag et al. 2005). This study could detect for the first time protofilament structures *in vivo*, which probably consist of bacterial tubulins (B.5.4). Thus, there is support that BtubAB are also able to polymerize *in vivo*.

The immunofluorescence staining results with recombinant *E. coli* cells (B.5.4.1) were basically consistent with the observations by Sontag et al. (2005), which also described rods running the length of the cell in *E. coli* expressing BtubAB. Although, no curvature of the cells was reported (Sontag et al. 2005). This study differed fundamentally concerning the expressed construct, which comprised also *bklc* and the wild type spacer sequences. Therefore, one possible explanation suggests that the curvature of the cells was indirectly mediated by Bklc. Bklc may directly modify the protofilament, consisting of alternating BtubA and BtubB, leading to a curvature of the cells.

The possibility of heterotrimeric protofilaments consisting of BtubA-BtubB-Bklc subunits seems improbable with respect to the expression analysis (SDS-PAGE and Western hybridizations) of recombinant *E. coli* (expressing the complete *btub*-operon) and *Prosthecobacter*. In all cases Bklc appeared to be expressed in a much lower amount than the tubulin genes are (B.5.3.2). Therefore, Bklc may have a regulative or modulating function.

During Western hybridizations with anti-BtubA and anti-Bklc antibodies additional higher molecular weight signals were detected (B.5.3.2). This also indicates that the gene products of the *btub*-operon are able to form tightly bound multimeric (homo- or heteropolymeric) proteins. It is supported by the fact that the Bklc protein sequence comprises several tetratricopeptide repeat (TPR) domains, which are often involved in protein-protein interactions (Blatch and Lassle 1999).

The localization studies of BtubAB in recombinant *E. coli* cells expressing the complete operon are an indication how these proteins could be localized in *Prosthecobacter*, where very similar rod-like structures could be detected in the center of the cell. However, the localization using immunogoldstaining in *Prosthecobacter* could not unambiguously identify specific structures in *Prosthecobacter* as bacterial tubulins, due to experimental limitations (B.5.4). In no case, the signals indicated a special localization at the division site. This does therefore not support a recruitment of the *btub*-operon gene products to the cell division machinery at the Z ring. In the future, cryo-electron tomography in combination with immunodetection could provide a powerful tool for better understanding the localization and functions of the gene products of the bacterial tubulin-operon. Given the peculiar stalked morphology of *Prosthecobacter*, it can be speculated that *btub*-genes are functionally involved in the determination of the cell shape. The localization of signals in immunodetection just below the membrane support such an involvement, as also other bacterial shape determining proteins run in spirals below the membrane (Ausmees et al. 2003; Michie and Lowe 2006; Shih and Rothfield 2006). However, in the stalked bacterium *Caulobacter crescentus* not only crescentin, a *Caulobacter*-specific protein, was shown to contribute to the comma-like cell shape. At least one other cytoskeletal protein, MreB, is involved in shape determination (Ausmees et al. 2003). It will be interesting to see in the future if and how the eukaryotic-like *Prosthecobacter* tubulins interact with typical bacterial proteins; maybe Bklc has the function of mediating such interactions.

B.6 Origin of Bacterial Tubulins

B.6.1 Phylogenetic relationships within the Tubulin/FtsZ superfamily

➲ Appendix D

To get hints about the origin of *Prosthecobacter* tubulins, comprehensive sequence data analyses within the FtsZ/Tubulin superfamily were performed. This distinct group of GTPases (A.1.1) comprises eukaryotic tubulins, bacterial tubulins, FtsZs, archaeal FtsZ-likes (Larsen et al. 2007; Vaughan et al. 2004) and bacilli Tubulin-likes (Larsen et al. 2007). The Tubulin family comprises ten eukaryotic subfamilies, alpha through kappa (A.1.1). Like alpha and beta tubulins, gamma tubulins are present in all eukaryotic organisms. The subfamilies delta and epsilon are present in many but not in all eukaryotic lineages. Zeta and eta are only known from few eukaryotic organisms. Three sequences from *Paramecium* represent the putative new tubulin subfamilies theta, iota and kappa (Dutcher 2003; Libusova and Draber 2006; Vaughan et al. 2000).

To perform phylogenetic analyses, a comprehensive ARB database was established comprising diverse members of the above mentioned groups. The ARB database was used to calculate a protein identity matrix of selected representatives (Appendix D, Figure 1, p. 172). Bacterial tubulins showed the highest identity values with the major eukaryotic tubulin subfamilies in the range 21-44%. The values were significantly lower if the bacterial tubulins were compared to FtsZs (14-17%), FtsZ-likes (13-20%) or Tubulin-likes (10-15%).

The topologies of different calculated global trees were analyzed. They showed that the representatives of the tubulin family, comprising the subdivisions alpha through kappa, formed always a stable monophyletic group together with the bacterial A and B tubulin sequences. The tree topology within the tubulin family was rather instable (see below). Archaeal and bacterial FtsZs were always recovered as a stable monophyletic group. In most of the trees, the Tubulin-like sequences of bacilli were monophyletic. The archaeal FtsZ-like sequences formed a less stable monophyletic group. A representative maximum likelihood tree is shown in Appendix D, Figure 2 (p. 173). These phylogenetic analyses firmly support that eukaryotic and bacterial tubulins are members of a single clade within the Tubulin/FtsZ superfamily.

Further sequence analyses were performed to elucidate the relationships within the tubulin family. The program PRINTS (Attwood et al. 2003) was used to detect motif similarities

among bacterial tubulins and the different subfamilies of eukaryotic tubulins (Appendix D, Table 1, p. 171). All bacterial B tubulin sequences had the highest hit score with the beta tubulin subfamily. On the contrary, bacterial A tubulin showed similar scores with alpha, beta and epsilon families depicting an unclear situation. This was also true for the identity values of bacterial tubulins with different tubulin subfamily representatives (Appendix D, Figure 1, p. 172). Thus, based on the identity matrix, also no significant associations of bacterial A and B tubulins could be detected to any of the eukaryotic subfamilies.

Tree topologies within the tubulin family in the ARB database used for the calculation of the global trees and in a second database differing slightly in the sequence alignment were analyzed. Although the dataset was very comprehensive, the evolutionary relationships among the different tubulin subfamilies were not unambiguously resolved. Also previous, less comprehensive studies indicated similar problems manifested by trees with low bootstrap values (Dutcher 2003; Jenkins et al. 2002; Keeling and Doolittle 1996). In all calculated trees *Paramecium* kappa tubulin consistently associated with alpha tubulins and *Paramecium* theta tubulin with beta tubulins, respectively. With these two exceptions, alpha, beta and gamma tubulin subfamilies as well as bacterial A and B tubulins were always recovered as monophyletic groups in all calculated trees. The epsilon and delta tubulin groups were monophyletic in most but not in all of the calculated trees. No clear associations between bacterial tubulins themselves or between any of the bacterial tubulins and known tubulin subfamilies could be recovered. In many trees, a monophyletic group comprising alpha, beta, gamma and bacterial A and B tubulins could be detected. A consensus tree is shown in Appendix D, Figure 3 (p. 174).

B.6.2 Alternative hypothesis on the origin of bacterial tubulins

➲ Appendix D

It was speculated by previous studies that *Prosthecobacter* acquired the bacterial tubulin genes by horizontal gene transfer (Jenkins et al. 2002; Schlieper et al. 2005; Sontag et al. 2005). The present study showed that by PCR or genome data screening (B.3) no tubulin genes could be detected in other *Verrucomicrobia*. This is a further strong support for horizontal gene transfer together with the following indirect evidences obtained in this study: (1)the presence of genuine *ftsZ* and other bacterial cell division genes in all *Verrucomicrobia* subdivisions with cultivable representatives (B.4); (2) the high divergence between *Prosthecobacter* FtsZ and bacterial tubulins (B.4); (3) the integration of bacterial tubulin operons in a set of genes functionally related among themselves but apparently not with the tubulin operon (B.5.1); (4) the different genomic environments of the different *btub*-operons (B.5.1); (5) the presence of two *btub*-operons in one *Prosthecobacter* species (B.5.1).

The level of conservation of bacterial tubulins among *Prosthecobacter* species and the functional studies on *Prosthecobacter* tubulins (Schlieper et al. 2005; Sontag et al. 2005; B.5) indicate that these genes are important, functional and evolutionary constrained in *Prosthecobacter*. This means that the gene products of the bacterial tubulin operon either represent an important but functionally independent unit or that they are capable of properly interacting with other typical bacterial proteins. Indeed, the analysis of the *P. dejongeii* genome (95% coverage) showed that, except the genes belonging to the *btub*-operon, very few genes with homologies to eukaryotic counterparts are present in this organism and none of them is related to the eukaryotic cytoskeleton (Staley et al. 2005).

Prosthecobacter tubulins belong to the tubulin family but clearly differ from previously known tubulin subfamilies and should therefore be considered as members of two novel tubulin subfamilies. If bacterial A and B tubulins originated from a modern eukaryotic donor, a topological association to a eukaryotic tubulin subfamily would have been expected in phylogenetic trees.

Schlieper *et al.* (2005) proposed that one or possibly two tubulin genes were transferred from a eukaryote to *Prosthecobacter* where they were modified not to form tight heterodimers and to fold without chaperones. A schematic representation of this hypothesis is provided in Appendix D, Figure 4A (p. 175). A direct derivation of bacterial A and B tubulins from modern eukaryotic tubulins (Schlieper et al. 2005) would imply that bacterial A and B tubulins lost the capability to form tight heterodimers, but obtained the capability to fold

without the help of chaperones. Moreover, together with the bacterial kinesin light chain, they would have been reorganized into a typical bacterial operon and likely shifted to a completely different function due to the novel bacterial proteomic environment. Subsequently, this novel function would have become essential for the bacterial host cell, thus, justifying the level of conservation observed among *Prosthecobacter* species. This event of trans-kingdom horizontal gene transfer would have occurred between organisms which are not involved in a symbiotic association. Although the hypothesis by Schlieper *et al.* (2005) cannot be disregarded; the present study suggests a different interpretation.

Our phylogenetic analyses do not support a derivation of bacterial tubulins from a specific eukaryotic tubulin subfamily. The structure of the bacterial tubulin operon, which is typical for that of prokaryotes suggest an alternative interpretation depicted in Appendix D, Figure 4B (p. 175). According to this scenario, bacterial A and B tubulins derived, as well as alpha, beta and other eukaryotic tubulins, from a common ancestor of the tubulin family after the separation from FtsZ/bacilli tubulin-likes/archaeal FtsZ-likes. The capability to form weak heterodimers and to fold without the help of chaperones would be an ancestral and not a derived character.

According to our hypothesis, the donor organism was a bacterium that still remains to be identified. It is well known that the majority of prokaryotes have yet not been isolated (Schleifer 2004). Nothing, except 16S rRNA, is known about most of these organisms and it could be speculated that some of them contain genes coding for bacterial tubulins that may have been transferred to *Prosthecobacter*.

This would have of course implications on present models of tubulin and eukaryotic cell evolution (Cavalier-Smith 2002; Embley and Martin 2006; Margulis et al. 2000; Martin and Muller 1998; Moreira and Lopez-Garcia 1998). It will be interesting to see if future genomic and metagenomic studies on yet uncultivable bacteria and protists will provide answers to these questions.

C SUMMARY

C.1 Summary

Tubulins were considered as typical eukaryotic features until tubulin genes were detected in the bacterial genus *Prosthecobacter*, member of the phylum *Verrucomicrobia*. These bacterial tubulin (*btub*) genes were more similar to eukaryotic tubulins than to the bacterial homolog FtsZ. FtsZ is the major bacterial cell division protein in bacteria, forming the Z ring at the division site. It is present in almost all *Bacteria* and *Euryarchaea*. Based on a genome sequence draft of *Prosthecobacter dejongeii*, *Verrucomicrobia* were supposed to lack FtsZ. It was hypothesized that bacterial tubulins in *Verrucomicrobia* took over the function of FtsZ. Interestingly, also the phyla *Planctomycetes* and *Chlamydiae* do not possess FtsZ; their phylogenetic relationship to *Verrucomicrobia* was discussed controversial.

To investigate the genomic environment of protein coding genes detected by PCR, the novel and highly efficient Two-Step Gene Walking method was developed. In other verrucomicrobial strains no tubulin-like genes could be detected. Therefore, *Prosthecobacter* strains were screened for the presence of *ftsZ* and other cell division genes. Unexpectedly, *ftsZ* and other genes of the division and cell wall gene cluster (*dcw*) could be identified in *Prosthecobacters* and in all verrucomicrobial subdivisions with cultivable representatives. Sequence and transcriptional analyses suggested that these genes are functional and phylogenetically constrained. An FtsZ-based cell division mechanism was proposed for all verrucomicrobial subdivisions and for the sister-phylum *Lentisphaerae*. 23S rRNA phylogeny supported that *Verrucomicrobia/Lentisphaerae* are related to *Planctomycetes* and *Chlamydiae*. The genomic organization of *dcw* cluster genes suggested that the last common ancestor of the mentioned phyla had an FtsZ-based cell division mechanism, which was independently lost. The analyses of the genomic organization of the *btub*-genes revealed that they are organized in a typical bacterial operon (*btub*-operon) together with a bacterial kinesin light chain-like (*bklc*) gene. RT-PCR and Western hybridizations demonstrated for the first time that all genes of the *btub*-operons are expressed in *Prosthecobacter*. Localization studies (TEM, immunofluorescence/immunogold staining) were performed with *Prosthecobacter* and with recombinant *E. coli*, expressing the *btub*-operon. The results indicate that bacterial tubulins can also polymerize *in vivo*, form protofilaments and may contribute to the peculiar shape of *Prosthecobacter*. Phylogenetic analyses on bacterial tubulins suggested that they constitute two new tubulin subfamilies. There are several strong hints that *Prosthecobacter* acquired the *btub*-genes by horizontal gene transfer. In contrast to a previous hypothesis, the present study favors a bacterial instead of a eukayotic donor organism.

C.2 Zusammenfassung

Tubuline galten solange als typische eukaryontische Merkmale, bis bakterielle Tubulin-Gene (*btub*) in der bakteriellen Gattung *Prosthecobacter* (*Verrucomicrobia*) gefunden wurden. Diese Gene zeigten höhere Ähnlichkeit zu eukaryontischen Tubulinen als zu FtsZ. FtsZ ist das wichtigste Zellteilungsprotein in Bakterien, da es den Z-Ring zwischen sich teilenden Zellen ausbildet. FtsZ kommt in fast allen Bakterien und Euryarchaeen vor. Auf Grund der Analyse der noch unvollständigen *Prosthecobacter dejongeii* Genom-Sequenz wurde angenommen, dass Verrucomicrobien kein *ftsZ* besitzen und dass dessen Funktion in Verrucomicrobien von den bakteriellen Tubulinen übernommen wurde. In der Vergangenheit wurde über die Verwandtschaft der bakteriellen Phyla *Planctomycetes* und *Chlamydiae* kontrovers diskutiert. Interesannterweise besitzen diese Phyla ebenfalls kein FtsZ.

Die neuartige, höchst effiziente Two-Step Gene Walking Methode wurde entwickelt, um die Umgebung von Protein-kodierenden Genen auf dem Genom zu untersuchen, die mittels PCR und degenerierten Primern entdeckt wurden. In anderen Verrucomicrobien-Stämmen konnten keine Tubulin-ähnlichen Gene gefunden werden. Deswegen wurde in *Prosthecobacter*-Stämmen nach *ftsZ* Genen gesucht. Unerwarteterweise konnten *ftsZ* und weitere Gene des Zellteilungsgenclusters (*dcw*) in *Prosthecobacter* und in allen verrucomicrobiellen Subdivisions mit kultivierbaren Vertretern identifiziert werden. Die Sequenz- und Transkriptionsanalysen deuteten darauf hin, dass diese Gene funktionell und phylogenetisch konserviert sind. Folglich kann ein auf FtsZ basierender Zellteilungsmechanismus für *Verrucomicrobia* sowie für das Schwesterphylum *Lentisphaerae* vorgeschlagen werden. Phylogenetische 23S rRNA Untersuchungen unterstützten eine Verwandtschaft von *Verrucomicrobia*/*Lentisphaerae* zu Planctomyceten und Chlamydien. Die Ergebnisse der Untersuchung der genomischen Anordnung der *dcw* Gene lassen schließen, dass der letzte gemeinsame Vorfahre der genannten Phyla einen auf FtsZ-basierenden Zellteilungsmechanismus aufwies, der wiederum unabhängig voneinander verloren wurde. Die genomische Anordnung der *btub*-Gene zeigte, dass diese in einem typischen bakteriellen Operon lokalisiert sind, zusammen mit einem bakteriellen Kinesin Light Chain-ähnlichem Gen (*bklc*). Mittels RT-PCR und Western Hybridisierungen wurde zum ersten Mal die Expression aller Gene des *btub*-Operons in *Prosthecobacter* nachgewiesen. Die Ergebnisse von Lokalisationsstudien (TEM, Immunofluoreszenz, Immunogold) in *Prosthecobacter* und rekombinanten *E. coli* deuten darauf hin, dass bakterielle Tubuline auch *in vivo* polymerisieren und Protofilamente bilden können. Dadurch könnten diese zur

charakteristischen Morphologie von *Prosthecobacter* beitragen. Auf Grund von phylogenetischen Untersuchungen der bakteriellen A und B Tubuline wurden zwei neue Tubulin-Subfamilien vorgeschlagen. Mehrere überzeugende Hinweise deuten an, dass *Prosthecobacter* die *btub*-Gene durch horizontalen Gentransfer erworben hat. Im Gegensatz zu einer früheren Hypothese schlägt die vorliegende Studie einen bakteriellen anstatt eines eukaryontischen Donor vor.

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APPENDICES

Appendix A

Coexistence of tubulins and *ftsZ* in different *Prosthecobacter* species

Martin Pilhofer, Giovanna Rosati, Wolfgang Ludwig, Karl-Heinz Schleifer, Giulio Petroni

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LETTERS

Coexistence of Tubulins and *ftsZ* in Different *Prosthecobacter* Species

Martin Pilhofer, Giovanna Rosati,† Wolfgang Ludwig,* Karl-Heinz Schleifer,* and Giulio Petroni†*

*Lehrstuhl für Mikrobiologie, Technical University Munich, Freising, Germany; and †Dipartimento di Biologia, University of Pisa, Pisa, Italy

Prosthecobacter, one of the few cultivable representatives of the bacterial phylum *Verrucomicrobia*, is of increasing interest to the scientific community due to the presence of tubulin genes in its genome and the apparent absence of the bacterial homologue FtsZ that is normally involved in prokaryotic cell division. These findings suggested the possibility of a vicarious takeover of the FtsZ function through these novel tubulins and opened new scenarios on the possible evolution of bacterial cytoskeleton and cell division. In the present manuscript, we report the characterization of *ftsZ* and *ftsA* homologues in different *Prosthecobacter* species that also possess tubulin genes. Based on these findings, we propose an FtsZ-based cell division mechanism in *Verrucomicrobia*. The analysis of available genome data of *Verrucomicrobia* suggests that tubulins are not a feature common to all members of this phylum. Therefore, it can be assumed that *Prosthecobacte*r acquired tubulins through horizontal gene transfer. The functional role of tubulins in *Prosthecobacter* remains enigmatic.

The hypothesis that bacteria contain a cytoskeleton that is related to the eukaryote cytoskeleton was first established when the bacterial Z-ring, which plays a key role during bacterial cell division, was visualized using green fluorescent protein–labeled FtsZ. FtsZ is a protein with a secondary structure that mirrors tubulin (Lowe and Amos 1998; Nogales et al. 1998) and displays in vitro similar dynamic properties (reviewed in Addinall and Holland 2002; Stricker et al. 2002). Although FtsZ is incapable to form microtubule-like structures, the combined structural and functional properties make it unlikely that FtsZ and tubulin proteins evolved twice (Erickson 1998); therefore, eukaryotic tubulin and bacterial FtsZ are considered to be homologous proteins.

Despite microtubule-like structures have been reported several times in bacteria (Bermudes et al. 1994; Petroni et al. 2000), the first molecular indications of the presence of tubulin genes in the bacteria are rather recent. In 2002, during the analysis of the genome sequence (95% completion) of *Prosthecobacter dejongeii*, Jenkins et al. reported the presence of 2 genes showing a higher similarity to eukaryotic tubulin than to bacterial *ftsZ*. These genes were referred to as bacterial A tubulin (*btubA*) and bacterial B tubulin (*btubB*) because of their apparent similarity to eukaryotic alpha and beta tubulins. However, no FtsZ genes were found in the genome sequence of *P. dejongeii* (Jenkins et al. 2002). Later biochemical studies showed that BtubA and BtubB are able to associate in vitro into heterodimers that form long filaments. (Schlieper et al. 2005; Sontag et al. 2005).

Prosthecobacter dejongeii is one of the few cultivable representatives of the still poorly investigated bacterial phylum *Verrucomicrobia*, which is phylogenetically related to *Chlamydiae* and *Planctomycetes* (Wagner and Horn 2006). Intriguingly, the latter 2 are the only bacterial phyla that do not possess FtsZ and rely on a yet unknown cell division mechanism (Read et al. 2000; Gloeckner et al. 2003; Horn et al. 2004; Strous et al. 2006).

These findings suggested the possibility of a vicarious takeover of the FtsZ function through the *btub*s in *Verrucomicrobia*, thus opening novel scenarios on the evolution of the eukaryotic cell. To evaluate this hypothesis, we accurately screened several *Prosthecobacter* species and their closest cultivated relative, *Verrucomicrobium spinosum*, for the presence of *ftsZ* and tubulin genes.

The complete nucleotide sequence coding for the 2 *btub* genes, *btubA* and *btubB*, of *P. dejongeii* was already published as well as the partial sequences of these genes in *Prosthecobacter vanneervenii* and *Prosthecobacter debontii* (Jenkins et al. 2002). We confirmed the presence of 1 A tubulin and 1 B tubulin gene in *P. vanneervenii*, and the sequence of both open reading frames together with a connecting spacer was completed. In addition, we could detect and completely sequence 2 further A and B tubulin genes in *P. debontii*. This finding was also confirmed by Southern blot and hybridization experiments. *Prosthecobacter debontii btubA* and *btubB* partial sequences characterized by Jenkins et al. (2002) do not exist as adjacent loci, but each of them is adjacent to the newly identified *btub* genes. Therefore, we renamed the *btub* genes in *P. debontii*. Henceforth, *P. debontii btubA* (Jenkins et al. 2002) is renamed *btubA2* and is followed by the newly characterized *btubB2*; the newly characterized *btubA1* precedes *P. debontii btubB* (Jenkins et al. 2002) that is renamed *btubB1* (table 1). Several combinations of primers were used in polymerase chain reaction (PCR) attempts to detect tubulin genes in *V. spinosum* but without any success (table 1). This negative result was later confirmed by Blast analysis of *V. spinosum* genome data (sequence complete and all gaps closed, update 7 May 2005).

Despite the apparent absence of *ftsZ* in *P. dejongeii* (Jenkins et al. 2002; Staley et al. 2005), we could detect a sequence coding for FtsZ in that organism using consensus degenerate hybrid oligonucleotide primers (Rose et al. 1998) in PCR. Moreover, *ftsZ* was also identified in *P. debontii* and *P. vanneervenii* (accession numbers AJ888907,

Key words: bacterial tubulin (*btub*), FtsZ, *Prosthecobacter*, *Verrucomicrobia*.

E-mail: gpetroni@biologia.unipi.it.

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Table 1
Presence of *btubA*, *btubB* and *ftsZ* in Representatives of *Verrucomicrobia*, *Chlamydiae*, and *Planctomycetes*

	btubA, *btubB*	Evidence	*ftsZ*	Evidence
Prosthecobacter vanneervenii	*btubA*, *btubB*	This study, Jenkins et al. 2002	+	This study
	btubA1	This study	+	This study
	btubB1	This study, Jenkins et al. 2002		
	btubA2	This study, Jenkins et al. 2002		
Prosthecobacter debontii	*btubB2*	This study		
Prosthecobacter dejongeii	*btubA*, *btubB*	Jenkins et al. 2002	+	This study
Prosthecobacter fusiformis	*btubA*, *btubB*	Jenkins et al. 2002	nd	
Verrucomircobium spinosum	—	Genome project	+	Genome project
Chlamydiae	—	Genome projects	−	Genome projects
Planctomycetes	—	Genome projects	−	Genome projects

Note.—+, gene present; −, gene(s) absent; nd, not determined; *Chlamydiae* stands for *Chlamydia*, *Chlamydophila*, *Protochlamydia*; *Planctomycetes* stands for *Candidatus* Kuenenia stuttgartiensis and *Rhodopirellula baltica*.

AJ888908, AM498604). The retrieved sequences were used to detect an open reading frame with protein sequence similarities to FtsZ also in the sequence data of the ongoing *V. spinosum* DSM 4136 genome project (TIGR_240016, contig 534) (table 1).

Prosthecobacter and *Verrucomicrobium* FtsZs exhibit most of the typical FtsZ features and some peculiar characteristics. Like typical bacterial FtsZ, they can be divided into the 4 domains (N-terminus, core, spacer, and C-terminus) as defined by Vaughan et al. (2004).

The sequences present the typical features of functional FtsZ. First, 6 out of 6 characteristic motifs of FtsZ were identified by PRINTS fingerprint scan (Attwood et al. 2003) (probability values between 3.4×10^{-49} and 3.9×10^{-44}; see table 2 and its extended version in supplementary fig. S1, Supplementary Material online). Second, the tubulin signature motif [S/A/G]GGTG[S/A/T]G (PROSITE motif PS00227) is always present and perfectly conserved (supplementary fig. S1, Supplementary Material online). Third, amino acids which contact guanosine diphosphate (Lowe and Amos 1998; Nogales et al. 1998) are conserved or conservatively exchanged with the exception of position N70H according to *Methanocaldococcus jannaschii* sequence (supplementary fig. S2, Supplementary Material online). Other nonconservative substitutions in the core domain are 1) position D235G (supplementary fig. S2, Supplementary Material online), a highly conserved position located within the T7-loop which is considered to be important for GTPase activity (Scheffers and Driessen 2001) and FtsZ polymerization (Cordell et al. 2003); and 2) the C-terminal end of the core domain, generally represented by the conserved tripeptide ATG and replaced in *Verrucomicrobia* by the tripeptide SSL. In all characterized *Verrucomicrobia*, the substituted amino acids are conserved, thus suggesting that functional constraints are still present at these positions although the substitutions are different from those occurring in other bacteria.

Residues that have been demonstrated to be involved in protein–protein interaction, for example, with FtsA (Yan et al. 2000; Haney et al. 2001) are located in the C-terminal domain of FtsZ. These amino acids are arranged in a nonapeptide and are followed by a stretch of variable length, which is rich in basic amino acids (Vaughan et al. 2004). This feature is considered typical of a functionally active FtsZ and is also present in *Verrucomicrobia*. Moreover, the nonapeptide of the investigated *Verrucomicrobia* shows a good conservation in comparison to the bacterial consensus sequence (Vaughan et al. 2004) especially in positions which, in *Escherichia coli*, have been shown to be important for the protein conformation (Mosyak et al. 2000)

Table 2
Sequence Analysis of Different FtsZ, Btub, and Eukaryotic Tubulin Protein Sequences

			FtsZ		Tubulin	
Organism	Phylogenetic group	Protein	No. of motifs	*P* value	No. of motifs	*P* value
Escherichia coli	*Proteobacteria*	FtsZ	**6 of 6**	$\mathbf{3.4 \times 10^{-79}}$	2 of 9	6.3×10^{-07}
Prosthecobacter dejongeii	*Verrucomicrobia*	FtsZ	**6 of 6**	$\mathbf{3.4 \times 10^{-49}}$	2 of 9	3.4×10^{-07}
Prosthecobacter vanneervenii	*Verrucomicrobia*	FtsZ	**6 of 6**	$\mathbf{2.1 \times 10^{-48}}$	2 of 9	1.6×10^{-06}
Prosthecobacter debontii	*Verrucomicrobia*	FtsZ	**6 of 6**	$\mathbf{1.0 \times 10^{-46}}$	2 of 9	2.6×10^{-07}
Verrucomicrobium. spinosum	*Verrucomicrobia*	FtsZ	**6 of 6**	$\mathbf{3.9 \times 10^{-44}}$	—	—
P. dejongeii	*Verrucomicrobia*	BtubA	2 of 6	8.3×10^{-06}	**9 of 9**	$\mathbf{1.4 \times 10^{-62}}$
P. dejongeii	*Verrucomicrobia*	BtubB	3 of 6	4.3×10^{-05}	**9 of 9**	$\mathbf{6.4 \times 10^{-73}}$
P. vanneervenii	*Verrucomicrobia*	BtubA	2 of 6	1.4×10^{-06}	**9 of 9**	$\mathbf{3.4 \times 10^{-61}}$
P. vanneervenii	*Verrucomicrobia*	BtubB	2 of 6	1.5×10^{-08}	**9 of 9**	$\mathbf{1.2 \times 10^{-72}}$
P. debontii	*Verrucomicrobia*	BtubA1	2 of 6	8.2×10^{-06}	**9 of 9**	$\mathbf{3.5 \times 10^{-61}}$
P. debontii	*Verrucomicrobia*	BtubB1	2 of 6	4.1×10^{-09}	**9 of 9**	$\mathbf{1.6 \times 10^{-72}}$
P. debontii	*Verrucomicrobia*	BtubA2	2 of 6	8.3×10^{-06}	**9 of 9**	$\mathbf{4.5 \times 10^{-58}}$
P. debontii	*Verrucomicrobia*	BtubB2	3 of 6	1.4×10^{-10}	**9 of 9**	$\mathbf{2.2 \times 10^{-73}}$
Arabidopsis thaliana	*Eukarya*	TUA3	2 of 6	1.1×10^{-08}	9 of 9	2.7×10^{-97}

Note.—Protein sequences analyzed with PRINTS (Attwood et al. 2003); *P* value, probability value (based on scoring matches to the motifs). In bold are reported the values for the FtsZ–FtsZ and bacterial tubulin-tubulin matches.

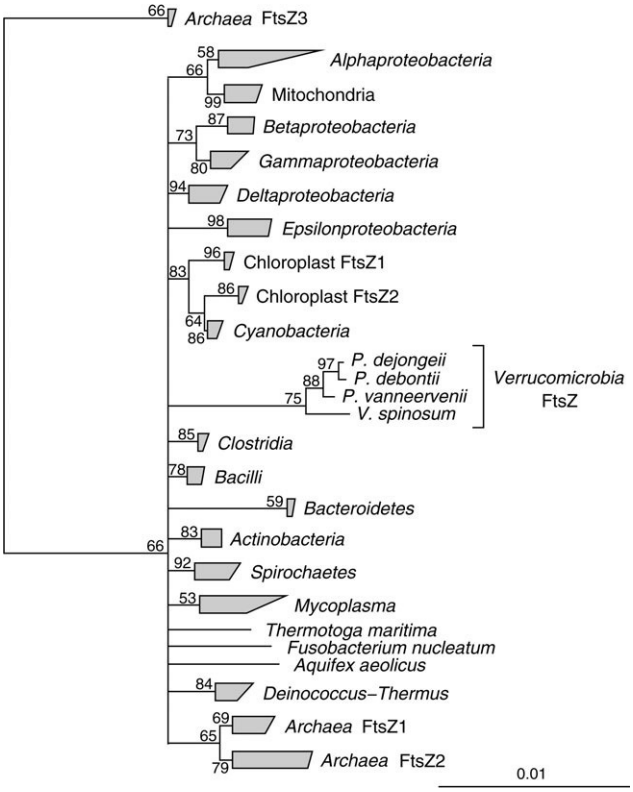

FIG. 1.—Comparative sequence analysis of FtsZ protein sequences of bacteria, archaea, and eukaryotic organelles representatives. Phylogenetic tree produced using Tree-Puzzle (Schmidt et al. 2002) (prot_30 filter, 1,000 puzzling steps, mixed rate of heterogeneity). Only the core domain was used for calculation. The *Prosthecobacter dejongeii* sequence was not complete. Archaeal FtsZ3 was used as outgroup. Numbers represent confidence values in percent. Verrucomicrobial FtsZs cluster together forming a monophyletic group, also the other major bacterial groups are recovered. Compared with the majority of other groups, verrucomicrobial FtsZs present a longer branch indicative of their sequence peculiarities.

or are thought to be involved in interactions with FtsA (Haney et al. 2001) (supplementary fig. S3, Supplementary Material online).

Phylogenetic analyses were performed on the core domain protein sequences using the ARB program package (Ludwig et al. 2004). They indicate a steady monophyly of verrucomicrobial FtsZ independently from the applied algorithm. One representative tree is shown in figure 1; the other calculated trees are available in FtsZ_ClustalW ARB database at http://www.arb-home.de. Calculated trees clearly indicate that the phylogenetic information retained by FtsZ is relatively limited and, in most cases, is not sufficient to resolve relationships above the phylum level, as it was also shown in earlier studies (Faguy and Doolittle 1998; Gilson and Beech 2001). Verrucomicrobial FtsZ always cluster together as independent lineage, thus supporting the existence of specific evolutionary constraints for these genes.

The genomic environment of *P. debontii* and *P. vanneervenii* FtsZ was additionally investigated. It shows the presence of an open reading frame similar to *ftsA*. FtsA is an actin homologue that is also involved in bacterial cell division. Moreover, the *V. spinosum* genome reveals a cluster of genes involved in cell division, comprising open reading

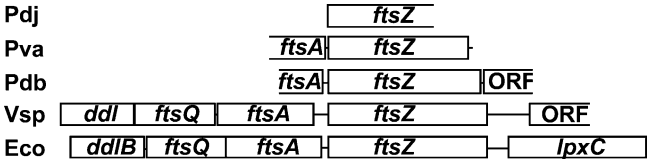

FIG. 2.—Detected *ftsZ* genes and their genomic environment in *Verrucomicrobia* and *Escherichia coli*. *Prosthecobacter vanneervenii* (Pva) and *Prosthecobacter debontii* (Pdb) show an open reading frame with similarities to *ftsA* upstream of *ftsZ*; *Verrucomicrobium spinosum* (Vsp) presents 3 open reading frames functionally related to cell division: *ftsA*, *ftsQ*, *ddl* (D-ala D-ala ligase). This gene order is conserved also in distantly related species, for example, in *E. coli* CFT073 (Eco). Partial *ftsZ* was characterized in *P. dejongeii* (Pdj).

frames with similarities to D-alanine-D-alanine-ligase, *ftsQ*, *ftsA*, and *ftsZ* (fig. 2). This gene order is highly conserved and also found in other distantly related organisms (e.g., *E. coli* CFT073) (Faguy and Doolittle 1998).

The following properties indicate that the identified *ftsZ* genes are functionally active in *Verrucomicrobia*: 1) all characteristics typical of functional FtsZ are present; 2) verrucomicrobial FtsZ is evolutionary constrained; and 3) other typical bacterial cell division genes are present in these organisms.

The simultaneous presence of functional FtsZ in *Prosthecobacter* spp. and *Verrucomicrobium* together with tubulin genes in the genus *Prosthecobacter* is a strong indication that FtsZ and not tubulin is the major protein involved in cell division in the *Verrucomicrobia*.

The comparison of *Prosthecobacter* tubulins and verrucomicrobial FtsZs shows only a low sequence similarity (see table 2 and its extended version in supplementary fig. S1, Supplementary Material online) and indicates that *Prosthecobacter* tubulins did not directly derive from *Prosthecobacter* FtsZ. The apparent absence of tubulin genes in *V. spinosum* and the great divergence between *Prosthecobacter* FtsZ and tubulins would favor the hypothesis that tubulin sequences were acquired by *Prosthecobacter* through horizontal gene transfer as it was already suggested by other authors (Schlieper et al. 2005). In any case, the origin and especially the function of *Prosthecobacter* tubulins and of those tubulins supposed to be present in other representatives of the phylum, that is, epixenosomes (Rosati et al. 1993; Petroni et al. 2000), remain to be elucidated.

Supplementary Material

Materials and Methods and figures S1–S3 are available at *Molecular Biology and Evolution* online (http://www.mbe.oxfordjournals.org/). The DNA sequences reported in this work have been deposited in the EMBL nucleotide database (accession numbers AJ888907, AJ888908, AM041148-AM041150, AM498604). ARB database is available at http://www.arb-home.de.

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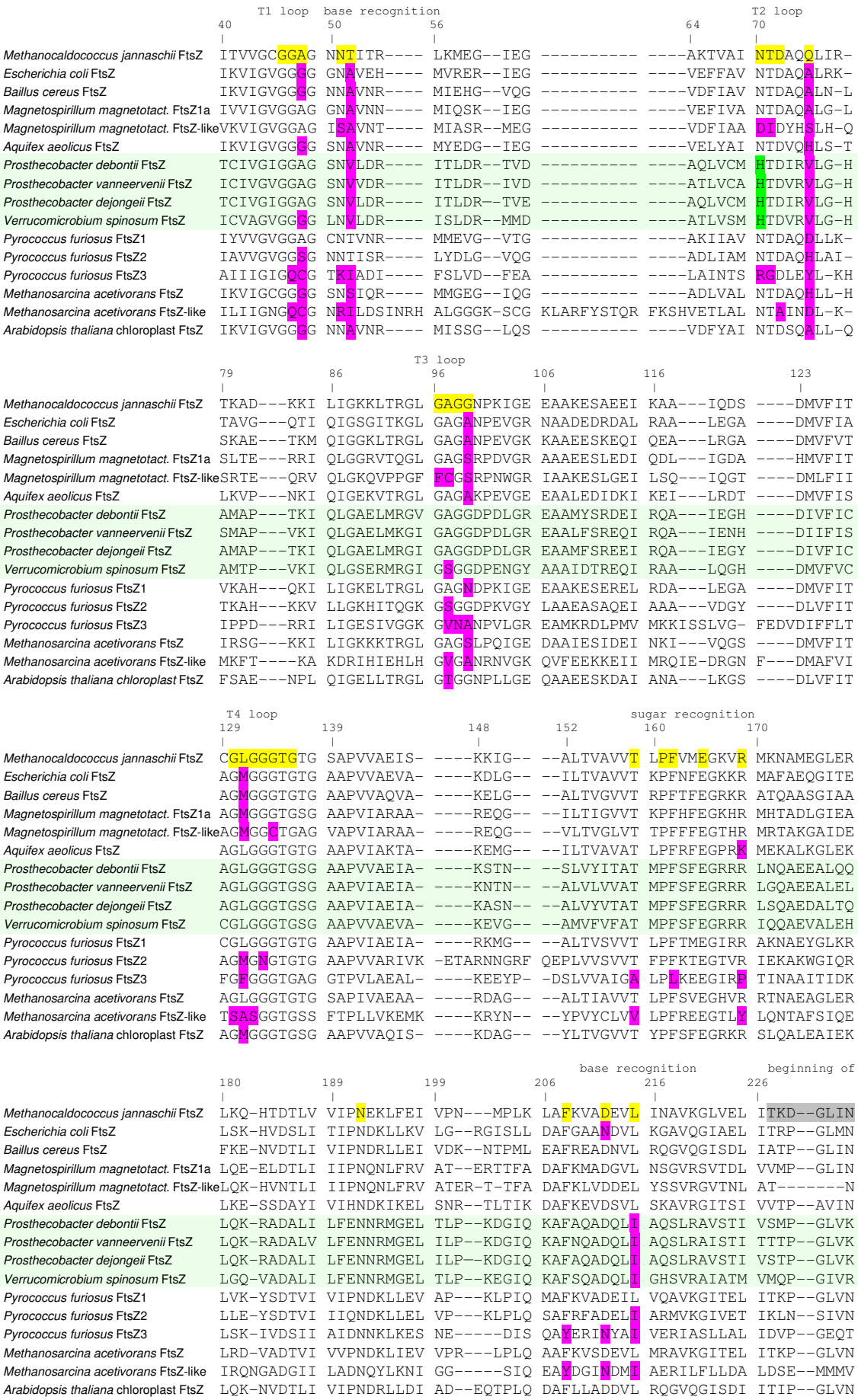
Supplementary Material

Figures S1-S3

Organism	Protein/ genome accession No.	Phylogenetic group	Protein	FINGERPRINTSCAN				Tubulin domain [S/A/G]GGTG[S/A/T]G
				No. of FtsZ motifs	P value	No. of Tubulin motifs	P value	
Methanocaldococcus jannaschii	Q57816	*Euryarchaeota*	FtsZ1	6 of 6	$1.2e^{-71}$	-	-	GGGTGTG
Escherichia coli	NP_414637	*Proteobacteria*	FtsZ	6 of 6	$3.4e^{-79}$	2 of 9	$6.3e^{-07}$	GGGTGTG
Bacillus cereus	NC_003909	*Firmicutes*	FtsZ	6 of 6	$1.2e^{-82}$	-	-	GGGTGTG
Bacillus cereus	NC_003909	*Firmicutes*	FtsZ-like	-	-	2 of 9	$2.9e^{-05}$	GGGTGTG
Mycoplasma genitalium	AAC71445	*Firmicutes*	FtsZ	3 of 6	$8.8e^{-21}$	-	-	G**K**GTGTG
Magnetospirillum magnetotacticum	ZP_00054722	*Proteobacteria*	FtsZ1a	6 of 6	$1.6e^{-79}$	-	-	GGGTGSG
Magnetospirillum magnetotacticum	ZP_00054263	*Proteobacteria*	FtsZ-like	5 of 6	$1.5e^{-45}$	2 of 9	$1.1e^{-05}$	GG**C**TGAG
Aquifex aeolicus	O66809	*Aquificae*	FtsZ	6 of 6	$2.4e^{-64}$	2 of 9	$5.2e^{-08}$	GGGTGTG
Prosthecobacter vanneervenii	CAI61965.1	*Verrucomicrobia*	FtsZ	6 of 6	$2.1e^{-48}$	2 of 9	$1.6e^{-06}$	GGGTGSG
Prosthecobacter debontii	CAI61968.1	*Verrucomicrobia*	FtsZ	6 of 6	$1.0e^{-46}$	2 of 9	$2.6e^{-07}$	GGGTGSG
Prosthecobacter dejongeii	CAM57305.1	*Verrucomicrobia*	FtsZ	6 of 6	$3.4e^{-49}$	2 of 9	$3.4e^{-07}$	GGGTGSG
Verrucomicrobium spinosum	TIGR_240016	*Verrucomicrobai*	FtsZ	6 of 6	$3.9e^{-44}$	-	-	GGGTGSG
Pyrococcus furiosus	NP_579717	*Euryarchaeota*	FtsZ1	6 of 6	$2.9e^{-67}$	2 of 9	$8.9e^{-09}$	GGGTGTG
Pyrococcus furiosus	NP_578254	*Euryarchaeota*	FtsZ2	6 of 6	$1.2e^{-54}$	-	-	G**N**GTGTG
Pyrococcus furiosus	NP_579236	*Euryarchaeota*	FtsZ3	4 of 6	$3.6e^{-13}$	-	-	GGGTGAG
Methanosarcina acetivorans	NP_619135	*Euryarchaeota*	FtsZ1	6 of 6	$5.2e^{-73}$	2 of 9	$8.0e^{-08}$	GGGTGTG
Methanosarcina acetivorans	NP_615906	*Euryarchaeota*	FtsZ-like	2 of 6	$2.2e^{-08}$	2 of 9	$3.6e^{-05}$	SGGTGS**S**
Methanopyrus kandleri	AAM02076	*Euryarchaeota*	FtsZ-like-1	-	-	-	-	GG**AV**G**YA**
Arabidopsis thaliana (chloroplast)	AAA82068	*Eukarya*	cpFtsZ	6 of 6	$9.7e^{-77}$	-	-	GGGTGSG
Arabidopsis thaliana	NP_197478	*Eukarya*	TUA3	2 of 6	$1.1e^{-08}$	9 of 9	$2.7e^{-97}$	GGGTGSG
Arabidopsis thaliana	NP_199247	*Eukarya*	TUB4	2 of 6	$2.7e^{-09}$	9 of 9	$9.3e^{-111}$	GGGTGSG
Prosthecobacter dejongeii	AAO12155	*Verrucomicrobia*	BtubA	2 of 6	$8.3e^{-06}$	9 of 9	$1.4e^{-62}$	GGGTGSG
Prosthecobacter dejongeii	AAO12159	*Verrucomicrobia*	BtubB	3 of 6	$4.3e^{-05}$	9 of 9	$6.4e^{-73}$	GGG**S**GSG

Figure S1. Sequence analysis of different FtsZ, FtsZ-like and tubulin protein sequences.
Protein sequences analyzed with PRINTS: Verrucomicrobial FtsZ (green), FtsZ and FtsZ-like from other bacteria (yellow), archaeal FtsZ and FtsZ-like (blue), eukaryotic chloroplast FtsZ (grey), bacterial and eukaryotic tubulins (white). FtsZ-like sequences (Vaughan et al. 2004) and non FtsZ sequences are highlighted in orange. The number of FtsZ and tubulin domains detected in each protein is reported. In the adjacent column the corresponding probability (P) values are displayed. P value is the probability value based on scoring matches to the motifs. The tubulin signature motif (PROSITE motif PS00227) is reported in the last column; mismatches in bold. FtsZ gene designations are according to Vaughan et al. (2004). Verrucomicrobial FtsZ presents all typical FtsZ motifs whereas *Prosthecobacter* tubulins present the typical tubulin motifs.


T1 loop
base recognition
T2 loop
T3 loop
T4 loop
sugar recognition
base recognition
beginning of
Methanocaldococcus jannaschii FtsZ
Escherichia coli FtsZ
Baillus cereus FtsZ
Magnetospirillum magnetotact. FtsZ1a
Magnetospirillum magnetotact. FtsZ-like
Aquifex aeolicus FtsZ
Prosthecobacter debontii FtsZ
Prosthecobacter vanneervenii FtsZ
Prosthecobacter dejongeii FtsZ
Verrucomicrobium spinosum FtsZ
Pyrococcus furiosus FtsZ1
Pyrococcus furiosus FtsZ2
Pyrococcus furiosus FtsZ3
Methanosarcina acetivorans FtsZ
Methanosarcina acetivorans FtsZ-like
Arabidopsis thaliana chloroplast FtsZ

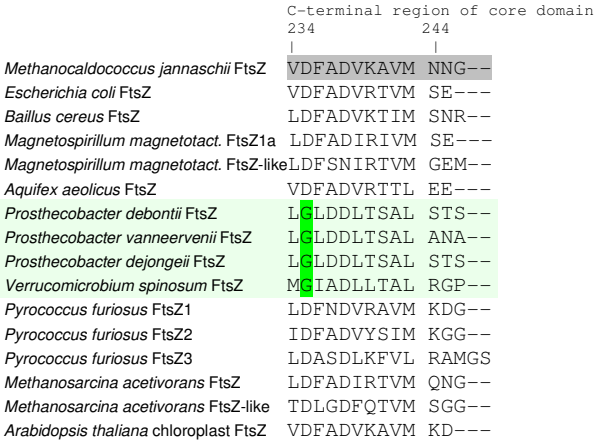


Figure S2. Sequence alignment of different FtsZ and FtsZ-like partial core sequences.
A selection of sequences from organisms presented in figure S1 (Supplementary Material online) is shown (partial core sequences; positions according to reference sequence *M. jannaschii*), tubulin and not alignable FtsZ-like sequences were excluded. FtsZ sequences from *Verrucomicrobia* are shaded in green. Residues highlighted in yellow make contact with GDP (Lowe and Amos 1998; Nogales et al. 1998), mismatches to these positions are highlighted in red. The beginning of the C-terminal region of the core domain is shaded in grey. Two highly conserved positions which are non-conservatively exchanged in *Verrucomicrobia* FtsZ (N70H, G235D) are highlighted in green. The third non conservative substitution (SSL versus ATG) is located at the end of the core domain and is not included in the figure.

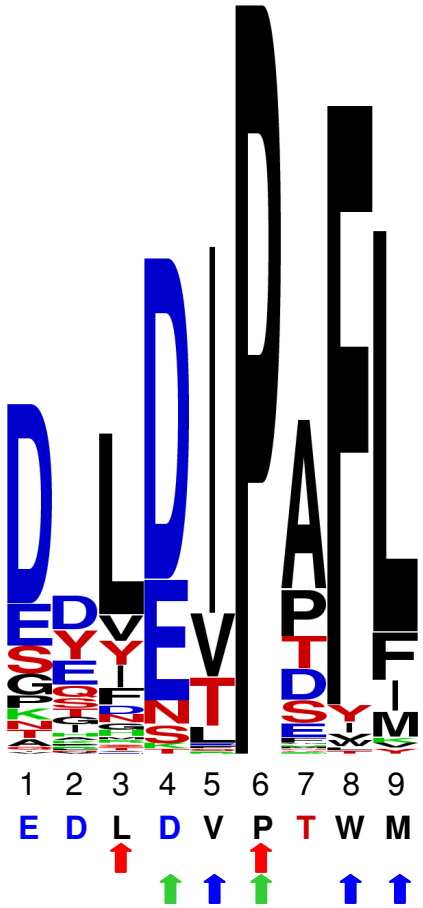


Figure S3. Comparison of the conserved C-terminus motif of *Verrucomicrobia* FtsZ and other FtsZ homologues.
The used dataset for the sequence logo production (Schneider and Stephens 1990; Crooks et al. 2004) were all FtsZ C-terminus sequences available at http://www.biomed2.man.ac.uk/addinall/vaughan_supp.html (version 26-08-2004) (Vaughan et al. 2004). The heights of the letters represent the frequency of the amino acid at that position (position 1 corresponds to position 616 of *P. debontii* FtsZ). The colors of the letters represent the different residue characteristics (blue: acidic, green: basic, red: neutral/polar, black: neutral/nonpolar). The motif of *Verrucomicrobia* FtsZs is shown below the positions. The conservation in the positions important for interaction with FtsA (red arrows) (Haney et al. 2001) and for the conformation of the peptide (green arrows) (Mosyak et al. 2000) is high. The positions considered to be important for interaction with ZipA (blue arrows) (Mosyak et al. 2000) appear less conserved.

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Supplementary Material

Materials and Methods

Bacterial Strains and Culture

Cultures of *Prosthecobacter dejongeii* DSM12251, *Prosthecobacter debontii* DSM14044, *Prosthecobacter vanneervenii* DSM12252 and *Verrucomicrobium spinosum* DSM4136 were obtained from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany) and grown at 28° C. *Prosthecobacter* strains were grown aerobically in DSM medium 628, *V. spinosum* was grown aerobically in DSM medium 607.

DNA Extraction

DNA from the *Prosthecobacter* strains and *V. spinosum* was extracted according to Wisotzkey, Jurtshuk and Fox (1990).

Detection and amplification of *ftsZ*

A major fragment of *ftsZ* in *Prosthecobacter* strains was initially amplified using Consensus Degenerate Hybrid Oligonucleotide Primers (CODEHOP) (Rose et al. 1998; Rose, Henikoff and Henikoff 2003) (5'-3') TGCGCTGCAGGGGCATGAYATGGTNTT respectively GATATTCGTGTGCTCGGNCAYGCNATG (forward) and GGGCTCTACGGCCAGNCCR AACATDAT respectively TTGATCCGCCTGAGCRAANGCYTTYTG (reverse) in PCR with 5 cycles with 30 sec. 45° C annealing and 1 min. 72° C extension, followed by 30 cycles with 15 sec. 55° C annealing and 1 min. 72° C extension.

Detection of tubulin genes in *Verrucomicrobia* by PCR

Attempts to detect tubulin genes in *Verrucomicrobia* were performed using PCR with a high number of different combinations of primers, designed according to different strategies [e.g. consensus primers, degenerate primers or CODEHOP (Rose et al. 1998; Rose, Henikoff and Henikoff 2003)].

Primers used for the search of tubulin genes.

Primers which were used to serch tubulin genes in *Verrucomicrobia* using PCR

Primer	Sequence	Description
DNEALF_R	RAAIAGIGCYTCRTTRTC	
EGAGGNFA_A_F	GARGGIGCIGGIGGNAAYTTYGC	
GGSGSG_F	GIGIGGITCIGNTCNG	
GGSGSG_R	CCIGAICCIGAICCNCCNCC	
GTP_BIND_Fi1	GGIGGIGGIACIGGNACNGG	
GTP_BIND_Fn1	GGNGNGGNACNGGNTCNGG	
GTP_BIND_Fn2	GGNGGNGGNACNGGNAGYGG	
GTP_BIND_Ri1	CCIGAICCIGTICCNCCNCC	
GTP_BIND_Rn1	CCNGANCCNGTNCCNCCNCC	
GTP_BIND_Rn2	CCRCTNCCNGTNCCNCCNCC	
PGAANNWA_B_F	GGIGCIGCIAAYAAYTGGC	**Primers targeting tubulin genes**
PGAANNWA_B_R	GCCCARTTRTTIGCNGCNCC	
PrM1_ex_Rn	TAYTGRTTYCCRCAYTGNCC	
PrM1_Fi	GGICARTGYGGNAAYCA	
PrM1_Fn	GNCARTYGNAAYCA	
PrM4_Fn	GAYAAYGARGCNYTNTT	
PrM4_Ri	YAAIARIGCYTCRTTRTC	
PrM4_Rn	YAANARNGCYTCRTTRTC	
TNLVPQP_A_R	GGYTIGIACNARRTTNGT	
YVPRA_VDLEP_F	TAYGTICCICGIGCGTIGAYYTNGARCC	
YWIPTAF_A_R	RAAIGCIGTIGGDATCCARTA	
Bac_Tub_AB_F183	TGGCARSTAYGTGCCACG	
Bac_Tub_AB_F209	TGGTGGAYYTGGAGCC	
Bac_Tub_AB_F420	GCACKCCATCGGTGGCGGT	
Bac_Tub_AB_F546	GGYKRYGGAGCCYTACAA	
Bac_Tub_AB_F1408	AARGCNTTYGCNAAYTGGTA	
Bac_Tub_AB_R142	ACYTTRTGRAARAANACYTCCAT	**Primers targeting bacterial tubulin genes**
Bac_Tub_AB_R183	CGTGGCACRTASYTGCCA	
Bac_Tub_AB_R209	GGTTCCARRTCCACCA	
Bac_Tub_AB_R298	ACRTTRTANCCGCCCARTTRTT	
Bac_Tub_AB_R420	ACCGCCACCGATGGMGTGC	
Bac_Tub_AB_R625	CYSRAAMAGCGCCTC	
DLEP_F_c1	AAGTACGTGCCCCGCGCCGTCATGGTGGAYCTNGARCC	
ANNWA_F_B_cod	AGCAGCATCGTCCGCAAGATCCCCGGTGCCGCNAAYAAYTGGGC	
DLEP_F_c2	AAGTACGTGCCCCGCGCCGTCATGGTGGAYTTRGARCC	
DNEA_R_A_cod	GTTACACTTGCGGTGGGCCAGATCGAAGAGNGCYTCRTTRTC	
DNEA_R_B_cod	GTTCAGCTTGGCTTTGGCGATGCGGAAGAGNGCYTCRTTRTC	**CODEHOP targeting tubulin genes**
EALF_F_c1	GATGCCGCCCTGATCTTGGATAATGARGCNCTNTTY	
EALF_F_c2	GATGCCGCCCTGATCTTGGATAATARGCNTTRTTY	
GFNA_F_A_cod	GCCAACCTATCTCCCGCACGAAGGTGCTGCCGGNAAYTTYGC	
YVPR_R_c1	GGCTCCAGGTCCACCATGACGGCGCGNGGNACRTA	

Southern blotting and hybridization

The presence of duplicated *btub* genes in *P. debontii* was confirmed by Southern blotting and hybridization (Sambrook and Russel 2001). Enzymatically digested *P. debontii* and *P. vanneervenii* DNA were loaded on agarose gel and were transferred to a positively charged nylon membrane after electrophoresis. The membrane was hybridized with two different digoxigenin labeled probes targeting *btubA1* and *btubB1* respectively. Hybridizations were performed at 37° C with formamide concentrations in the hybridization buffer ranging between 20 and 50% according to requested stringency conditions.

Cloning and sequencing

PCR products were cloned using a commercially available TOPO TA cloning kit (Invitrogen, Carlsbad, USA). Sequencing of the plasmid inserts was performed by MWG (Ebersberg, Germany).

Primer walking

After cloning and sequencing of the PCR products, the genomic environment was characterized using a PCR based gene walking strategy. PCR reactions were performed using only one specific outward pointing primer. The PCR steps were as follows: 30 cycles at high stringency condition and 3 min. 72° C extension (linear amplification), one cycle at low stringency condition (annealing temperature 40° C) and 3 min. 72° C extension (to allow unspecific binding of the primer, thus it can also function as reverse primer) and 30 cycles at high stringency condition and 3 min. 72° C extension (exponential and stringent amplification of the amplified fragments). The PCR products were directly sequenced using nested specific primers by MWG (Ebersberg, Germany).

Sequence Analyses

FtsZ, FtsZ-like, tubulin protein sequences were analyzed using the program PRINTS (http://umber.sbs.man.ac.uk/cgi-bin/dbbrowser/fingerPRINTScan/muppet/FPScan.cgi) (Attwood et al. 2003) in order to detect motifs and to calculate a probability value for the FtsZ and for the tubulin fingerprint.

The sequence logo of the C-terminus region of FtsZ sequences was produced using WEBLOGO (http://weblogo.berkeley.edu) (Schneider and Stephens 1990; Crooks et al. 2004) including all FtsZ C-terminus sequences available at http://www.biomed2.man.ac.uk/addinall/vaughan_supp.html (update 26-08-2004) (Vaughan et al. 2004).

Open reading frames in the genomic environment of *ftsZ* genes were detected with the ORF-finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) (Wheeler et al. 2003) and similarities to

known protein sequences were searched using protein-protein BLAST (http://www.ncbi.nih.gov/BLAST/) (Altschul et al. 1997).

The cell division gene cluster in *V. spinosum* was detected by TBLASTN of *P. debontii* FtsZ against the genome sequence of *V. spinosum*. Preliminary sequence data were obtained from The Institute for Genomic Research website at http://www.tigr.org. Sequencing of *Verrucomicrobium spinosum* DSM 4136 was accomplished with support from National Science Foundation.

Phylogenetic analysis of FtsZ sequences

The FtsZ protein core sequence dataset (269 amino acid entries) which was used for phylogenetic analyses of FtsZ was imported from http://www.biomed2.man.ac.uk/addinall/vaughan_supp.html (version 26-08-2004) (Vaughan et al. 2004) and was completed by verrucomicrobial FtsZ sequences obtained in this study. The initial alignment was produced by using ClustalW (Thompson, Higgins and Gibson 1994) of the ARB program package (Ludwig et al. 2004) and was refined manually. The database FtsZ_ClustalW is available at http://www.arb-home.de.

Two minimum similarity filters prot_10 and prot_30 were calculated which retained only positions conserved respectively in at least 10% or 30% of selected 49 sequences representatives of the major bacterial groups.

Phylogenetic analyses were performed using distance matrix methods [programs ARB neighbor joining and Phylip UPGMA (Sneath and Sokal 1973), FITCH (Fitch and Margoliash 1967), and KITSCH (Felsenstein 1988)], maximum parsimony [program Phylip PROTPARS (Eck and Dayhoff 1966)], maximum likelihood [program Phylip PROML (Felsenstein 1988) and TREE-PUZZLE (Schmidt et al. 2002)]. All programs are implemented in the ARB program package (Ludwig et al. 2004). For distance matrix methods and maximum likelihood method the used model of substitution was Dayhoff PAM matrix (Dayhoff, Schwartz and

Orcutt 1978), for TREE-PUZZLE method Muller-Vingron model of substitution (Muller and Vingron 2000) was used.

Each analysis was repeated using the two filters prot_10 and prot_30. Neighbor joining and maximum parsimony trees were bootstrap resampled (1000 respectively 100 bootstraps) number of puzzling steps for TREE-PUZZLE trees was 1000. The *P. dejongeii* FtsZ sequence is partial and was therefore not used for calculation of all trees. The topologies of the 14 produced trees were compared. Database FtsZ_ClustalW comprises all produced trees.

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Appendix B

Characterization of bacterial operons consisting of two tubulins and a kinesin-like gene by the novel Two-Step Gene Walking method

Martin Pilhofer, Andreas Peter Bauer, Martina Schrallhammer, Lothar Richter, Wolfgang Ludwig, Karl-Heinz Schleifer, Giulio Petroni

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Characterization of bacterial operons consisting of two tubulins and a kinesin-like gene by the novel Two-Step Gene Walking method

Martin Pilhofer[1], Andreas Peter Bauer[1], Martina Schrallhammer[2], Lothar Richter[3], Wolfgang Ludwig[1], Karl-Heinz Schleifer[1] and Giulio Petroni[2,*]

[1]Lehrstuhl für Mikrobiologie, Technical University Munich, Am Hochanger 4, D-85354 Freising, Germany, [2]Dipartimento di Biologia, University of Pisa, via A. Volta 4/6, I-56126 Pisa, Italy and [3]Machine Learning and Data Mining in Bioinformatics, Technical University Munich, Boltzmannstraße 3, D-85748 Garching b. München, Germany

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ABSTRACT

Tubulins are still considered as typical proteins of *Eukaryotes*. However, more recently they have been found in the unusual bacteria *Prosthecobacter* (*btubAB*). In this study, the genomic organization of the *btub*-genes and their genomic environment were characterized by using the newly developed Two-Step Gene Walking method. In all investigated Prosthecobacters, *btubAB* are organized in a typical bacterial operon. Strikingly, all *btub*-operons comprise a third gene with similarities to kinesin light chain sequences. The genomic environments of the characterized *btub*-operons are always different. This supports the hypothesis that this group of genes represents an independent functional unit, which was acquired by *Prosthecobacter* via horizontal gene transfer. The newly developed Two-Step Gene Walking method is based on randomly primed polymerase chain reaction (PCR). It presents a simple workflow, which comprises only two major steps—a Walking-PCR with a single specific outward pointing primer (step 1) and the direct sequencing of its product using a nested specific primer (step 2). Two-Step Gene Walking proved to be highly efficient and was successfully used to characterize over 20 kb of sequence not only in pure culture but even in complex non-pure culture samples.

INTRODUCTION

Sequencing of DNA using a specific primer requires a template, which must consist of a maximum of target-DNA and a minimum of non-target DNA. Any kind of non-target DNA can lead to unspecific binding of the sequencing primer. The standard methods to shift the ratio towards target-DNA are either to amplify the target-DNA using the PCR technique (1–3) or to clone the target-DNA into a vector (phage or plasmid), which is highly amplified in a bacterial host and subsequently specifically isolated. If the target-DNA is represented by a specific gene, the typical approach to accomplish its characterization is to design two degenerated primers, targeting highly conserved regions of the gene. The intervening region is then amplified, cloned and sequenced. This approach, which is nowadays a routine procedure, rapidly provides sequence data on yet uncharacterized genes belonging to known gene families. The main limit of this approach is that both extremities of the investigated gene remain unknown. Despite several methods have been proposed, the characterization of a sequence adjacent to a known region (gene walking or chromosome walking) still remains a laborious and time-consuming task.

In the past, there were reports that suggested the possibility to perform direct sequencing of genomic DNA to characterize unknown regions adjacent to known ones without any kind of preliminary amplification (4,5). Despite the theoretical advantages of this approach, they never became standard and have been applied only in a very restricted number of cases. The reported main problems concern: (i) the high amount of DNA used (several micrograms) (4,5); (ii) the unspecific binding of the sequencing primer due to the bad ratio of target-DNA over non-target DNA resulting in very short read lengths or in complete failure of the sequencing run (4,5); (iii) the target organisms' genomes, which must possess a G + C ratio in a certain range (5); and (iv) the specific set-up of the sequencing instruments and software, which hampers the use of custom sequence services (4). For these reasons,

*To whom correspondence should be addressed. Tel: +39 050 2211384; Fax: +39 050 2211393; Email: gpetroni@biologia.unipi.it

PCR-based gene walking methods are generally applied and largely favored over direct sequencing of genomic DNA for the characterization of unknown sequences adjacent to known ones.

PCR-based gene walking methods can be divided into three groups (based on their underlying methods): (i) inverse PCR (6–9), (ii) ligation-mediated PCR (10–19) and (iii) randomly primed PCR (20–34). Using inverse PCR and ligation-mediated PCR, as a first step DNA has to be digested with restriction enzymes. Inverse PCR amplifies unknown DNA adjacent to known DNA fragments after its intramolecular circularization, which is performed by a ligase at low DNA concentrations. The region of interest is then amplified using outward pointing primers located at both ends of the known fragment. In ligation-mediated PCR, synthetic adapter-DNAs, which are ligated to restriction fragments, are used as primer annealing sites for PCR. The region of interest is then amplified by a known sequence-specific outward-pointing primer and a primer reverse complementary to the ligated adapter. A disadvantage of ligation-mediated PCR methods is the requirement of relatively high amounts of target DNA. Furthermore, ligation-mediated PCR and inverse PCR are dependent upon time-consuming and labor-intensive enzymatic modifications of the target DNA (e.g. restriction digest and ligation reactions).

Randomly primed PCR methods use the ability of oligonucleotides (primers) to bind unspecifically in the unknown fragment at low stringency conditions (20). There are two possibilities to facilitate priming in the unknown region. Some methods use different types of walking primers to bind in the unknown region (20,22–24,26,27,29,31,33,34). Other experimental approaches utilize only one primer in PCR, which binds specifically in the known sequence stretch and in very low stringency cycles also in the unknown region (21,25,28,30,32). The unspecific binding of these primers causes the facilitation of unspecific PCR products. Therefore, the existing randomly primed PCR methods include cloning and/or enrichment procedures (e.g. immobilization of specific products on paramagnetic beads). The derived PCR products or clones must be intensively screened for specificity, e.g. using modified oligonucleotides in hybridization experiments.

In this study, a straightforward Two-Step Gene Walking method was developed. It consists of a Walking-PCR (step 1) and direct sequencing of the PCR product (step 2). There is no need for enrichment, cloning or screening procedures; the amount of template DNA for Walking-PCR is minimal (50 ng). The new method was developed for the characterization of the genomic environment of bacterial tubulin genes in *Prosthecobacter* and was proven to work even in complex non-pure culture samples.

Tubulins are typical eukaryotic proteins. The first molecular evidences for real tubulin genes in bacteria were reported by Jenkins *et al.* (35) and later confirmed and extended by Pilhofer *et al.* (36) in different *Prosthecobacter* species. Bacterial A and B tubulin genes (*btubAB*) exist as adjacent loci on the genome. Jenkins *et al.* (35) described the presence of a gene downstream of *btubB* in *Prosthecobacter dejongeii*, which has similarity to kinesin light chain. But to date, no sequence information was published. Therefore, recent intensive biochemical studies on the bacterial tubulin genes of *Prosthecobacter* could not include that third gene (37,38), which may be functionally related to the bacterial tubulin genes. The aim of this study was to characterize and comparatively analyze the genomic organization and the genomic environment of *btub* genes in different *Prosthecobacter* species using the newly developed Two-Step Gene Walking technique.

MATERIALS AND METHODS

Cultures

Cultures of *Prosthecobacter vanneervenii* DSM12252 and *Prosthecobacter debontii* DSM14044 were obtained from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany) and grown aerobically at 28°C in DSM medium 628. *Euplotidium itoi* with epixenosomes as ectosymbionts was grown according to Rosati *et al.* (39). *Paramecium caudatum* infected with *Caedibacter caryophilus* was cultured in Sonneborn's *Paramecium* medium (ATCC medium 802) inoculated with *Klebsiella planticola*.

DNA Extraction

DNA from *Prosthecobacter* was isolated according to Wisotzkey *et al.* (40). DNA from epixenosomes was extracted using a modified protocol to inhibit high nuclease activity. The lysis of the cells was directly performed in high SDS concentration without previous lysozyme treatment. SDS (10%) was added in ratio 1:1 to the cell resuspension, followed by the addition of Proteinase K (0.625 mg/ml) and RNAse (0.25 mg/ml). The mixture was incubated for 1 h at 55°C. Following steps were as described by Wisotzkey *et al.* (40).

Prior to DNA extraction of *C. caryophilus*, the *Paramecium* culture was starved for ten days to minimize contaminations by food bacteria. Twenty liters of paramecia were harvested with a cream separator (Westfalia Separator AG) and further concentrated with an oil-testing centrifuge for 3 min at 300 *g*. The concentrated paramecia were mechanically homogenized and the crushed cells were washed in phosphate-buffered saline (PBS). The pellet was resuspended in 1:1 PBS and 90% Percoll (Amersham), and applied on the top of a Percoll gradient. The gradient was build up of 90%, 70% and 50% Percoll (in 1× PBS). Gradient centrifugation was carried out at 10 000 *g* at 4°C for 20 min. *Caedibacter caryophilus* and other bacteria were recovered from the fraction between 70 and 90% and washed again in PBS. DNA was extracted from the bacteria contained in this fraction according to Wisotzkey *et al.* (40).

Two-Step Gene Walking Procedure

The Two-Step Gene Walking method consists of a Walking-PCR (step 1) and direct sequencing of the

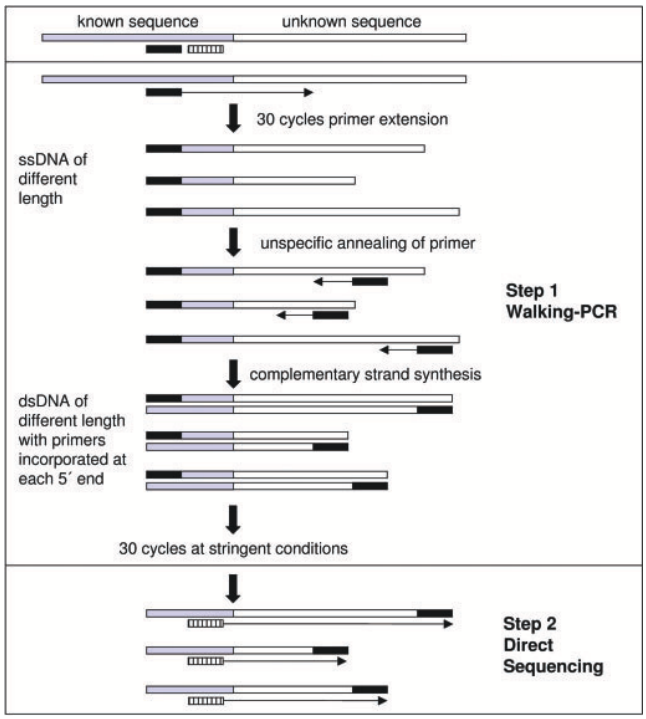

Figure 1. Basic principle of the Two-Step Gene Walking procedure. Known sequence stretch is shown in gray; unknown sequence stretch is shown in white; Walking-PCR primer is shown in black; specific nested sequencing primer is shown striped. In first 30 cycles, the PCR primer binds at stringent conditions; specific ssDNA of different length (caused by different drop off sites of polymerase) is produced. One subsequent cycle at low annealing temperature allows unspecific binding of primer at different sites on ssDNA as reverse primer. dsDNA of different length with primer sequence incorporated at each 5-prime end is produced. Thirty cycles at stringent conditions specifically and exponentially amplify dsDNA. PCR product is sequenced directly by using a specific nested primer.

PCR product (step 2). The basic principle is outlined in Figure 1. Primers were obtained from MWG Biotech AG (Germany) and their sequences are listed in supporting material. Walking-PCRs were performed using 0.25 µl (100 µM) of one specific primer, 0.25 µl Ex-Taq (Takara Bio Inc., Japan), 5 µl Ex-*Taq* buffer (10×), 5 µl dNTP mixture (2.5 mM each), 38.5 µl ultra pure water and 1 µl template DNA (50 ng). PCRs were performed using a Primus 96 Plus (MWG Biotech AG, Germany) and the cycling program shown in Table 1.

Aliquots of Walking-PCR products were examined through agarose gel electrophoresis. The rest was purified using Perfectprep Kit (Eppendorf, Germany) to remove the remaining primers of the Walking-PCR. Typically, 5 µl of purified PCR product were directly sequenced by MWG Biotech AG (Germany) using a specific unmodified nested primer (supporting material) and an ABI 3730 XL system. For partially characterized PCR products, complete sequence was obtained through successive sequencing of the PCR product with newly designed nested primers (primer walking).

Chromatograms were analyzed and sequence stretches overlapping with known sequences were searched using CHROMAS (Version 2.01, www.technelysium.com.au/chromas.html). Contigs were assembled manually and open reading frames were searched using ORF-Finder (41). Similarities to known protein sequences were searched using protein–protein BLAST (42).

Sequences with similarities to kinesin light chain were analyzed using the program PRINTS in order to detect motifs of the kinesin light chain fingerprint (43). Multiple EM for Motif Elicitation (MEME, http://meme.sdsc.edu/meme/website/meme.html) (44) was used to discover motifs in the bacterial kinesin light chain sequences. The Neuronal Network Promoter Prediction software (45) was used to identify putative promoter sequences. The prediction of Rho-independent terminators was performed with the program FindTerm (www.softberry.com/berry.phtml?topic=findterm&group=programs&subgroup=gfindb).

Table 1. Walking-PCR cycling program

Round	Cycles	Temperature	Time	Action
		94°C	4 min	Primary denaturation
1	30	94°C 56°C 72°C	30 s 30 s 3 min	Specific primer extension; ssDNA synthesis
2	1	94°C 40°C 72°C	30 s 30 s 3 min	Unspecific binding of primer; dsDNA synthesis
3	30	94°C 56°C 72°C	30 s 30 s 3 min	Specific exponential amplification
		72°C	10 min	Final elongation

RESULTS AND DISCUSSION

Technical facts and theory of Two-Step Gene Walking

Initially, the authors attempted gene walking in *Prosthecobacter* genomes based on inverse PCR (6), Arbitrary PCR (22), SiteFinding-PCR (34) (randomly primed PCR methods) and ligation-mediated gene walking (13) (data not shown). To overcome the poor results and the low efficiency of these methods and to perform gene walking also in non-pure culture samples, the Two-Step Gene Walking technique was developed. Altogether, over 20 kb of sequence were characterized by Two-Step Gene Walking in very different sample systems.

Over 85% of the performed attempts were successful using Two-Step Gene Walking. In the other cases, there was either no detectable Walking-PCR product or unreadable sequence raw data. But in every case it was possible to keep on walking replacing the Walking-PCR primer, the sequencing primer or both of them.

Walking-PCR primers and sequencing primers that produced unambiguous results meet the following criteria: (i) C-rich sequence at the 3-prime end (preferably CC or CCC), (ii) size of 18 to 25 bp and (iii) melting temperature of 60 to 63°C. Primer design according to these criteria

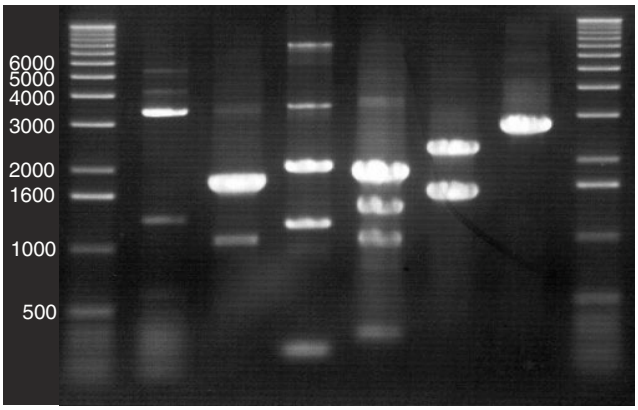

Figure 2. Typical walking-PCR product patterns. Different Walking-PCR products, which were successfully used for direct sequencing with nested specific primers, are shown. Patterns show big variety based on different templates and different primers. 5 µl aliquots were loaded on a 1% agarose gel and subjected to electrophoresis. 1 kb DNA Ladder (Invitrogen, USA) is shown in first and in last lane. Numbers stand for corresponding fragment lengths of respective bands.

allowed the development of a standard Walking-PCR program (Table 1). The annealing temperature is adjusted to 56°C in specific-annealing cycles and to 40°C in the unspecific-annealing cycle. For confident contig assembly, a minimal distance of 70 bp is required between the priming site of the sequencing primer and the end of the known sequence.

The Walking-PCR products analyzed on agarose gels showed in most cases a pattern representing fragments of different sizes (Figure 2). This can be explained by different drop off sites of the polymerase and due to different binding sites of the primer during the unspecific-annealing cycle. It results in a mixture of specific PCR products, which are heterogeneous in length. Also, a fraction of unspecific fragments is expected, because the primer could also bind unspecifically in other regions of the genome during the low stringency cycle. To estimate the portion of unspecific fragments produced during the Walking-PCR, cloning experiments were performed (data not shown). Some Walking-PCR products, with which gene walking as described above was successful, were selected. The proportion of clones with specific inserts did not exceed the proportion with unspecific inserts. This suggests that there is a significant fraction of unspecific amplification during the Walking-PCR, nevertheless the unspecific products do not interfere with the following step of direct sequencing. Therefore, direct sequencing with a specific nested primer has to be favored over a cloning and sequencing approach.

The chromatograms of successful sequencing attempts showed different typical features. In most cases, the sequence quality was high up to 600–900 bp (Figure 3A). Partially sequenced PCR products could be completely characterized using a newly designed nested primer, which was used for sequencing of the same PCR product (primer walking). In most cases, the Walking-PCR products were long enough to use them in multiple successive sequencing reactions. Thus, there is no need for the performance of a new Walking-PCR before the sequence of a Walking-PCR product is completely characterized through primer walking. This is crucial for the high efficiency of the Two-Step Gene Walking method.

A second typical feature of chromatograms is the type of sequence at the 3-prime end of a Walking-PCR product. Regularly, the reverse complement Walking-PCR primer sequence could be detected at the end of a sequenced fragment (Figure 3B). This is in accordance with the theory, which proposes unspecific binding of the Walking-PCR primer during the unspecific-annealing cycle. Therefore, the reverse complement sequence of the Walking-PCR primer has to be removed at the 3-prime sequence end of a completed PCR product. Further gene walking steps can elucidate the real genomic sequence, to which the Walking-PCR primer bound unspecifically. As expected, the genomic site of unspecific annealing of the Walking-PCR primer was in most cases quite complementary to the Walking-PCR primer (e.g. 12 complement base pair of 21 bp primer length; Figure 3B), especially at the 3-prime end that is fundamental for the proper binding of the polymerase. Also, the Walking-PCR product patterns on agarose gels (Figure 2) support this, showing mainly multiple sharp bands instead of a smear.

Another chromatogram feature was only sporadically observed, but illustrates the mechanism of Two-Step Gene Walking very clearly. Less than 3% of the obtained sequence raw data showed a short stretch (length of the Walking-PCR primer plus 1–2 bp) of clear peaks and double peaks within the sequence (Figure 3C). Afterwards, the chromatogram proceeded with peaks of high quality but with lower amplitudes. The region of ambiguities is produced by the concomitant presence of: (i) the 3-prime end of a short fragment that contains the reverse complement Walking-PCR primer sequence (generally, like in the case of Figure 3C, the higher peaks); (ii) the genomic sequence of the longer PCR products. The decrease of the peak amplitudes after the end of the shorter fragment can be explained by lower template amount. One or two double peaks adjacent to the primer-binding region emerge due to the terminal transferase 'A' activity of the used polymerase.

Genomic organization of bacterial tubulin genes

Initially, Jenkins *et al.* (35) reported the presence of bacterial tubulin (*btub*) genes in three *Prosthecobacter* species. Although no sequence data were released, the authors described the existence of a third gene, located downstream of bacterial B tubulin in *P. dejongeii*, that was referred to as kinesin light chain homologue. Pilhofer *et al.* (36) confirmed the presence of bacterial tubulin genes in *Prosthecobacter vanneervenii* and *P. debontii* and discovered a duplication of *btub* genes in the latter one.

Two-Step Gene Walking was used in this study to characterize the genomic environment of all *btub*-genes in *P. vanneervenii* and *P. debontii*. The EMBL nucleotide database entries AM041148 to AM041150 were extended with the new sequence data. Characterized genes and genomic environments are shown in Figure 4.

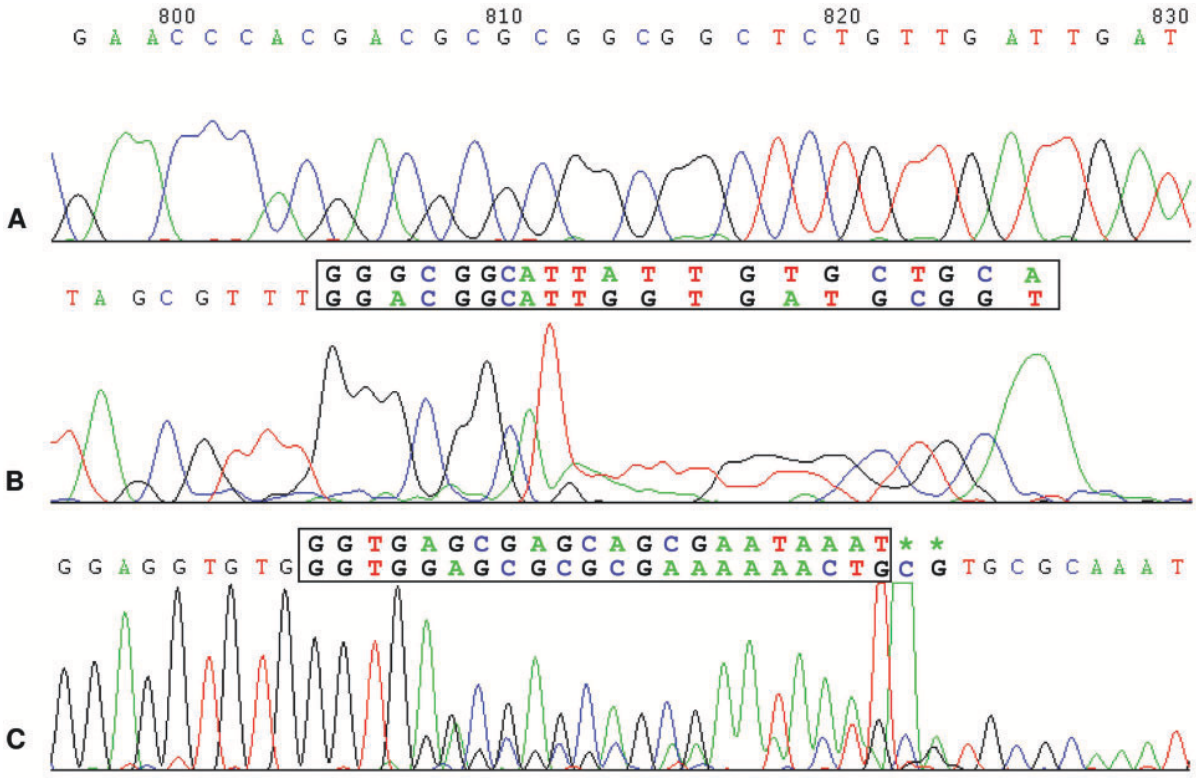

Figure 3. Features of sequence raw data. Panels show sequence raw data of three different Walking-PCR products, sequenced directly with specific nested primers. (**A**) displays high-quality sequence in region 800–830 bp; numbers indicate sequenced base pairs. (**B**) shows the sequence raw data at the end of a Walking-PCR fragment. Boxed sequence highlights Walking-PCR primer-binding region. Upper sequence displays reverse complement sequence of PCR primer, which is mainly recovered by peaks. Lower sequence displays real genomic sequence obtained through another walking step. (**C**) shows raw data of sequence at the end of one specific shorter Walking-PCR fragment and continuing with the sequence of one specific longer Walking-PCR fragment. This feature only occurs in <3% of all sequences. Boxed sequence highlights the binding region of the Walking-PCR primer. Upper sequence displays reverse complement sequence of the Walking-PCR primer; identical to higher peaks. Lower sequence displays real genomic sequence obtained through sequencing of reverse strand of same PCR product; identical to lower peaks. One or two additional double peaks at 3-prime end emerge due to terminal transferase activity (of adenosines) of used polymerase (indicated with asterisk).

All *Prosthecobacter* B tubulin genes characterized by Pilhofer *et al.* (36) are also followed by an open reading frame that shows some similarity to kinesin light chain. According to the original designation (35), this third gene will be referred to as *bklc* for bacterial kinesin light chain. The presence of *bklc* genes downstream of all bacterial B tubulin genes so far characterized clearly suggests that the bacterial kinesin light chain is an essential part of a functional unit, likely an operon, represented by one bacterial A tubulin, one bacterial B tubulin and one bacterial kinesin light chain [in accordance with (35)]. Henceforth, this cluster of genes will be referred to as bacterial tubulin operon (*btub*-operon).

Also gene expression features (e.g. promoters, ribosomal binding sites and terminators) support that these three genes are part of a single typical bacterial operon (see updated features of database entries AM041148 to AM041150) as well as RT–PCR results on *P. dejongeii* (35).

The analysis of the genomic environment of the *btub*-operon shows that the genes upstream of bacterial A tubulins always appear functionally related to those downstream of the bacterial kinesin light chain gene (Figure 4). None of the bordering genes indicated a functional relationship to cell cycle or cytoskeleton. In all three cases the *btub*-operon appears as an insert interrupting functionally related, but always different, genes.

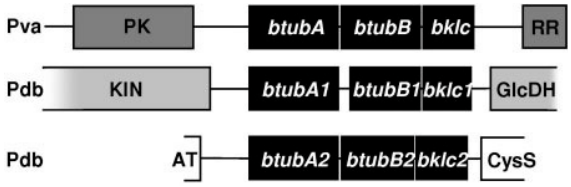

Figure 4. Bacterial tubulin operons including their genomic environment. Besides the same setup of all three bacterial tubulin operons (black), their genomic environments are different and show no functional relationship to cell division, cytoskeleton or typical eukaryotic genes. The two bordering genes of each operon show a functional relationship in any case (genes in dark gray involved in signal transduction mechanisms; genes in light gray involved in carbohydrate transport and metabolism; genes in white involved in amino acid transport and metabolism; PK: serine/threonine-protein kinase; RR: response regulator; KIN: pyruvate/orthophosphate dikinase; GlcDH: glucose/sorbosone dehydrogenase; AT: aspartate aminotransferase; CysS: cysteine synthase). Pva: *Prosthecobacter vanneervenii*, Pde: *Prosthecobacter debontii*

Genes showing low similarities to eukaryotic kinesin light chains have been described in *Bacteria* since 1997 [*Plectonema boryanum*; (46)], but nothing is known about their function. All *Prosthecobacter* bacterial kinesin light chain sequences show very low similarity to eukaryotic and bacterial kinesin light chain sequences detected using BLASTP ($3.7\,e^{-6}$ to $4.4\,e^{-7}$). Fingerprint analysis (43) of verrucomicrobial kinesin light chain sequences could recover only two or three out of the six typical kinesin light chain motifs. A tandem repeat of 4 respectively 5 tetratricopeptide repeat (TPR) domains could be detected in all *Prosthecobacter* Bklc sequences. TPR typically contains 34 amino acids and is found in both bacteria and eukaryotes; it is involved in many functions including protein–protein interactions (47). The function of *bklc* in *Prosthecobacter* still remains to be elucidated, but a functional relationship to *btub*-genes can be supposed based on its genomic organization within the *btub*-operon and the presence of TPR domains.

Application of Two-Step Gene Walking on non-pure culture systems

To our knowledge, the existing gene walking methods were only used for pure culture systems. Two-Step Gene Walking was successfully applied on two different complex non-pure culture sample systems, using the standardized protocol without any optimization.

Epixenosomes are episymbiotic bacteria inhabiting the surface of the hypotrich ciliate *Euplotidium* (48). PCR-analysis on the epixenosome rRNA operon with specific primers targeting 16S and 23S rRNA genes resulted in no amplification product (P. G., unpublished data). This suggested that 16S and 23S rRNA genes are not joined in this organism. Two-Step Gene Walking was applied to characterize the upstream region of the 23S rRNA gene by gene walking. Up to now, these organisms are not available as pure culture and can only be grown together with their host. Therefore, the sample used for DNA preparation also included the host organism *Euplotidium*, as well as several free-living bacteria that were present in the host culture and some of the algae used as food source for the host. Nevertheless, Two-Step Gene Walking proved to be successful to characterize a 1522 bp region upstream of the 23S rRNA gene. An open reading frame coding for a transposase, a $tRNA^{ile}$ gene and a $tRNA^{ala}$ gene (from 5-prime to 3-prime) could be detected. The nucleotide sequence was submitted to GenBank with accession number EF650087.

Caedibacter caryophilus are endosymbionts localized in the macronucleus of *P. caudatum* (49,50). *Paramecium caudatum* cannot be grown axenically and, in addition to regularly added food bacteria, a community of diverse free-living bacteria is present in the culture medium. These contaminants complicate each molecular approach. The 16S rRNA gene of *C. caryophilus* shows an unusual insertion of 194 bp within the 5-prime-terminal region. This sequence is not present in mature rRNA and is therefore called internal excised element (IEE) (51,52). Two-Step Gene Walking starting from the IEE was used to characterize the upstream region of the 16S rRNA gene. Two-Step Gene Walking was successfully used to characterize 936 bp of sequence. The sequence comprised the 5-prime end of the 16S rRNA gene of *C. caryophilus* and 767 bp of the upstream region. An open reading frame coding for a putative soluble lytic murein transglycosylase precursor was detected. The nucleotide sequence was submitted to EMBL nucleotide database with accession number AM743196.

CONCLUSIONS

In comparison to other gene walking techniques, Two-Step Gene Walking has striking advantages, which are detailed below. It proved to be a very simple gene walking method, applicable to very different organisms. The required minimal amount of DNA (50 ng) makes it also feasible to perform gene walking in organisms for which a limited amount of biomass is available, such as slowly growing or even uncultivable organisms. To our knowledge, Two-Step Gene Walking is the first gene walking technique, which was shown to work in complex non-pure culture samples. The method was always processed using the standardized protocol without any optimization. The two-step workflow can be processed within one day, comprising only one Walking-PCR with one specific primer and direct sequencing with one specific nested primer. Over 20 kb of sequence were characterized exclusively by Two-Step Gene Walking, which is more than it is reported for other gene walking techniques. The derived sequence reads showed very high quality up to 900 bp.

Concluding, Two-Step Gene Walking overcomes the major disadvantages of other gene walking techniques. Inverse PCR and ligation-mediated PCR require time-consuming and labor-intensive enzymatic modifications of the target DNA, a relatively high amount of target DNA (several micrograms) and other DNA modifying enzymes in addition to DNA polymerase. The advantages of Two-Step Gene Walking compared to other randomly primed PCR methods are the independence of (i) modified oligonucleotides (18,19,23,27,29,31), (ii) special walking primers (20,23,24,26,31,33,34), (iii) successive PCRs (20,23–27,29,31–34), (iv) cloning steps (21,25,26,28, 29,31,33,34), (v) procedures to screen for specificity or to enrich specific fragments (20,21,25,27–29,31–34) and (vi) other enzymatic modifications than the DNA polymerase (27). Some methods are restricted and optimized to only one walking step starting always from the same known sequence (e.g. an inserted transposon) (28,30). In contrast, the developed method enables theoretically to completely characterize one DNA molecule using successive gene walking steps and primer walking on Walking-PCR products. Potential applications of Two-Step Gene Walking are (i) rapidly obtaining full gene sequences, finding the corresponding promoters and regulatory elements, (ii) characterization of complete operons starting from a small known DNA fragment, (iii) characterization of primer binding sequences (particularly important when the used primers work suboptimal), (iv) identification of transposon integration

sites in known or unknown genomes for gene function studies and (v) gap closure in genome sequencing projects. The latter one could be also performed in a high-throughput scale due to the simple and standardized protocol of Two-Step Gene Walking.

The characterization of the genomic environment of *Prosthecobacter* tubulin genes using Two-Step Gene Walking revealed a typical bacterial operon structure. Strikingly, in addition to *btubA* and *btubB*, the *btub*-operon comprises also a third, kinesin-like gene (*bklc*). The sequence for *bklc* is reported here for the first time.

The *btub*-operons are integrated in a set of genes functionally related among themselves but apparently not with the *btub*-operon. The genomic environments of the different *btub*-operons are always different (Figure 4). These two facts further support the hypothesis of a horizontal gene transfer of *btub*-genes to *Prosthecobacter* (36,37), but the theory has now to be extended to the whole *btub*-operon including also *bklc*. This of course leads to a different view on these peculiar bacterial genes and will influence future functional studies on bacterial tubulins.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR online.

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Conflict of interest statement. None declared.

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Name	Sequence	Target region	Target organism	PCR	Sequencing
23S_Epix_62R	CTTGTGTCGCCAAGGCATCC	23S rRNA	epixenosomes		+
23S_Epix_96R	GGAAATTCCCGGGTCGAAGC	23S rRNA	epixenosomes	+	
23SEpixup1	CAAGAAATAGATCGAGCAGTCAATACC	23S rRNA upstream	epixenosomes	+	
Epix23S_up3	CACTGCGTCAGCTTCGCGTTTGAATCC	23S rRNA upstream	epixenosomes		+
Epix23Sup150	GAAACTTCTGGCCTGACGAAAACTT	23S rRNA upstream	epixenosomes	+	
Epix23S_up5	GCGAACCCTACCCATCACCCC	23S rRNA upstream	epixenosomes		+
IEE_Caedi_R2	AAGAATTGACACCCTTTCCC	16S rRNA	*C. caryophilus*	+	
IEE_Caedi_R3	CTCGGTACCTTTCCTTTCC	16S rRNA	*C. caryophilus*		+
PvAup7	GACGAGCACGCGATCCAGA	KIN	*P. vanneervenii*		+
PvAup6	CTCGCGCTCCTCAGTCAGAA	KIN	*P. vanneervenii*		+
PvAup5	CCCCATGAGCATCCATGCAC	KIN	*P. vanneervenii*		+
PvAup3	GAGTCCAAGCAAATTCCGCCC	*btubA* upstream	*P. vanneervenii*		+
PdevA2R295	AAGTTCCCGCCAGCACCTTC	*btubA*	*P. vanneervenii*		+
PvAr282	TCCAGACGGCTCATCACT	*btubA*	*P. vanneervenii*	+	
PdevB1F655	AGCCCGAATTACATGGAC	*btubB*	*P. vanneervenii*	+	
PvB+113F	CATACCACGATCCTATTCG	*bklc*	*P. vanneervenii*	+	+
PvbtubBeF	AAATCGCCTCCGCCTCCC	*btubB* downstream	*P. vanneervenii*		+
PdevkinesinF1	TGGTGGAGCAGATCAGTGTG	*bklc*	*P. vanneervenii*		+
Pvbklc_dwn1	CTTGGAGGAGCAAGACGCC	*bklc* downstream	*P. vanneervenii*		+
PvBdownF	GTCATCATAGGAGATGTCCAGAAC	*bklc* downstream	*P. vanneervenii*		+
PdePPDKup2	AACTTCAGAGCGGCAGCGG	PPDK	*P.debontii*		+
PdePPDKup1	ATGTCGTCACGGCTGATGCC	PPDK	*P.debontii*	+	+
PdeA1R91	TCCGTGCGCGAGATCAAA	*btubA1*	*P.debontii*		+
PdevA2R295	AAGTTCCCGCCAGCACCTTC	*btubA1*	*P.debontii*	+	
PdeA1up1	CGGTGCTGAGTCACTCTATCCC	*btubA1* upstream	*P.debontii*	+	
PdeA1up2	TGCCTTGGGTTAAGCTCCTTCC	*btubA1* upstream	*P.debontii*		+
PdeA1up3	GGACATTCTTTTCAGCAGCACC	PPDK	*P.debontii*	+	
PdePPDKR1/2	GTCGCCACCGATGGTGCC	PPDK	*P.debontii*		+
PdeB1down5	TCGGCACCAACCTGAACAGC	GlcDH	*P.debontii*		+
PdeB1down3	GCAAAACGGATGGTGAATACAGC	*bklc1*	*P.debontii*		+
PdeB1down	AAGCCGTGGACTCCCCTC	*bklc1*	*P.debontii*	+	+
PdeB1F602	TCATTTTGGATAACGAGGCGCTC	*btubB1*	*P.debontii*	+	
PdeA2up1	CGCCTTCTCTTTCTCTTACACC	*btubA2* upstream	*P.debontii*		+
PdeA2R255	GCTGGATTGAACAAGGAGCCC	*btubA2*	*P.debontii*		+
PdeA2R308	TGTAACCCACGGCGAAGTTCC	*btubA2*	*P.debontii*	+	
PdeB2down1	GCGTCCTGGCGAGTCTTC	*bklc2*	*P.debontii*	+	+
PdeB2down6	GGGAAACATCTACAACAACATCGTC	*bklc2* downstream	*P.debontii*		+
PdeB2+537F	GAACTTGGATGTCGAACACGCC	*bklc2*	*P.debontii*		+
PdeB2F655	AGCCCCACTTACATGGAC	*btubB2*	*P.debontii*	+	

Appendix C

Characterization and evolution of cell division and cell wall synthesis genes in the bacterial phyla *Verrucomicrobia*, *Lentisphaerae*, *Chlamydiae* and *Planctomycetes* and phylogenetic comparison with rRNA genes

Martin Pilhofer, Kristina Rappl, Christina Eckl, Andreas Peter Bauer, Wolfgang Ludwig, Karl-Heinz Schleifer, Giulio Petroni

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Characterization and Evolution of Cell Division and Cell Wall Synthesis Genes in the Bacterial Phyla *Verrucomicrobia*, *Lentisphaerae*, *Chlamydiae*, and *Planctomycetes* and Phylogenetic Comparison with rRNA Genes▽†

AQ: A

Martin Pilhofer,[1]* Kristina Rappl,[1] Christina Eckl,[1] Andreas Peter Bauer,[1] Wolfgang Ludwig,[1] Karl-Heinz Schleifer,[1] and Giulio Petroni[2]

Lehrstuhl für Mikrobiologie, Technical University Munich, Am Hochanger 4, D-85354 Freising, Germany,[1] and Dipartimento di Biologia, University of Pisa, via A. Volta 4/6, I-56126 Pisa, Italy[2]

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In the past, studies on the relationships of the bacterial phyla *Planctomycetes*, *Chlamydiae*, *Lentisphaerae*, and *Verrucomicrobia* using different phylogenetic markers have been controversial. Investigations based on 16S rRNA sequence analyses suggested a relationship of the four phyla, showing the branching order *Planctomycetes*, *Chlamydiae*, *Verrucomicrobia*/*Lentisphaerae*. Phylogenetic analyses of 23S rRNA genes in this study also support a monophyletic grouping and their branching order—this grouping is significant for understanding cell division, since the major bacterial cell division protein FtsZ is absent from members of two of the phyla *Chlamydiae* and *Planctomycetes*. In *Verrucomicrobia*, knowledge about cell division is mainly restricted to the recent report of *ftsZ* in the closely related genera *Prosthecobacter* and *Verrucomicrobium*. In this study, genes of the conserved division and cell wall (*dcw*) cluster (*ddl*, *ftsQ*, *ftsA*, and *ftsZ*) were characterized in all verrucomicrobial subdivisions (1 to 4) with cultivable representatives. Sequence analyses and transcriptional analyses in *Verrucomicrobia* and genome data analyses in *Lentisphaerae* suggested that cell division is based on FtsZ in all verrucomicrobial subdivisions and possibly also in the sister phylum *Lentisphaerae*. Comprehensive sequence analyses of available genome data for representatives of *Verrucomicrobia*, *Lentisphaerae*, *Chlamydiae*, and *Planctomycetes* strongly indicate that their last common ancestor possessed a conserved, ancestral type of *dcw* gene cluster and an FtsZ-based cell division mechanism. This implies that *Planctomycetes* and *Chlamydiae* may have shifted independently to a non-FtsZ-based cell division mechanism after their separate branchings from their last common ancestor with *Verrucomicrobia*.

Fn2

In the past decade, our view of the bacterial cytoskeleton has basically changed. Bacterial homologues of the three major eukaryotic cytoskeletal families, actin, tubulin, and intermediate filaments, have been identified: FtsA/MreB/ParM, FtsZ/BtubAB, and crescentin (reviewed in reference 9). Another bacterial homologue of tubulin (TubZ) was very recently detected in *Bacillus*. It was shown to polymerize in dynamic, linear polymers, which were suggested to be important for plasmid maintenance (21). Bacterial cell division is generally based on the tubulin homologue FtsZ, which is the structural protein forming a Z ring at the division site (5). In *Escherichia coli*, FtsA is the first protein known to be recruited to the Z ring and is thought to be important for stabilization of FtsZ (56, 57). FtsZ is found in almost all bacteria studied so far. However, the analysis of genome-sequencing data suggested that it may be absent in representatives of the three bacterial phyla *Verrucomicrobia*, *Chlamydiae*, and *Planctomycetes* (10, 15, 18, 37, 46).

The report of genuine tubulin genes in the genus *Prosthecobacter* (18), representatives of *Verrucomicrobia*, might have suggested a vicarious takeover of the FtsZ function through the bacterial tubulins in the phylum *Verrucomicrobia*. However, *ftsZ* genes were recently detected and characterized in three different *Prosthecobacter* species and in the incomplete genome sequence data for their closest relative, *Verrucomicrobium spinosum* (36).

Generally, *ftsZ* is located in a cluster of genes involved in cell division and in the synthesis of peptidoglycan precursors (2, 54). A prominent feature of this *d*ivision and *c*ell *w*all (*dcw*) cluster is that it is conserved in many bacterial genomes over a broad taxonomic range (33, 47, 55). This is remarkable, as genome organization, gene content, and gene order within operons or gene clusters are very dynamic in evolutionary terms (reviewed in reference 11). Further, a strong relationship between the organization/conservation of the *dcw* cluster and cell morphology has been detected. In contrast to cocci, the majority of rod-shaped and filamentous bacteria have been found to possess a conserved *dcw* cluster (47). The correlation suggests that cotranslational assembly of some *dcw* cluster gene products links gene order with cell morphology; this mechanism was referred to as genomic channeling (30, 31). As the last common ancestor of extant bacteria has been suggested to have had a rod shape (20), it was inferred that it also had a large *dcw* cluster (similar to that of *E. coli*), which has been maintained in some lineages and has been reduced in others (33). It has been speculated that the ancestral cluster might have arisen from the blending of a primordial *mur* operon with three other gene subsets, differing in their functional roles (30). Alternatively, but very unlikely, the complex gene structure of the *dcw* cluster might have been a recent acquisition

* Corresponding author. Mailing address: Lehrstuhl für Mikrobiologie, Technical University Munich, Am Hochanger 4, D-85354 Freising, Germany. Phone: 498161715456. Fax: 498161715475. E-mail: martin-pilhofer@web.de.

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that has arisen independently by convergent evolution in phylogenetically distant bacterial groups. The possibility that the *dcw* cluster spread through the bacterial domain by horizontal gene transfer is improbable because the comparative sequence analyses of the individual genes matches the 16S rRNA phylogeny (30). Indeed, genome analysis of deeply branching bacteria also strongly supports the possibility that the last common ancestor of extant bacteria had a large *dcw* cluster, which was secondarily reduced in different lineages (30).

The *dcw* cluster comprises 16 genes in *E. coli*. The first two genes at the 5-prime terminus (*mraWZ*) code for proteins with unknown functions (6). The *mur* genes, together with *mraY* and *ddl*, are essential and are involved in the synthesis of murein (peptidoglycan) precursors (52). In addition, the *dcw* cluster comprises six other *fts* genes that are essential for cell division; their products have been shown to localize at the division site during septation (27). Gene order and content are conserved to different extents in the different regions of the *dcw* cluster. A variable 5′ region is followed by a conserved block comprising *ftsI* to *murG*, followed by another variable region that may contain the genes *murC*, *murB*, and *ddl*; finally, there is another highly conserved block comprising the division genes *ftsQAZ* (47).

The present knowledge about cell division in the *Verrucomicrobia* is restricted to the characterization of *ftsZ* only in the very closely related genera *Prosthecobacter* and *Verrucomicrobium* (36, 64). However, the phylum *Verrucomicrobia* (13, 16) comprises five subdivisions consisting of very heterogeneous free-living and symbiotic species with only very few cultivable representatives.

On the basis of 16S rRNA gene phylogeny, *Verrucomicrobia*, *Chlamydiae*, and *Planctomycetes* form a monophyletic cluster, also comprising *Lentisphaera* and two additional candidate phyla (*Poribacteria* and OP3). Despite some studies that disagree (12, 16, 59), the establishment of the new PVC (*P*lanctomycetes-*V*errucomicrobia-*C*hlamydiae) superphylum has been proposed on the basis of 16S rRNA phylogeny (58). We tested the hypothesis of the PVC superphylum using comparative sequence analyses of 23S rRNA genes. We characterized the major bacterial cell division gene *ftsZ* and other *dcw* cluster genes in all verrucomicrobial subdivisions with cultivable representatives. Comprehensive sequence analyses and the comparison of the presence of *dcw* genes and their gene order in representatives of all phyla of the PVC superphylum is interpreted here in relation to the cell division mechanism in their last common ancestor.

MATERIALS AND METHODS

Cultures. All strains were cultured with shaking in 50 ml liquid media. Cultures of *Prosthecobacter dejongeii* DSM12251, *Prosthecobacter debontii* DSM14044, *Prosthecobacter vanneervenii* DSM12252, and *Opitutus terrae* DSM11246 were obtained from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Germany). *Akkermansia muciniphila* CIP107961 was obtained from the Pasteur Institute Collection (France). *Chthoniobacter flavus* (43) and isolate Ellin514 (44) were obtained from Peter H. Janssen (University of Melbourne, Melbourne, Australia). *Prosthecobacter* species were grown aerobically at 28°C in DSM medium 628. *O. terrae* was grown anaerobically at 30°C in DSM medium 298g. *A. muciniphila* was grown anaerobically at 30°C in brain heart infusion medium (Becton Dickinson and Company). Media for anaerobic cultures were degassed using a vacuum, and the gas atmosphere was exchanged for dinitrogen. *C. flavus* and strain Ellin514 were grown aerobically at 30°C in medium VL55 (41).

DNA extraction. DNA from *Prosthecobacter* was isolated according to the method of Wisotzkey et al. (63). DNA from *A. muciniphila*, *C. flavus*, *O. terrae*, and isolate Ellin514 was extracted using a modified protocol to inhibit high nuclease activity. Liquid cultures were centrifuged, and the pellet was resuspended in TE buffer (10 mM Tris/HCl, 1 mM EDTA disodium salt, pH 8). The lysis of the cells was directly performed in a high sodium dodecyl sulfate concentration without previous lysozyme treatment. Sodium dodecyl sulfate (10%) was added in a 1:1 ratio to the cell resuspension, followed by the addition of proteinase K (0.625 mg/ml) and RNase (0.25 mg/ml). The mixture was incubated for 1 h at 55°C. The subsequent steps were as described by Wisotzkey et al. (63).

Sequencing of 23S rRNA genes. 23S rRNA genes were characterized for *P. debontii*, *P. dejongeii*, *P. vanneervenii*, *A. muciniphila*, *C. flavus*, isolate Ellin514, and *O. terrae*.

Partial rRNA operons were amplified using the primers listed in the supplemental material. The PCR program was as follows: 35 cycles with 30 s of 50°C annealing and 4 min of 72°C extension. Direct sequencing of the 23S rRNA genes was performed by MWG Biotech AG (Germany) using the sequencing primers listed in the supplemental material.

Sequencing of *ddl* genes. In our previous study, consensus degenerate hybrid oligonucleotide primers (CODEHOP) (39, 40) were used to detect *ftsZ* in *Prosthecobacter* (36). Attempts to detect *ftsZ* in other *Verrucomicrobia* using a high number of different combinations of CODEHOP in PCR failed.

Therefore, CODEHOP were designed to target conserved domains of the D-Ala:D-Ala ligase (*ddl*) gene, which is usually located upstream of the *ftsQAZ* cluster. A fragment of the *ddl* gene was initially amplified using the same forward primer for all investigated species, targeting the *ddl* nucleotide sequences coding for the deduced protein domain GEDG. The reverse primers targeted the *ddl* nucleotide sequences coding for the deduced protein domains FYDI (*P. debontii* and *P. dejongeii*), FYDY (*P. vanneervenii*), EINT (*A. muciniphila*), GKEL (*C. flavus*), and NTIP (strain Ellin514 and *O. terrae*). Primer sequences are listed in the supplemental material. The PCR program was as follows: 5 cycles with 30 s of 45°C annealing and 1 min of 72°C extension, followed by 30 cycles with 15 s of 55°C annealing and 1 min of 72°C extension. PCR products were analyzed on 2% agarose gels. Selected fragments were cut from the gel, and DNA was extracted using the Perfectprep kit (Eppendorf, Germany). Fragments were cloned using a commercially available TOPO TA cloning kit (Invitrogen), and inserts were sequenced by MWG Biotech AG (Germany) using vector-specific primers.

Sequencing of the genomic environment of *ddl* and *ftsZ*. In *P. dejongeii* and *P. debontii*, specific primers targeting *ddl* and *ftsZ* were used in PCR to specifically amplify and subsequently sequence the intervening region comprising *ftsQA*. For the remaining investigated *Verrucomicrobia*, two-step gene walking, as described by Pilhofer et al. (35), was applied to characterize the genomic environment of the *ddl* gene. Attempts to walk downstream of the *O. terrae ddl* gene were not performed, as there are two ongoing genome projects on *Opitutaceae* isolates. The sequencing results were verified, and chimera formation during gene walking was excluded using both specific primers and genomic DNA as templates in PCR and Southern hybridization experiments (data not shown). The procedure was described previously (35).

Detection of *dcw* cluster genes in genome project data. The Integrated Microbial Genomes System (28) and BLASTP (1) were used to screen for the presence of *dcw* cluster genes in available genome project data.

At present, genome sequence data (unpublished genomes) for five isolates and strains belonging to *Verrucomicrobia* or *Lentisphaerae* (the closest relatives of *Verrucomicrobia*) are publicly available. The strains are *V. spinosum* (*Verrucomicrobia*, subdivision 1, *Verrucomicrobiae*), isolate DG1235 (*Verrucomicrobia*, subdivision 4, *Opitutaceae*), isolate TAV2 (*Verrucomicrobia*, subdivision 4, *Opitutaceae*), *Lentisphaera araneosa* (*Lentisphaerae*), and *Victivallis vadensis* (*Lentisphaerae*). The sequence data for isolate TAV2 and *Victivallis vadensis* were produced by the U.S. Department of Energy Joint Genome Institute (http://www.jgi.doe.gov/). Preliminary sequence data for isolate DG1235 and *L. araneosa* were obtained from the J. Craig Venter Institute or from the Gordon and Betty Moore Foundation Marine Microbial Genome Sequencing Project website (https://research.venterinstitute.org/moore/).

For *Chlamydiae*, the published genomes of *Chlamydia pneumoniae*, *Chlamydia trachomatis*, *Chlamydophila caviae*, *Chlamydophila felis*, *Chlamydophila abortus*, and "*Candidatus* Protochlamydia amoebophila" were analyzed (3, 15, 19, 37, 38, 51). For *Planctomycetes*, the genomes of *Rhodopirellula baltica* (10), "*Candidatus* Kuenenia stuttgartiensis" (46), *Planctomyces maris* (unpublished), and *Blastopirellula marina* (unpublished) were analyzed. Preliminary sequence data for *Planctomyces maris* and *Blastopirellula marina* were obtained from the J. Craig Venter Institute website (http://www.jcvi.org/).

RNA isolation and transcription analyses. Total RNA was isolated from *Prosthecobacter* cultures (20 ml; mid-log phase) by using Trizol reagent (Invitrogen). The protocol of the manufacturer was modified. After cells were pelleted by centrifugation, the cells were lysed by adding 1 ml Trizol reagent. The cells

were resuspended by vortexing them for 30 s and then incubated for 5 min at 20°C and for 1 h at −80°C. The subsequent steps were as described in the instructions of the manufacturer.

DNase (RQ1 RNase-Free DNase; Promega) treatment of total RNA was performed according to the instructions of the manufacturer. Subsequently, reverse transcription was carried out with the Revert Aid First Strand cDNA kit (Fermentas, Germany) according to the instructions of the manufacturer and by using random hexamer primers. One microliter of the obtained cDNA was used for PCR (25-μl final volume), together with specific primers (see the supplemental material) for the genes *ddl*, *ftsQ*, *ftsA*, and *ftsZ*.

Sequence analyses. Open reading frames (ORFs) were detected using the ORF-Finder at http://www.ncbi.nlm.nih.gov (62). Similarities to known protein sequences and to conserved protein domains were searched using protein-protein BLAST (1). The program MOTIF Search (http://motif.genome.jp/) was used in order to detect conserved domains in Ddl and FtsQAZ protein sequences.

Phylogenetic sequence analyses. Comparative sequence analyses were performed using the ARB program package (24). The 23S rRNA ARB database comprised the new 23S rRNA sequences obtained in this study and sequences of the major bacterial groups. For tree calculation, 96 (860 for RAxML) sequences were selected representing all available sequences of *Verrucomicrobia*, *Lentisphaerae*, *Chlamydiae*, *Planctomycetes*, and selected species of the major bacterial groups, some *Archaea*, and some 28S rRNA sequences of *Eukarya*. Minimum-similarity filters were calculated, which retained only positions conserved in at least 0%, 20%, or 50% of the selected sequences. The phylogenetic analyses were performed using each filter in combination with each treeing program. The treeing methods used were distance matrix methods (Phylip NEIGHBOR and FITCH), maximum parsimony (Phylip DNAPARS), and maximum likelihood (RAxML, AxML, and PHYML DNA). All programs are implemented in the ARB program package (24). Maximum-parsimony and maximum-likelihood trees were bootstrap resampled (1,000 bootstraps). As a nucleotide substitution model, the Kimura-2-Parameter was used for distance matrix methods and PHYML; the HKY85 model was used for RAxML.

The FtsZ_ClustalW ARB database published by Pilhofer et al. (36) was extended with the new sequences obtained in this study. A Ddl ARB database was established importing the Ddl protein sequences obtained in this study and Ddl proteins of other major bacterial groups. The initial alignment was produced by using ClustalW (50) of the ARB program package (24) and was refined manually. Minimum-similarity filters were calculated in each database, which retained only positions conserved in at least 0% or 30% of the selected sequences, respectively. Phylogenetic analyses were performed using distance matrix methods (the programs ARB neighbor joining and Phylip UPGMA, FITCH, and KITSCH), maximum parsimony (Phylip PROTPARS), and maximum likelihood (Phylip PROML and TREE-PUZZLE [45]). All programs are implemented in the ARB program package (24). For distance matrix methods and the maximum-likelihood method, the model of substitution used was the Dayhoff PAM matrix (8); for the TREE-PUZZLE method, the Muller-Vingron model of substitution (32) was used. Maximum-parsimony trees were bootstrap resampled (10,000 bootstraps); the number of puzzling steps for TREE-PUZZLE trees was 100,000.

Nucleotide sequence accession numbers. Nucleotide sequences were submitted to the EMBL nucleotide database with accession numbers AJ96882 to AJ96884 and AM905423 to AM905426. All characterized nucleotide sequences of the *dcw* clusters were submitted to the EMBL nucleotide database with accession numbers AM905291 to AM905296. Entries AJ888907 and AJ888908 were updated.

RESULTS

Phylogenetic sequence analyses of 23S rRNA genes. 23S rRNA genes were characterized for *P. dejongeii*, *P. debontii*, *P. vanneervenii*, *A. muciniphila*, *C. flavus*, Ellin514, and *O. terrae*. For the remaining investigated *Verrucomicrobia* and *Lentisphaerae*, 23S rRNA gene sequences were retrieved from the genome project data. The data set used for calculation of phylogenetic trees included all available 23S rRNA sequences of members of the *Verrucomicrobia*, *Lentisphaerae*, *Chlamydiae*, and *Planctomycetes* and selected representatives of the major bacterial groups, some *Archaea*, and some *Eukarya*. Trees were calculated using 0%, 20%, and 50% minimum-similarity filters in combination with the different treeing methods (see Materials and Methods). (For one representative maximum-likelihood tree, see Fig. 4A; a further selection of trees is available as supplemental material.) All 18 calculated trees showed a monophyly of all included members of the PVC superphylum. The tree topology within the PVC superphylum was also stable, showing the monophyletic groups always branching in the order *Planctomycetes*, *Chlamydiae*, *Lentisphaerae*, and *Verrucomicrobia*. The verrucomicrobial subdivisions (1 to 4) also showed a monophyletic and stable treeing pattern.

Overall, the 23S rRNA trees were in absolute agreement with many published 16S rRNA phylogenies (7, 16, 43, 58) in regard to the monophyly of the PVC superphylum, the branching order of the different phyla within the PVC superphylum, and the tree topology within *Verrucomicrobia*.

Detection and genomic organization of *ddl* and *ftsQAZ* in *Verrucomicrobia* and *Lentisphaerae*. Sequences with similarities to *ddl* and *ftsZ* could be detected in all verrucomicrobial subdivisions with cultivable representatives.

With the exception of *P. vanneervenii* and *A. muciniphila*, the intervening region of *ddl-ftsZ* (generally *ftsQA*) was shown to be conserved (Fig. 1). Downstream of the *P. vanneervenii ddl* gene, an ORF with similarities to *rarD* genes (coding for predicted permeases) (26) could be detected. Upstream of the complete *ftsQ* sequence, an ORF with similarities to the conserved protein domain duf1501, a protein family with unknown function, was identified (Fig. 1). In *A. muciniphila*, *ftsQ* was detected downstream of the *ddl* gene, followed by a gene with similarities to *recA* (a bacterial recombination protein). Downstream of the *recA* homolog, a single ORF with similarities to both *amiA* (*N*-acetylmuramoyl-L-alanine amidase; N terminal) and *lipB* (lipoyltransferase; C terminal) was detected (Fig. 1). F1

ddl, *ftsQ*, *ftsA*, and a sequence with some similarities to *ftsZ* were also detected in *L. araneosa* (*Lentisphaerae*, closest relatives of *Verrucomicrobia*), but the single genes were organized isolated at different locations on the genome (Fig. 1). In the incomplete *V. vadensis* (*Lentisphaerae*) genome sequence data, only the *ddl* gene could be identified (Fig. 1).

Genomic organization of *dcw* cluster genes in the phyla *Verrucomicrobia*, *Lentisphaerae*, *Chlamydiae*, and *Planctomycetes*. The upstream region of *ddl* in *Verrucomicrobia*/*Lentisphaerae* showed either *murB* (*A. muciniphila* and Ellin514), a *murCB* fusion (DG1235), or the *ddl* gene fused with *murB* or *murBC* (*V. vadensis* and *L. araneosa*, respectively) (Fig. 1). In some other cases the upstream region was not conserved and apparently not related to the *dcw* cluster (*P. debontii*, *P. dejongeii*, and *V. spinosum*) (Fig. 1).

The only verrucomicrobial organism for which the available sequence data could prove the conservation of the gene order of the complete *dcw* cluster was isolate DG1235 (Fig. 2). Notably, the gene content and order were highly similar to the proposed ancient *dcw* cluster type (Fig. 2) (48). The only differences from the ancient type were (i) the replacement of *ftsL* through a hypothetical protein, (ii) the insertion of *lysM* between *murD* and *ftsW*, and (iii) the *murC-murB* fusion (Fig. 2). The first feature is shared with *L. araneosa* and all *Chlamydiae*; the second feature is shared only with *Chlamydiae*. F2

Ongoing and finished genome projects of the phyla *Lentisphaerae*, *Chlamydiae*, and *Planctomycetes* were also investigated for the presence of *dcw* cluster genes. With the exception of *ftsL*, *L. araneosa* was found to also possess all genes of the proposed ancient *dcw* cluster; however, the genes generally

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Organism	Phylogenetic group	Cell morphology	Evidence	Genomic organization of some *dcw* cluster genes
Prosthecobacter debontii	*Verrucomicrobia* subdivision 1 *Verrucomicrobiae*	fusiform vibroid rod	This study Pilhofer et al., 2007	*abg* *ddl* *ftsQ* *ftsA* *ftsZ* PS-DA
Prosthecobacter vanneervenii	*Verrucomicrobia* subdivision 1 *Verrucomicrobiae*	fusiform rod	This study Pilhofer et al., 2007	*ddl* *rarD* * hyp. protein duf1501 *ftsQ* *ftsA* *ftsZ*
Prosthecobacter dejongeii	*Verrucomicrobia* subdivision 1 *Verrucomicrobiae*	fusiform rod	This study Pilhofer et al., 2007	*abg* *ddl* *ftsQ* *ftsA* *ftsZ*
Verrucomicrobium spinosum	*Verrucomicrobia* subdivision 1 *Verrucomicrobiae*	prosthecate rod	Genome project	*narK* *ddl* *ftsQ* *ftsA* *ftsZ*
Akkermansia muciniphila	*Verrucomicrobia* subdivision 1 *Verrucomicrobiae*	oval	This study	*murB* *ddl* *ftsQ* *recA* *amiA-lipB*
Chthoniobacter flavus	*Verrucomicrobia* subdivision 2 *Spartobacteria*	oval rod	This study	*ddl* *ftsQ* *ftsA* *ftsZ*
Isolate Ellin514	*Verrucomicrobia* subdivision 3	cocci	This study	*murB* *ddl* *ftsQ* *ftsA* *ftsZ* *corB*
Opitutus terrae	*Verrucomicrobia* subdivision 4 *Opitutaceae*	cocci	This study	*ddl*
Isolate TAV2	*Verrucomicrobia* subdivision 4 *Opitutaceae*	cocci	Genome project	*ddl* *ftsQ* *ftsA* *ftsZ*
Isolate DG1235	*Verrucomicrobia* subdivision 4 *Opitutaceae*	cocci	Genome project	*murCB* *ddl* *ftsQ* *ftsA* *ftsZ*
Uncultivated species	*Verrucomicrobia* subdivision 5			
Victivallis vadensis	*Lentisphaerae*	cocci	Genome project	*murB-ddl*
Lentisphaera araneosa	*Lentisphaerae*	sphere	Genome project	*murBC-ddl* * *ftsQ* * *ftsA* * *ftsZ*

FIG. 1. Genomic organization of the 3′-terminal part of the *dcw* cluster in *Verrucomicrobia* and *Lentisphaerae*. The genes shaded in gray or black are generally organized within the *dcw* cluster. The genes in white are not functionally related to and normally not organized within the *dcw* cluster. The asterisk indicates that contigs are isolated on the chromosome. The dashed lines indicate that the sequence of the ORF is partial. With two exceptions, *Verrucomicrobia* show the conserved gene order *ddl-ftsQAZ*, whereas in *Lentisphaerae*, the individual genes are isolated on the chromosome.

located in the 3′-terminal region of the cluster (*ftsQ*, *ftsA*, and *ftsZ*-like) were dispersed in the genome (Fig. 2).

In *Chlamydiae* representatives, *ftsZ*, *ftsA*, *ftsQ* (with the exception of "*Ca*. Protochlamydia"), *ftsL*, and *mraZ* are absent. The remaining *dcw* genes are arranged in two isolated clusters, which are remarkably more similar to DG1235 than to the ancestral cluster (*ftsL* is replaced by a gene insertion, *lysM*, encoding a hypothetical protein). *Chlamydia* and *Chlamydophila* show a fused *murC-ddl* gene, whereas in "*Ca*. Protochlamydia," *murC* is followed by *ftsQ* and the *ddl* gene is isolated (Fig. 2).

In representatives of *Planctomycetes*, the *dcw* gene content and order conservation vary widely between the different representatives. Most of the genes of the ancient *dcw* cluster are present in "*Candidatus* Kuenenia stuttgartiensis." They are organized in a cluster that is very similar to that predicted for the ancestral type; *ftsA*, *ftsZ*, *murD*, and *murB* are absent, and three ORFs coding for hypothetical proteins are inserted. In contrast, *R. baltica* and *B. marina* genome data present only some isolated *mur* genes and none of the *fts* genes (*ftsQ* in *B. marina*). *P. maris* possesses most of the *dcw* genes, but they are clustered at different locations in the genome (Fig. 2).

Transcription analyses. The transcription of *ddl*, *ftsQ*, *ftsA*, and *ftsZ* was verified in *P. dejongeii* and *P. vanneervenii* using reverse transcription of mRNA (Fig. 3). Total RNA was isolated from *Prosthecobacter* cultures and was subsequently treated using DNase. Total RNA was reverse transcribed using random hexamers. The cDNA was subsequently used as a template in PCR with gene-specific primers. Control reactions for each PCR were one reaction without template and one control sample processed without reverse transcriptase (Fig. 3). Specific amplification from cDNA was detected for all investigated genes in both species. F3

Search for conserved protein domains and residues. In order to verify the sequence similarities detected using BLASTP, Ddl and FtsQAZ protein sequences of *Verrucomicrobia*/*Len-*

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	Organism	*dcw* cluster gene organization
	ancestral cluster	*mraZ mraW ftsL ftsI murE murF mraY murD ftsW murG murC murB ddl ftsQ ftsA ftsZ*
V	*V. spinosum*	*mraW ftsI murE mraY murD ftsW murG murC-murB ddl ftsQ ftsA ftsZ*
V	Isolate DG1235	*mraZ mraW* H *ftsI murE murF mraY murD lysM ftsW murG murC-murB ddl ftsQ ftsA ftsZ*
L	*Lentisphaera araneosa*	*mraZ mraW* H *ftsI murE murF mraY* | *murD* | *ftsW* | *murG* | *murB-murC--ddl ftsQ ftsA ftsZ*
C	*Chlamydia* spp.	*mraW* H *ftsI murE murF mraY murD lysM ftsW murG murC----------ddl*
C	*Chlamydophila* spp.	*mraW* H *ftsI murE murF mraY murD lysM ftsW murG murC----------ddl*
C	*Cand.* P. amoebophila	*mraW* H *ftsI murE murF mraY murD lysM ftsW murG murC ftsQ ddl-ddl*
P	*Cand*. K. stuttgartiensis	*mraZ mraW ftsL ftsI* H *murE murF mraY ftsW* H *murG* H *murC ddl ftsQ*
P	*Rhodopirellula baltica*	*mraW murE murG murB ddl*
P	*Blastopirellula marina*	*mraZ murE murB ftsQ*
P	*Planctomyces maris*	*mraZ mraW lysM ftsI murE murF mraY ftsW murG murC murB ddl*

FIG. 2. Genomic organization of *dcw* genes in *Verrucomicrobia*, *Lentisphaerae*, *Chlamydiae*, and *Planctomycetes*. Comparison of *dcw* gene content and gene order conservation in the PVC superphylum. The hypothetical ancestral cluster (47) is shown in the first line. The first column designates phylogenetic groups: V, *Verrucomicrobia*; L, *Lentisphaerae*; C, *Chlamydiae*; P, *Planctomycetes*. The vertical lines indicate isolated organization on the chromosome. Hyphens denote gene fusions. H stands for hypothetical protein. DG1235 shows a good conservation of the *dcw* cluster with some peculiarities compared to the ancestral type. *L. araneosa* presents most of the *dcw* genes, whereas the gene order is lost. *Chlamydiae dcw* clusters are disrupted in two parts but are remarkably similar to DG1235, except that they contain no *fts*(*Q*)*AZ* genes. *Planctomycetes* show high variability in the conservation of gene content and gene order.

tisphaerae were searched for conserved domains of the Pfam library (4). The matching scores of the query sequences to the respective Pfam domains are listed as E values in Table 1. All detected Ddl sequences showed matches to both D-Ala:D-Ala ligase Pfam domains with comparable high scores, except for two partial sequences (*O. terrae* and TAV2). For all available investigated FtsQA sequences, there were matches to the POTRA domain FtsQ type (42) or to the cell division protein FtsA domain, respectively. Due to the high sequence divergence of these proteins, the scores for the matches were generally lower than Ddl matches (Table 1). All complete verrucomicrobial FtsZ sequences showed a match to the tubulin/FtsZ family GTPase domain, with comparable high scores. The sequence of *L. araneosa* (*Lentisphaerae*) with similarities to FtsZ did not match the tubulin/FtsZ family GTPase Pfam domain, nor did it match the FtsZ PROSITE pattern, the BLOCKS database, or the PRINTS motif (the search was performed using MOTIF search [http://motif.genome.jp/]). However, the sequence was initially detected using BLASTP and showed similarities to the conserved domain FtsZ (cd02191) (26) and to FtsZ from *P. debontii* with E values of 5e−06 and 6e−04, respectively.

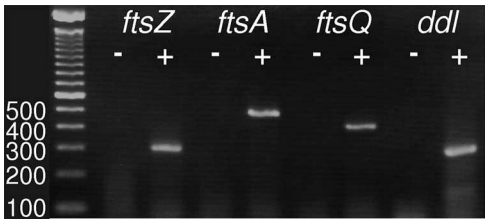

FIG. 3. Transcriptional analyses of *dcw* cluster genes in *Prosthecobacter*. Five-microliter aliquots of PCR products were loaded on a 2% agarose gel. The template for PCR was the cDNA sample (+) or a control sample processed without reverse transcriptase (−). A 100-bp ladder (Invitrogen) was loaded on the left side; the numbers refer to fragment lengths in base pairs. The expected sizes of the fragments were 296 bp (*ftsZ*), 435 bp (*ftsA*), 354 bp (*ftsQ*), and 283 bp (*ddl*). Transcription was detected for all investigated genes in *P. dejongeii* (shown) and *P. vanneervenii*.

FtsZ sequences were additionally searched for the tubulin signature motif (S/A/G)GGTG(S/A/T)G (PROSITE motif PS00227), which is present and perfectly conserved in all investigated verrucomicrobial FtsZ proteins. In contrast, the putative *L. araneosa* FtsZ homolog did not show the tubulin signature motif. A similar observation was made during the analysis of amino acid positions that contact GDP (23, 34). The analysis performed by Pilhofer et al. (36) was extended using the newly obtained FtsZ sequences (data not shown). All important positions were conserved among *Verrucomicrobia*, even if the new FtsZ sequences of subdivisions 2 to 4 were added. The *L. araneosa* FtsZ-like protein showed no conservation in the majority of these amino acid residues. BlastP analysis of *ddl* ORFs revealed that *Verrucomicrobia* show the common structure comprising one Ddl domain. Whereas, similar to *ddl* genes of *Verrucomicrobia*, *ddl* genes of *Planctomycetes* comprise only one Ddl domain, Ddls of *Lentisphaerae* and *Chlamydiae* present different types of fused genes (Fig. 2). *ddl* genes were found to be fused with *murBC* (*L. araneosa*), *murB* (*V. vadensis*), and *murC* (*Chlamydia* and *Chlamydophila*) or with another *ddl* ("*Ca*. Protochlamydia").

Appendix C

TABLE 1. E values[a] of *dcw* genes for Pfam domains

Isolate	E value				
	Ddl		FtsQ (POTRA domain; FtsQ type)	FtsA (cell division protein FtsA)	FtsZ (tubulin/FtsZ family; GTPase)
	D-Ala:D-Ala ligase C terminus	D-Ala:D-Ala ligase N terminus			
P. dejongeii	9.8e−53	2.1e−15	0.01	1.3e−38	5.9e−49b
P. debontii	2.9e−59	1.5e−16	0.0042	7.8e−40	3.3e−50
P. vanneervenii	2.3e−64	2.0e−14	4.7e−11	8.9e−42	5.8e−54
V. spinosum	2.0e−69	2.1e−11	1.5e−07	1.3e−42	5.4e−48
A. muciniphila	3.5e−56	2.8e−18	7.9e−06		
C. flavus	9.7e−54	3.4e−13	5.4e−10	2.4e−50	5.5e−60b
Ellin514	3.5e−57	3.7e−19	1.8e−09	2.0e−46	7.1e−67
O. terrae	1.2e−48b	—[b,c]			
TAV2	—[b,c]	—[b,c]	5.4e−12	1.2e−19	0.27b
DG1235	2.6e−45	1.3e−13	4.5e−12	1.7e−24	1.2e−53
V. vadensis	5.8e−53	9.6e−21			
L. araneosa	3.4e−47	1.3e−26	3.4e−15	7.0e−05	—[c]
E. coli	3.4e−119	2.9e−47	8.7e−37	6.7e−79	7.8e−91

[a] Based on scoring matches to the motifs.
[b] Partial sequence.
[c] No significant hit.

In BLASTP analyses, the first hits detected for *Verrucomicrobia*, *Planctomycetes*, *Chlamydia*, and *Chlamydophila* Ddl sequences were other bacterial Ddls (data not shown). Notably, the "*Candidatus* Protochlamydia amoebophila" sequence comprising two Ddl domains showed the best matches (E value, 3e−161) to a nuclear-encoded gene in *Physcomitrella patens* (Viridiplantae), a moss for which *ddl* and related genes seem to be important for plastid division (25).

Phylogenetic analyses using Ddl and FtsZ sequences. The newly obtained FtsZ and Ddl sequences were used for phylogenetic-tree reconstructions. The FtsZ_ClustalW ARB database published by Pilhofer et al. (36) was extended with the new sequences obtained in this study. FtsZ sequences of *Verrucomicrobia* and *Lentisphaerae* (excluding partial TAV2 FtsZ) were used to produce two different minimum-similarity filters. Phylogenetic trees were calculated using both filters in combination with distance matrix methods, maximum parsimony, and maximum likelihood. Attempts to calculate global trees could recover a relationship of *L. araneosa* FtsZ-like proteins and verrucomicrobial FtsZs in most cases, whereas the relationships between the other major bacterial groups were unstable (data not shown; compare reference 36). Therefore, subsequent phylogenetic analyses were restricted to *Lentisphaerae* and *Verrucomicrobia*. All calculated trees showed stable tree topologies, with stable monophyletic groups representing the different subdivisions. In all trees, the branching patterns of the FtsZ sequences of the different verrucomicrobial subdivisions (1, 2, 3, and 4) were consistent with the pattern obtained in rRNA phylogeny (Fig. 4) (7, 16, 43, 58). One representative maximum-likelihood tree is shown in Fig. 4B.

A Ddl ARB database was established by importing the Ddl protein sequences obtained in this study and Ddl sequences of other bacterial groups from the EMBL nucleotide database. Initial attempts to calculate global trees using different methods and filters showed that in most cases the major bacterial groups could be recovered, whereas the relationships between the major bacterial groups were unstable (data not shown), as is also the case for FtsZ phylogeny (36). Therefore, subsequent phylogenetic analyses were restricted to *Lentisphaerae* and *Verrucomicrobia*. Notably, all global Ddl trees showed a stable relationship of "*Candidatus* Protochlamydia amoebophila" Ddl with *P. patens* (Viridiplantae) Ddl (nuclear encoded). The "*Ca*. Protochlamydia" Ddl was duplicated in the database, so each of the Ddl domains could be aligned once.

Ddl sequences of *Verrucomicrobia* and *Lentisphaerae* (excluding partial TAV2 Ddl) were used to calculate two minimum-similarity filters. Phylogenetic trees were reconstructed using both filters in combination with distance matrix methods, maximum parsimony, and maximum likelihood. As the isolate TAV2 Ddl sequence is partial and therefore very short, it was added separately to each tree using ARB Interactive Parsimony and a special filter considering the length of TAV2 Ddl. One representative maximum-likelihood tree is shown in Fig. 4C. All calculated trees showed stable tree topology, with stable monophyletic groups representing the two phyla *Verrucomicrobia* and *Lentisphaerae*. Within the *Verrucomicrobia*, each subdivision (1, 2, 3, and 4) was recovered monophyletically; however, the bootstrap support for the subdivision 4 cluster was not high. The branching pattern is consistent with the pattern obtained using comparative sequence analyses of 16S rRNA genes (7, 16, 43, 58), 23S rRNA genes (Fig. 4A), and FtsZ proteins (Fig. 4B).

DISCUSSION

Phylogenetic relationships between *Planctomycetes*, *Chlamydiae*, *Lentisphaerae* and *Verrucomicrobia*. A stable monophyly of *Planctomycetes*, *Chlamydiae*, *Lentisphaerae* and *Verrucomicrobia* could be detected using 23S rRNA phylogeny (Fig. 4A). Also, the bootstrap support was high for this clade (14). In the past, there were also other studies, addressing the phylogenetic relationships between *Planctomycetes*, *Chlamydiae*, and *Verrucomicrobia* based on 16S rRNA (7, 13, 22, 58, 61), 23S rRNA (59), or protein sequences (12, 17, 46, 49, 60), in which the formation of a monophyletic cluster of all three lineages was controversial. The study by Ward et al. (59), also using 23S

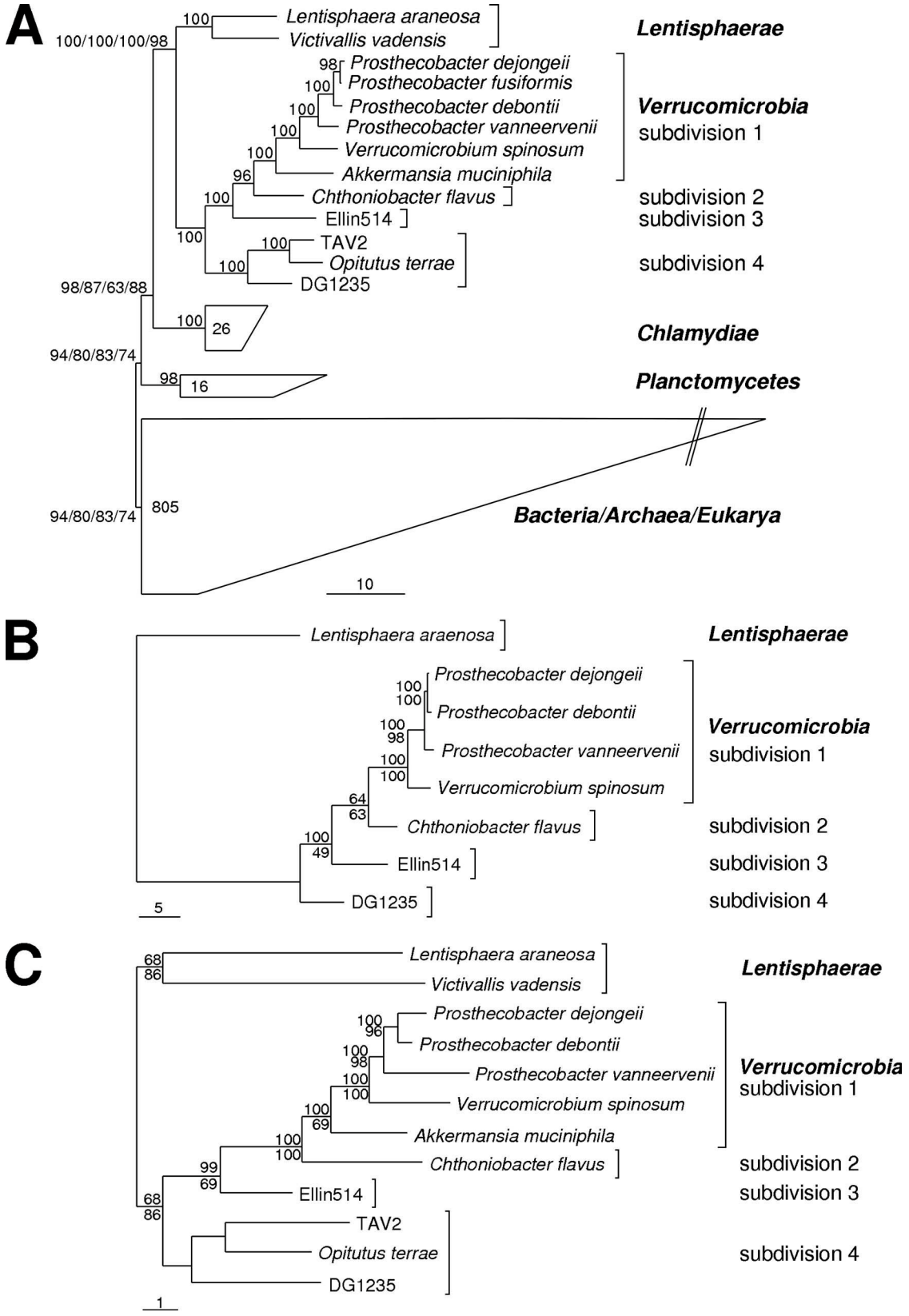

FIG. 4. Comparative sequence analyses using 23S rRNA (A), FtsZ (B), and Ddl (C). All calculated 23S rRNA trees showed a stable monophyletic group of *Planctomycetes*, *Chlamydiae*, *Lentisphaerae*, and *Verrucomicrobia* with a stable branching order within this PVC superphylum. Verrucomicrobial subdivisions were recovered monophyletically and showed a branching order matching the 16S rRNA trees. Other 23S rRNA trees are available in the supplemental material. Verrucomicrobial FtsZ and Ddl trees show the same tree topologies as 16S and 23S rRNA trees. (A) Phylogenetic 23S rRNA

rRNA as a phylogenetic marker, could not detect a significant relationship of the three phyla. However, the data set was rather limited: *Planctomycetes* were represented by 13 sequences (16 representatives in this study), *Chlamydiae* by one sequence (26 representatives in this study), and *Verrucomicrobia* by three sequences (11 representatives in this study), and *Lentisphaerae* were completely lacking (2 representatives in this study). The much more comprehensive phylogenetic analyses in this study showed very good agreement between trees derived from 23S and 16S rRNAs (Fig. 4). Therefore, these analyses support the suggestion of a PVC superphylum based on 16S rRNA phylogeny in which the sister phyla *Lentisphaerae* and *Verrucomicrobia* are more closely related to *Chlamydiae* than to *Planctomycetes* (58).

Origin of the "*Candidatus* Protochlamydia amoebophila" *ddl-ddl gene*. An intriguing feature derives from the analysis of "*Candidatus* Protochlamydia" Ddl-Ddl. This gene shows unexpectedly high similarity to plant Ddl-Ddl, involved in chloroplast division (25), and apparently low similarity to bacterial Ddl. The plant *ddl* genes have been characterized up to now in *Arabidopsis*, in *Oryza*, and in the moss *Physcomitrella*. For the first two organisms, genomic data are available, and they show the presence of many introns interrupting the coding region of the *ddl* genes. The observed high similarity between plants and "*Ca*. Protochlamydia" *ddl* suggests that either plants acquired *ddl* from a "*Ca*. Protochlamydia" ancestor and later inserted introns in the coding region or "*Ca*. Protochlamydia" acquired *ddl* from plants, most likely via reverse-transcription-mediated mechanisms (due to the "necessity" to remove introns). The latter case would imply that some bacterial genes of the *dcw* cluster might be lost and reacquired through horizontal gene transfer.

FtsZ-based cell division mechanism in *Verrucomicrobia* and *Lentisphaerae*. In contrast to previous assumptions, *ftsQAZ* and *ddl* genes could be detected in representatives of all verrucomicrobial subdivisions. The failure to detect *ftsZ* in *A. muciniphila* and *O. terrae* by PCR is not significant, due to the sequence divergence of *ftsZ* (see Materials and Methods); we predict that these organisms also possess *ftsZ*. Phylogenetic sequence analyses using the obtained FtsZ and Ddl sequences are in full agreement with the rRNA trees (Fig. 4) (7, 16, 43, 58). This consistency suggests the absence of horizontal gene transfer influence on FtsZ and Ddl evolution in *Verrucomicrobia* and strongly indicates that the genes are conserved due to functional constraints. This also applies to the isolated genes of the nonconserved *dcw* clusters (in terms of gene order) of *P. vanneervenii* and *A. muciniphila* (Fig. 1). Comprehensive analyses of the FtsZ sequences revealed that all verrucomicrobial FtsZ proteins matched the criteria of functional FtsZ as defined by Vaughan et al. (53). The sequences showed a match to the conserved Pfam domain tubulin/FtsZ family GTPase, the tubulin signature motif could always be detected without mismatches, and the analysis of amino acid positions that contact GDP (23, 34) also showed good conservation. The constant presence of *ftsQAZ* in *Verrucomicrobia*, the phylogenetic conservation of FtsZ, the fulfilment of all criteria of functional FtsZ within the gene sequence, and the proof of transcription of *ftsQAZ* in actively growing cultures provide a strong indication of functionality and suggest an FtsZ-based cell division mechanism in all members of the phylum *Verrucomicrobia*.

Concerning the phylum *Lentisphaerae*, represented by the cultivated species *V. vadensis* and *L. araneosa*, the situation is less clear. Considering that the genome sequence of *V. vadensis* is still unfinished (324 contigs), the present inability to detect *ftsQAZ*-like sequences in the genome data is not significant. Indeed, its closest relative, *L. araneosa*, possesses *ftsQ*, *ftsA*, and an *ftsZ*-like gene, although all of them are spread out among different positions on the chromosome. Concerning *L. araneosa* FtsZ, similarities to *Prosthecobacter* FtsZ and to the conserved domain FtsZ could be clearly detected, whereas the criteria of functional FtsZ (53) are not fulfilled at all. *L. araneosa* FtsQA have matches to the respective Pfam motifs with high E values (Table 1), which could be an indication of functionality. Up to now, no bacteria that had a non-FtsZ-based cell division mechanism and at the same time possessed *ftsA* (encoding the first protein recruited to the Z ring and directly interacting with FtsZ) could be identified. In summary, the present data may suggest that *Lentisphaerae* also perform FtsZ-based cell division like *Verrucomicrobia* and the majority of other bacteria. However, the failure to meet the criteria of functionality could indicate a modified mechanism or even that the gene is (at least in cell division) nonfunctional. Future studies of the *L. araneosa* FtsZ-like protein could verify whether the fundamental criteria of functional FtsZ (53) have to be revised.

Conservation of the gene content and gene order of the *dcw* cluster in *Verrucomicrobia* and *Lentisphaerae*. The analyses of the available sequence data demonstrated that in at least one verrucomicrobial organism (DG1235) the *dcw* cluster is nearly perfectly conserved in comparison to the ancient type of clus-

tree produced using the maximum-likelihood method (RAxML) and a minimum-similarity filter (50%). Selected representatives of major bacterial groups, *Archaea*, and *Eukarya* were used as outgroups. Note that the tree is unrooted. The branching pattern within the group "Bacteria/Archaea/Eukarya" is shown in the supplemental material. The numbers in closed groups denote the numbers of sequences included. The numbers at the nodes represent bootstrap values (1,000 bootstraps) (percent). Four bootstrap values are shown for the branchings of the phyla (maximum likelihood, 0% minimum-similarity filter/maximum likelihood, 50% minimum-similarity filter/maximum parsimony, 0% minimum-similarity filter/maximum parsimony, and 50% minimum-similarity filter); one bootstrap value is shown for the remaining nodes (maximum parsimony, 50% minimum-similarity filter). The bar represents the estimated evolutionary distance (percent). (B) Phylogenetic FtsZ (core domain) tree produced using the maximum-likelihood method and a minimum-similarity filter (30%). The numbers represent bootstraps/confidence values of a maximum-parsimony tree (lower numbers, 10,000 bootstraps) and a TREEPUZZLE tree (upper numbers, 100,000 puzzling steps) (percent). The bar represents the estimated evolutionary distance (percent). (C) Phylogenetic Ddl tree produced using the maximum-likelihood method and a minimum-similarity filter (30%). The numbers represent bootstraps/confidence values of a maximum-parsimony tree (lower numbers, 10,000 bootstraps) and a TREEPUZZLE tree (upper numbers, 100,000 puzzling steps) (percent). The bar represents the estimated evolutionary distance (percent).

ter, which comprises 16 genes (Fig. 2) (48). In at least five other cases (all investigated subdivision 1 organisms), gene order in the cluster showed variations (Fig. 1). With one exception (*ftsL*), *Lentisphaera* maintained the gene content, whereas the gene order was conserved only in the 5′-terminal part of the *dcw* cluster (Fig. 2).

A comparison of the *dcw* clusters from several bacterial genomes showed that there is a relationship between the organization of the gene cluster and the shape of the cells, so that the cluster tends to be conserved in rod-shaped and filamentous bacteria and tends to be disrupted in bacteria with other shapes (48). This observation is supported in this study, as none of the *Verrucomicrobia*/*Lentisphaerae* with dispersed *dcw* cluster genes formed classical cylindrical rods with hemispherically capped ends. The types of morphology in *Verrucomicrobia*/*Lentisphaerae* range from spheres (*L. araneosa*), cocci (*V. vadensis*, DG1235, TAV2, *O. terrae*, and Ellin514), oval rods (*C. flavus*), oval shapes (*A. muciniphila*), and prosthecate rods (*V. spinosum*) to fusiform rods (*Prosthecobacter*) (Fig. 1). It is notable that there are two different types of *dcw* cluster organizations (concerning the generally highly conserved 3′ region) within the genus *Prosthecobacter* that have very similar morphologies (*P. vanneervenii*-*P. dejongeii* and *P. debontii*) (Fig. 1). Mingorance and Tamames and Mingorance et al. (30, 31) proposed a model, called genomic channeling, in which the selective pressure to maintain the *dcw* cluster arises from the need to efficiently coordinate the process of elongation and septation in rod-shaped bacteria. Although *Prosthecobacter* and *Verrucomicrobium* have rod-like morphologies, they also possess one or many prosthecae. It can be suspected that the presence of these prosthecae is likely to force a growth mechanism rather divergent from the one found in classical rods. Thus, it is hard to predict if and how peptidoglycan elongation and septation machineries compete. Therefore, it seems likely that genomic channeling of the *dcw* cluster does not play a major role in these organisms, and it is very probable that other mechanisms for the determination of cell shape exist in *Prosthecobacter*.

Conservation of gene content and gene order of the *dcw* cluster in *Planctomycetes* and *Chlamydiae* compared to *Verrucomicrobia*/*Lentisphaerae*. The high similarity of *dcw* clusters within *Chlamydiae* could be derived from adaptation to their similar intracellular lifestyles and to the consequent functional constraints. Although up to now the existence of peptidoglycan in *Chlamydiae* has not been proven, the genes coding for a nearly complete pathway for peptidoglycan synthesis were suggested to be functional (reviewed in reference 29). In contrast to *Chlamydiae*, *Planctomycetes* show a high degree of variability in the *dcw* cluster, which could be related to the very different lifestyles of its representatives.

The hypothesis that the last common ancestor of extant bacteria harbored a conserved *dcw* cluster and that some lineages lost this gene order during evolution (33) is strongly supported by the observations obtained in this study. In comparison to the ancestral *dcw* cluster type, DG1235 shows only one gene replacement, one gene insertion, and one gene fusion; *Chlamydiae* show the gene order conservation of many *dcw* genes; and 12 out of 16 genes are conserved in the *Planctomycetes* representative "*Candidatus* Kuenenia stuttgartiensis" (Fig. 2). These results are also a strong indication that the last common ancestor of the PVC superphylum possessed a conserved *dcw* cluster. Combined with the phylogenetic analyses, these data clearly support the view that many representatives of *Planctomycetes*, *Chlamydiae*, and *Lentisphaerae* independently lost some of the *dcw* genes or the gene order within the *dcw* cluster. An exception to this might be the replacement of *ftsL* by a hypothetical protein in DG1235 (*Verrucomicrobia*), *L. araneosa* (*Lentisphaerae*), and all *Chlamydiae*; this replacement might represent a characteristic of the last common ancestor of *Verrucomicrobia*-*Lentisphaerae*-*Chlamydiae*. In the same way, the insertion of *lysM* between *murD* and *ftsW* might represent a synapomorphic feature of *Verrucomicrobia* and *Chlamydiae* (the absence of *lysM* in *V. spinosum* in the expected position could be interpreted as a secondary loss).

In the scenario postulated here, after the separate branchings of *Chlamydiae* and *Planctomycetes* from their last common ancestor with *Verrucomicrobia*, their cell division mechanisms most probably shifted independently from an FtsZ-based mechanism to a non-FtsZ-based mechanism. The major cell division gene *ftsZ* was completely lost or diverged so far from the original *ftsZ* that a relationship is no longer recognizable. FtsZ could have been replaced by two different division mechanisms (concerning the structural protein of the division ring) in the different phyla and therefore does not necessarily have to be homologous. Another possibility is that there was an independent shift to the same division mechanism in *Chlamydiae* and *Planctomycetes*. If this was the case, it seems probable that the mechanism could have already been present in the last common ancestor of *Planctomycetes*/*Chlamydiae*/*Lentisphaerae*/*Verrucomicrobia*. To the best of our knowledge, two *Ureaplasma* species are the only examples in bacteria besides *Chlamydiae* and *Planctomycetes* that were identified to lack *ftsZ*. Thus, a special predisposition of the last common ancestor of the PVC superphylum to evolve an FtsZ-independent type of cell division can be hypothesized.

The single presently identifiable *L. araneosa ftsZ*-like gene (in the unfinished genome sequence) shows the highest similarities to verrucomicrobial FtsZs. At the same time, the sequence divergence in comparison to other bacterial FtsZ proteins is higher than that of verrucomicrobial FtsZ. Thus, from an evolutionary perspective, *L. araneosa* could be seen as an organism on the way to developing an FtsZ-independent cell division mechanism or one that has already developed such a mechanism.

ACKNOWLEDGMENTS

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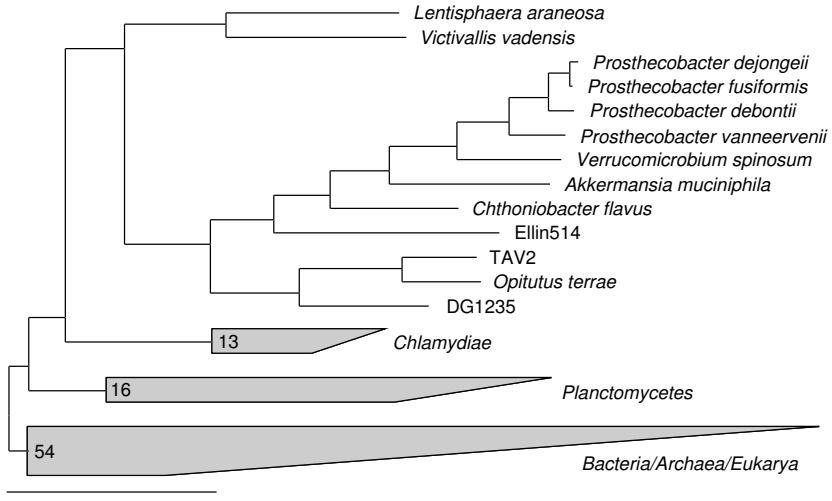
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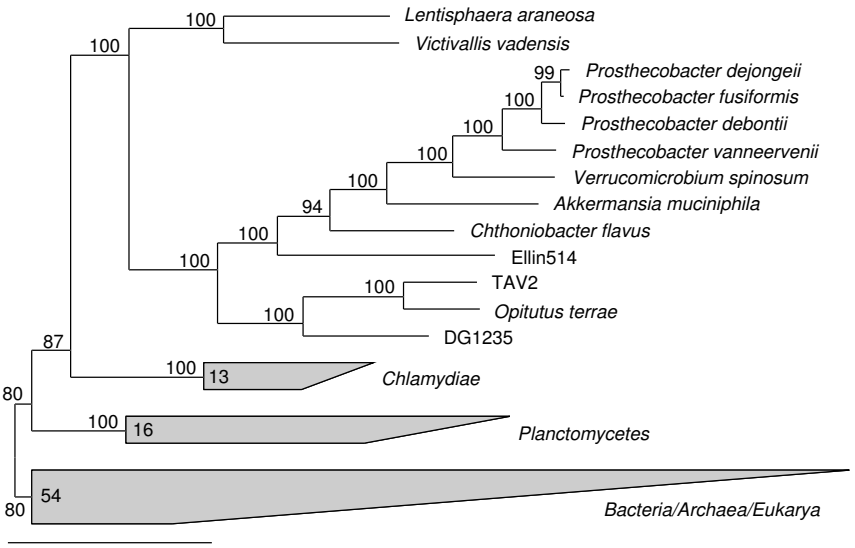
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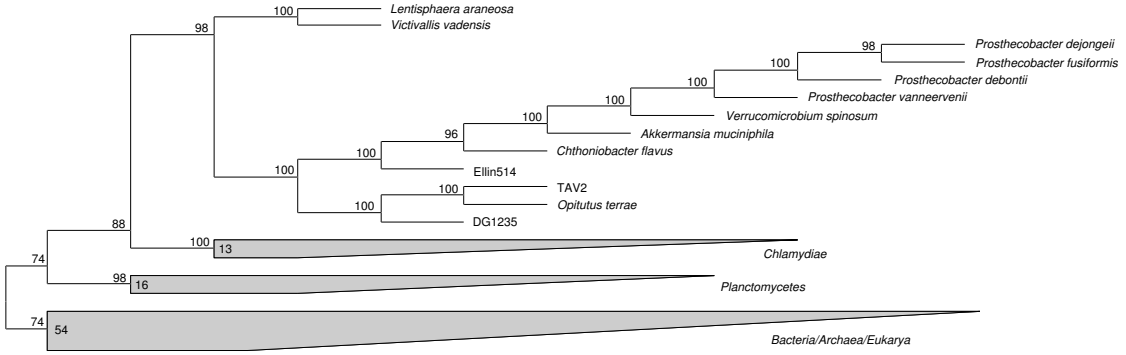
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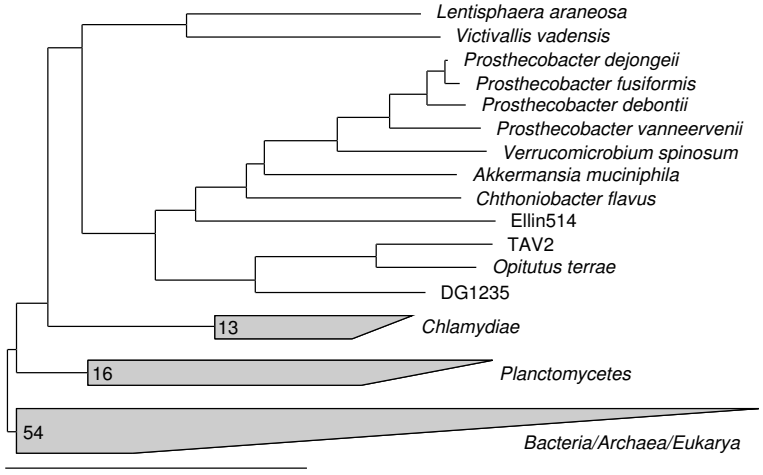
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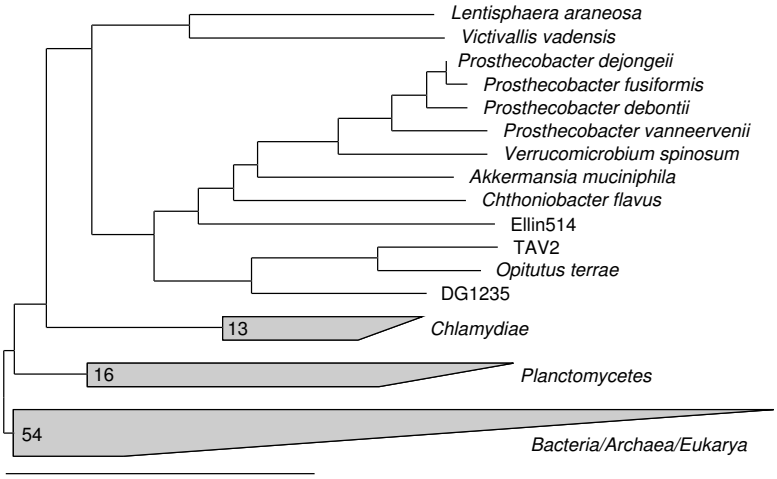
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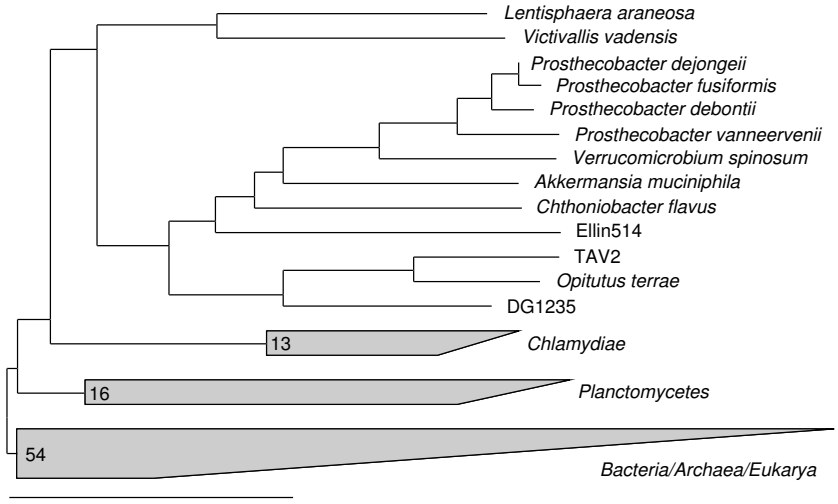
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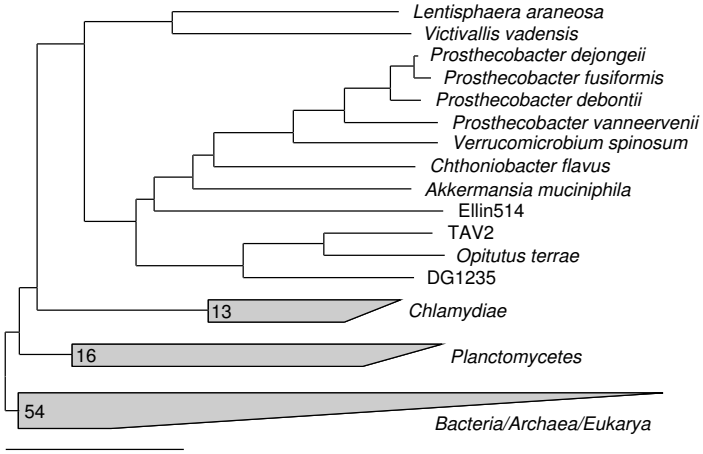
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(H)

Supplementary Material – Representative selection of 23S rRNA trees.

Phylogenetic 23S rRNA trees were produced using the treeing methods and minimum similarity filters listed below. Numbers in closed groups denote the number of included sequences. Numbers at nodes represent bootstrap values in percent. Bar represents 10% estimated evolutionary distance. Except for Panel A, the group of other *Bacteria* / *Archaea* / *Eukarya* was scaled down graphically.

(A) RAxML; 50%; same tree as Figure 4A with group *Bacteria*/*Archaea*/*Eukarya* opened

(B) AxML; 50%

(C) PHYML; 50%; 1000 bootstraps

(D) DNAPARS; 50%; 1000 bootstraps

(E) FITCH; 50%

(F) NEIGHBOR; 50%

(G) NEIGHBOR; 20%

(H) NEIGHBOR; 0%

Appendix D

Phylogenetic relationships of bacterial tubulins within the Tubulin/FtsZ superfamily: Implications on their evolutionary origin

Martin Pilhofer, Wolfgang Ludwig, Karl-Heinz Schleifer, Giulio Petroni

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concept by MP and GP
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phylogenetic analyses by MP and GP
writing by MP and GP with editorial help of the co-authors

Phylogenetic relationships of bacterial tubulins within the Tubulin/FtsZ superfamily: Implications on their evolutionary origin

Martin Pilhofer[1], Wolfgang Ludwig[1], Karl-Heinz Schleifer[1], Giulio Petroni[2]

1 Lehrstuhl für Mikrobiologie, Technical University Munich, Am Hochanger 4, D-85350 Freising, Germany

2 Dipartimento di Biologia, University of Pisa, via A. Volta 4/6, I-56126 Pisa, Italy

Abstract

Tubulins were considered as typical eukaryotic features until genes with high similarities to tubulin (bacterial A and B tubulins) were detected in the genus *Prosthecobacter*, members of the bacterial phylum *Verrucomicrobia*.

To get hints about the origin of *Prosthecobacter* tubulins, comprehensive sequence data analyses within the FtsZ/Tubulin superfamily were performed comprising bacterial tubulins, all known eukaryotic tubulin subfamilies, bacterial and archaeal FtsZ, archaeal FtsZ-likes and Bacilli Tubulin-likes. Bacterial A and B tubulins clearly belong to the eukaryotic tubulin family. The phylogenetic relationships within the tubulin family were rather instable. No clear associations between bacterial A and B tubulins or between bacterial tubulins and any other tubulin subfamily could be detected. BtubA and BtubB are therefore suggested to represent two novel bacterial subfamilies within the eukaryotic tubulin clade.

A PCR- and genome data screening in this study demonstrated the absence of tubulin-like genes in other *Verrucomicrobia*. This and the results of previous studies support that *Prosthecobacter* tubulins were acquired by horizontal gene transfer. The present hypothesis suggests a trans-kingdom transfer of tubulin genes from a Eukaryote to *Prosthecobacter*. Based on the results of this study, an alternative hypothesis is proposed in which *Prosthecobacter* tubulins originated, together with eukaryotic tubulins, from an ancestral tubulin present in an ancestral organism shared between Eukaryotes and some bacterial lineages. *Prosthecobacter* spp. acquired bacterial tubulins through horizontal gene transfer from a bacterial descendant.

Introduction

Bacteria contain a complex cytoskeleton which is more diverse than it was previously considered. Through advances in cellular imaging, it has become clear that the subcellular environments of bacterial cells are highly organized (Gitai, 2007; Pogliano, 2008). Bacterial homologs of the three major eukaryotic cytoskeletal families (actin, tubulin and intermediate filaments) have been identified (Larsen et al., 2007; Gitai, 2007).

FtsZ is the major bacterial cell division protein (Bi and Lutkenhaus, 1991) and found in nearly all *Bacteria* and *Euryarchaea*, as well as in several eukaryotic organelles (Margolin, 2005). Structural analyses suggested FtsZ being the bacterial/euryarchaeal homolog of eukaryotic tubulin (Lowe and Amos, 1998; Erickson, 1998; Nogales et al., 1998), constituting a Tubulin/FtsZ superfamily, a distinct family of GTPases. Recently, plasmid-encoded genes were characterized in four *Bacillus* species, which have similarities to both tubulin and FtsZ and which cluster together on the phylogenetic tree. In *B. thuringiensis* this gene is referred to as *tubZ* and was suggested to be important for plasmid stability (Larsen et al., 2007). A second group of sequences which is also only distantly related to FtsZ and tubulin was detected in the *Archaea*. The respective species also have one or more additional genes very closely related to FtsZ. Thus, they do probably not function as the major cell division protein (Larsen et al., 2007; Vaughan et al., 2004). Most of the archaeal protein sequences which are only distantly related to FtsZ form a monophyletic group in phylogenetic trees. It is not clear how tubulin, FtsZ, TubZ-like sequences and archaeal FtsZ-like sequences are related to each other (Larsen et al., 2007).

The Tubulin family comprises 10 eukaryotic subfamilies, alpha through kappa. Alpha and beta tubulins are the building blocks of microtubules, consisting of heterodimers which polymerize into protofilaments. Like alpha and beta tubulins, gamma tubulins are present in all eukaryotic organisms. The subfamilies delta and epsilon, are present in many but not in all eukaryotic lineages. Zeta and eta are only known from few eukaryotic organisms. Three

sequences from *Paramecium* represent the putative new tubulin subfamilies theta, iota and kappa. The function of most of the additional tubulin subfamilies remains to be elucidated; some were shown to be important for basal body development or C-tubule assembly (Vaughan et al., 2000; Dutcher, 2003; Libusova and Draber, 2006).

Tubulins were considered as a typical eukaryotic feature, until genes were detected in the bacterial genus *Prosthecobacter*, which were more similar to tubulin than to FtsZ (Pilhofer et al., 2007b; Jenkins et al., 2002). *Prosthecobacter* belongs to the phylum *Verrucomicrobia*, in which FtsZ was detected in all subdivisions with cultivable representatives (Pilhofer et al., 2007b; Pilhofer et al., 2008). In *Prosthecobacter* apparently functional FtsZ coexists with the bacterial tubulin genes (Pilhofer et al., 2007b), which may contribute to the spindle shape of the cells (Sontag et al., 2005; Schlieper et al., 2005). The bacterial tubulin genes are organized in a typical bacterial operon, comprising one bacterial A tubulin (*btubA*), one bacterial B tubulin (*btubB*) and one kinesin light chain-like gene (*bklc*). In *P. debontii* even two *btub*-operons could be identified (Pilhofer et al., 2007a).

Several results of previous studies supported the hypothesis that *Prosthecobacter* acquired the *btub*-genes by horizontal gene transfer (Pilhofer et al., 2007a; Sontag et al., 2005; Schlieper et al., 2005; Jenkins et al., 2002; Pilhofer et al., 2007b; Pilhofer et al., 2008). Schlieper *et al.* (2005) hypothesized, that one or possibly two tubulin genes were transferred from a eukaryote to *Prosthecobacter*.

This study screened for the presence of tubulins in other *Verrucomicrobia*, amongst others in the microtubule-like structure producing Epixenosomes (Petroni et al., 2000). The relationships of the *Prosthecobacter* tubulins within the tubulin superfamily were investigated by comprehensive sequence analyses including diverse members of the Tubulin/FtsZ superfamiliy and members of all known tubulin subfamilies. Based on the results, an alternative hypothesis on the origin of the bacterial tubulins in *Prosthecobacter* is proposed.

Results

Screening for tubulin genes in *Verrucomicrobia*

The following verrucomicrobial strains were screened for tubulin genes using PCR: *Akkermansia muciniphila* (subdivision 1), *Chthoniobacter flavus* (subdivision 2), isolate Ellin514 (subdivision 3), *Opitutus terrae*, *Opitutus* sp. strain VeGlc2 and Epixenosomes (all subdivision 4). Attempts to detect tubulin genes were performed using PCR with a high number of different combinations of primers, which were published previously (Pilhofer et al., 2007b). Using some combinations of these primers in PCR it was possible to amplify simultaneously eukaryotic alpha and beta tubulin as well as *Prosthecobacter* tubulins. However, no tubulin genes could be identified in any of the investigated strains.

Because of host and culture derived contaminations, a subtractive PCR approach using subtractive hybridization in microplates (Zwirglmaier et al., 2001) was performed on the Epixenosome-host system. Alpha, beta and gamma tubulin genes from the ciliate host (M. Hartmann and G. Petroni, unpublished data) were used as subtractor-DNA. In no case, genes similar to *Prosthecobacter* tubulins were found.

The absence of tubulin genes in *Verrucomicrobia* (except *Prosthecobacter*) was further supported by a later BLAST analysis of unfinished genome project data which became very recently available for *Verrucomicrobium spinosum*, *Akkermansia muciniphila* (both subdivision 1), isolate TAV2 (subdivision 4) and isolate DG1235 (subdivision 4).

Phylogenetic relationships within the Tubulin/FtsZ superfamily

To perform phylogenetic analyses in the Tubulin/FtsZ superfamily a comprehensive ARB database was established comprising archaeal and bacterial FtsZs, archaeal FtsZ-likes, Bacilli Tubulin-likes (Larsen et al., 2007), bacterial A and B tubulins (Jenkins et al., 2002; Pilhofer et al., 2007b) and different eukaryotic tubulins. Representatives of tubulins were the subfamilies alpha, beta, gamma, delta, epsilon, zeta, eta, theta, iota, kappa, a beta-related sequence and a cryptic eta-zeta-like sequence.

The ARB database was used to calculate a protein identity matrix of selected representatives (Figure 1). Bacterial tubulins showed the highest identity values with the major eukaryotic tubulin subfamilies in the range 21-44%. The values were significantly lower if the bacterial tubulins were compared to FtsZs (14-17%), FtsZ-likes (13-20%) or Tubulin-likes (10-15%).

Global trees including a selection of 174 representatives were calculated using different minimum similarity filters in combination with different treeing methods (see Materials and Methods). In all calculated trees, the representatives of the tubulin family, comprising the subdivisions alpha through kappa, formed a stable monophyletic group together with the bacterial A and B tubulin sequences. The tree topology within the tubulin family was rather instable (see below). Archaeal and bacterial FtsZs were always recovered as a stable monophyletic group. In most of the trees, the Tubulin-like sequences of Bacilli were monophyletic. In single cases the sequence of *Bacillus megaterium* clustered in a different position. The archaeal FtsZ-like sequences formed a less stable monophyletic group but if it was monophyletic, FtsZ-likes and FtsZ clustered together in most of the cases. Figure 2 shows a representative maximum likelihood tree. These phylogenetic analyses firmly support that eukaryotic and bacterial tubulins are members of a single clade within the Tubulin/FtsZ superfamily.

Phylogenetic relationships within the tubulin family.

All bacterial tubulin protein sequences were analyzed with the program PRINTS (Attwood et al., 2003) to detect motif similarities among bacterial tubulins and the different subfamilies of eukaryotic tubulins (Table 1). All bacterial B tubulin sequences had the highest hit score with the beta tubulin subfamily. On the contrary, bacterial A tubulin showed similar scores with alpha, beta and epsilon families thus depicting an unclear situation (Table 1).

This was also true for the identity values of bacterial tubulins with different tubulin subfamily representatives (Figure 1). BtubA sequences showed similar top values with alpha (37-40%) and beta (37-38%) tubulins; likewise the BtubB values with alpha (41-44%) and beta (40-41%) tubulins. The difference to the values with other tubulin subfamilies and to the value between BtubA and BtubB (34-38%) was also not significant in all cases.

The evolutionary relationships of bacterial tubulins within the tubulin family were further investigated by phylogenetic analyses using the ARB database used for the calculation of the global trees and a second database differing slightly in the sequence alignment (see Materials and Methods). The topologies within the tubulin family of all trees in both databases were compared.

Although the dataset was very comprehensive, the evolutionary relationships among the different tubulin subfamilies were not unambiguously resolved. Previous, less comprehensive studies indicated similar problems manifested by trees with low bootstrap values (Dutcher, 2003; Jenkins et al., 2002; Keeling and Doolittle, 1996). In all trees *Paramecium* kappa tubulin consistently associated with alpha tubulins branching either basally or internally to the group. In all calculated trees, *Paramecium* theta tubulin consistently associated with beta tubulins branching internally to the group but never associated with *Paramecium* beta tubulin. With these two exceptions, alpha, beta and gamma tubulin subfamilies were always recovered as monophyletic groups in all calculated trees, as well as bacterial A and B tubulins. The epsilon and delta tubulin groups were monophyletic in most but not in all of the calculated

trees. No clear associations between bacterial tubulins themselves or between any of the bacterial tubulins and known tubulin subfamilies could be recovered. In many trees, a monophyletic group comprising alpha, beta, gamma and bacterial A and B tubulins could be detected. A consensus tree is shown in Figure 3.

Discussion

It was speculated by previous studies that *Prosthecobacter* acquired the bacterial tubulin genes by horizontal gene transfer (Pilhofer et al., 2007a; Sontag et al., 2005; Schlieper et al., 2005; Jenkins et al., 2002; Pilhofer et al., 2007b; Pilhofer et al., 2008). This study failed to detect tubulin genes in other *Verrucomicrobia* by PCR or genome sequence screening. This is a further strong support for horizontal gene transfer together with the following indirect evidences: the presence of genuine *ftsZ* and other bacterial cell division genes in all *Verrucomicrobia* subdivisions with cultivable representatives (Pilhofer et al., 2007b; Pilhofer et al., 2008); the high divergence between *Prosthecobacter* FtsZ and bacterial tubulins (Pilhofer et al., 2007b); the integration of bacterial tubulin operons in a set of genes functionally related among themselves but apparently not with the tubulin operon (Pilhofer et al., 2007a); the different genomic environments of the different *btub*-operons (Pilhofer et al., 2007a); the presence of two *btub*-operons in one *Prosthecobacter* species (Pilhofer et al., 2007b).

The presence of repetitive elements delimiting a genomic region is a widely recognized indication for a recent acquisition through horizontal gene transfer. The lack of repetitive elements separating bacterial tubulin operons from the bordering genes (Pilhofer et al., 2007a) coupled with the presence of *btub*-operons in all *Prosthecobacter* species so far analyzed, suggest that the transfer of *btub*-operons into the genome of *Prosthecobacter* was not recent.

The level of conservation of bacterial tubulins among *Prosthecobacter* species and the functional studies on *Prosthecobacter* tubulins (Sontag et al., 2005; Schlieper et al., 2005) indicate that these genes are functional, important and evolutionary constrained in *Prosthecobacter*. This means that the products of the bacterial tubulin operon either represent an important but functionally independent unit or that they are capable of properly interacting with other typical bacterial proteins. Indeed, the analysis of the *P. dejongeii* genome (95% coverage) showed that, except the genes belonging to the *btub*-operon, very few genes with

homologies to eukaryotic counterparts are present in this organism and none of them is related to the eukaryotic cytoskeleton (Staley et al., 2005).

Interestingly, the third protein of the operon, the bacterial kinesin light chain, was shown to comprise five repetitions of a tetratricopeptide domain (Pilhofer et al., 2007a). This domain, also present in eukaryotic kinesin light chain, is generally involved in protein-protein interactions and in chaperone complexes (Gindhart and Goldstein 1996; Blatch and Lassle 1999). The role of bacterial kinesin light chain has not been investigated up to now, but its constant presence in the operons clearly suggests that it is essential for the proper functioning of bacterial A tubulin and bacterial B tubulin. A role of bacterial kinesin light chain in the assembly/stabilization of bacterial tubulin A-B heterodimer or in mediating its interaction with other proteins could be speculated.

Prosthecobacter tubulins belong to the tubulin family but clearly differ from previously known tubulin subfamilies (Figure 2, Figure 3) and should therefore be considered as members of two novel tubulin subfamilies. If bacterial A and B tubulins originated from a modern eukaryotic donor, a topological association to a eukaryotic tubulin subfamily would have been expected in phylogenetic trees.

Schlieper *et al.* (2005) proposed that one or possibly two tubulin genes were transferred from a eukaryote to *Prosthecobacter* where they were modified not to form tight heterodimers and to fold without chaperones, whose function in eukaryotic tubulin folding could be regulatory. A schematic representation of this hypothesis is provided in Figure 4A.

A direct derivation of bacterial A and B tubulins from modern eukaryotic tubulins (Schlieper et al., 2005) would imply that bacterial A and B tubulins lost the capability to form tight heterodimers, but obtained the capability to fold without the help of chaperones. Moreover, together with the bacterial kinesin light chain, they would have been reorganized into a typical bacterial operon and would likely have shifted to a completely different function due to the novel bacterial proteomic environment deprived of the typical proteins they usually

interact with. Subsequently, this novel function would have become essential for the bacterial host cell, thus, justifying the level of conservation observed among *Prosthecobacter* species. This event of trans-kingdom horizontal gene transfer would have occurred between organisms, which are not involved in a symbiotic association. Although the hypothesis by Schlieper *et al.* (2005) cannot be disregarded; our findings suggest a different interpretation.

The results of the phylogenetic analyses, which do not support a derivation of bacterial tubulins from eukaryotic tubulins, and the structure of the bacterial tubulin operon, which is typical of prokaryotes (the genes are tightly associated always in the same order, co-transcribed and no introns are present) suggest an alternative interpretation depicted in Figure 4B. Bacterial A and B tubulins derived, as well as alpha, beta and other eukaryotic tubulins, from a common ancestor of the tubulin family after the separation from FtsZ/Bacilli Tubulin-likes/archaeal FtsZ-likes. The capability to form weak heterodimers and to fold without the help of chaperones is an ancestral and not a derived character.

According to our hypothesis, the donor organism (from which Prosthecobacters acquired the *btub*-genes) was a bacterium that still remains to be identified. It is well known that the majority of prokaryotes have yet not been isolated (Schleifer, 2004). Nothing, except 16S rRNA, is known about most of these organisms and it could be speculated that some of them possess genes coding for bacterial tubulins that may have been transferred to *Prosthecobacter*.

Although our hypothesis could represent an interesting integration to most models of eukaryotic cell evolution (Embley and Martin, 2006; Martin and Muller, 1998; Moreira and Lopez-Garcia, 1998; Margulis et al., 2000; Cavalier-Smith, 2002), at this state of knowledge, we firmly believe that it is of fundamental importance to discuss different possible scenarios of tubulin evolution. It will be interesting to see if future genomic and metagenomic studies on yet uncultivable bacteria and protists will provide answers to these questions.

Materials and Methods

Verrucomicrobial strains and culture

Cultures of *P. debontii* DSM14044, *P. vanneervenii* DSM12252, *Opitutus terrae* DSM11246, *Opitutus sp.* VeGlc2 DSM14424 were obtained from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Germany) and grown at 28°C. *Prosthecobacter* strains were grown aerobically in DSM medium 628, *V. spinosum* was grown aerobically in DSM medium 607, *O. terrae* and *Opitutus* sp. VeGlc2 were grown anaerobically in DSM medium 298g and 503, respectively. *Euplotidium itoi* with Epixenosomes as ectosymbionts was grown according to Rosati et al. (1999).

DNA extraction

DNA from the *Prosthecobacter* strains was extracted according to Wisotzkey et al. (1990). DNA from *Opitutus* strains and Epixenosomes strains was extracted using a modified protocol to inhibit high nuclease activity. The lysis of the cells was directly performed in high SDS concentration without previous lysozyme treatment. SDS (10%) was added in ratio 1:1 to the cell resuspension, followed by the addition of Proteinase K and RNAse. The mixture was incubated for 1 h at 55° C. Following steps were as described by Wisotzkey et al. (1990).

Subtraction hybridization in microplates

A modified protocol for subtraction hybridization in microplates (Zwirglmaier et al., 2001) was used to eliminate fragments amplified from the Epixenosomes host genomic DNA and from other eukaryotic contaminants. Alpha, beta and gamma tubulin PCR fragments derived from the Epixenosomes host were used as subtractor-DNA and were therefore immobilized. Modified CODEHOP (Rose et al., 1998; Rose et al., 2003) with a random sequence (M1 or M2) at the 5'-end instead of the clamp were used in PCR to generate DNA fragments for

hybridization. PCR using the primers M1 and M2 was performed with 5 µl of removed supernatant to amplify putative PCR products which did not hybridize. Primers were M1-DNEA CTACCAACAGTCCCAATCCCAGNGCYTCRTTRTC, M2-DLEP1 CCCACCAGATGACCAAGAGTAGTGGAYCTNGARCC, M2-DLEP2 CCCACCAGATGACCAAGAGTAGTGGAYTTRGARCC, M1 CTACCAACAGTCCCAATCCC, M2 CCCACCAGATGACCAAGAGTA. Hybridization attempts were performed at increasing stringency conditions.

Sequence analyses

Protein sequences were analyzed using the program PRINTS (Attwood et al., 2003) in order to detect motifs and to calculate a probability value for the tubulin fingerprint.

To perform phylogenetic sequence analyses, two different databases were established using the ARB program package (Ludwig et al., 2004).

The two databases, Tubulin_ClustalW and Tubulin_Conserved_Domain, contained 194 entries which represent bacterial and archaeal FtsZs, different eukaryotic tubulin subfamilies, bacterial A and B tubulins, Bacilli Tubulin-likes and archaeal FtsZ-likes.

For the Tubulin_ClustalW database, amino acid sequences were first aligned using ClustalW (Thompson et al., 1994). For the Tubulin_Conserved_Domain database sequences were aligned according to the tubulin alignment available at the Conserved Domain Database (Marchler-Bauer et al., 2005); therefore additional 30 amino acid sequences were imported. In both databases the amino acid alignments were refined manually.

The identity matrix for a selection of representatives was generated using the ARB program package (Ludwig et al., 2004).

For tree calculations, two filters were produced (prot_10, prot_30), each retaining only positions conserved respectively in at least 10% or 30% of the selected sequences. Phylogenetic analyses were performed using distance matrix methods [programs ARB neighbor joining and Phylip UPGMA (Sneath and Sokal, 1973), FITCH (Fitch and

Margoliash, 1967)], maximum parsimony [program Phylip PROTPARS (Eck and Dayhoff, 1966)], maximum likelihood [programs Phylip PROML, PHYML (Felsenstein, 1988) and TREE-PUZZLE (Schmidt et al., 2002)]. All programs are implemented in the ARB program package (Ludwig et al., 2004). Each analysis was repeated using the different treeing methods in combination with both filters. For the TREE-PUZZLE method a smaller selection of sequences was used due to calculation time limits. Neighbor joining and maximum parsimony trees were bootstrap resampled (1000 respectively 100 bootstraps), number of puzzling steps for TREE-PUZZLE trees was 1000. For distance matrix methods and the maximum likelihood method the used model of substitution was Dayhoff PAM matrix, for the TREE-PUZZLE method the Muller-Vingron model of substitution (Muller and Vingron, 2000) was used. The topologies of all trees were compared to recover the most stable associations.

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Figure legends

Figure 1. Identity matrix of different representatives of the Tubulin/FtsZ superfamily

Identity matrix of protein sequences was produced using the ARB program package. Identity values in percent. Identities between the bacterial tubulins and eukaryotic tubulins are shaded in grey. Pva, *Prosthecobacter vanneervenii*; Pdb, *Prosthecobacter debontii*; Pdj, *Prosthecobacter dejongeii*; Pte, *Paramecium tetraurelia*; Ddi, *Dictyostelium discoideum*; Hsa, *Homo sapiens*; Bth, *Bacillus thuringiensis*; Bce, *Bacillus cereus*; Hal, *Halobacterium* sp.

Bacterial A and B tubulins have the highest similarities with the different tubulin subfamilies. No significant high similarity of BtubA or BtubB to a specific tubulin subfamily could be detected.

Figure 2. Phylogenetic tree of different representatives of the Tubulin/FtsZ superfamily

A representative maximum likelihood tree is shown, which was calculated in combination with a 10% minimum similarity filter. Bar represents 1% estimated evolutionary distance. Number of included sequences is indicated for closed groups. For single sequences accession numbers are provided.

The families Tubulin and FtsZ were always recovered as monophyletic groups. Bacterial A and B tubulins clustered always within the Tubulin family. The groups Tubulin-likes and FtsZ-likes were not always stable. Often, FtsZ and FtsZ-likes clustered together. The topology within the Tubulin family was rather instable (Figure 3).

Figure 3. Phylogenetic relationships within the tubulin family

The presented phylogenetic tree is a consensus of 28 trees produced using two different alignments, seven treeing algorithms and two filters. The consensus tree shows only associations recovered in the majority of the trees. Support values for six trees of the Tubulin_ClustalW database are reported at the branches; from left to right: maximum parsimony (100 bootstraps), neighbor joining (1000 bootstraps), TREE-PUZZLE (1000 puzzling steps); filters prot_30 (upper numbers) and prot_10 (lower numbers); asterisk denotes a node which was not recovered in the respective tree. Numbers within closed groups refer to the number of included sequences; due to calculation limits TREE-PUZZLE trees were calculated using a reduced number of sequences (number in parentheses).

All major tubulin subfamilies (alpha, beta, gamma, epsilon, bacterial A tubulin, bacterial B tubulin) were recovered as monophyletic groups with relatively high support; delta tubulins were sometimes split in two groups. No specific associations between any of the major tubulin subfamilies could be detected.

Figure 4. Schematic representation of the alternative hypotheses on the origin of bacterial tubulins

(A) Hypothesis proposed by Schlieper *et al.* (2005). One or two eukaryotic tubulin genes were horizontally transferred from a eukaryotic donor to *Prosthecobacter* spp. where they underwent regressive evolution.

(B) Hypothesis proposed by the authors. Bacterial A and B tubulins originated, together with eukaryotic tubulins, from an ancestral tubulin present in an ancestral organism shared between eukaryotes and some bacterial lineages. *Prosthecobacter* spp. acquired bacterial tubulins through horizontal gene transfer from a bacterial descendant.

Table 1. Tubulin and FtsZ sequence motifs identified in *Prosthecobacter* tubulins.

Protein	Group	No. of motifs	*P* value	Group	No. of motifs	*P* value
bacterial A tubulin	*P. dejongeii* (Jenkins et al. 2002)			*P. debontii* operon 1		
	Tubulin	9 of 9	$1.4\ e^{-62}$	Tubulin	9 of 9	$3.5\ e^{-61}$
	alpha tubulin	**7 of 13**	**$5.9\ e^{-17}$**	**epsilon tubulin**	**4 of 10**	**$2.5\ e^{-15}$**
	beta tubulin	6 of 13	$1.4\ e^{-14}$	beta tubulin	6 of 13	$6.3\ e^{-15}$
	epsilon tubulin	4 of 10	$1.2\ e^{-15}$	alpha tubulin	7 of 13	$6.6\ e^{-15}$
	delta tubulin	3 of 12	$1.9\ e^{-12}$	delta tubulin	4 of 12	$1.2\ e^{-13}$
	gamma tubulin	5 of 8	$3.3\ e^{-11}$	gamma tubulin	5 of 8	$6.5\ e^{-11}$
	FtsZ	2 of 6	$8.3\ e^{-6}$	FtsZ	2 of 6	$8.2\ e^{-6}$
	P. debontii operon 2			*P. vanneervenii*		
	Tubulin	9 of 9	$4.5\ e^{-58}$	Tubulin	9 of 9	$3.4\ e^{-61}$
	beta tubulin	**7 of 13**	**$1.5\ e^{-15}$**	**beta tubulin**	**8 of 13**	**$4.2\ e^{-16}$**
	epsilon tubulin	4 of 10	$1.8\ e^{-15}$	epsilon tubulin	4 of 10	$2.0\ e^{-14}$
	alpha tubulin	5 of 13	$1.4\ e^{-14}$	alpha tubulin	6 of 13	$6.4\ e^{-14}$
	gamma tubulin	4 of 8	$1.2\ e^{-7}$	delta tubulin	3 of 12	$1.2\ e^{-13}$
	FtsZ	2 of 6	$8.3\ e^{-6}$	gamma tubulin	5 of 8	$1.2\ e^{-11}$
	delta tubulin	2 of 12	$1.5\ e^{-5}$	FtsZ	2 of 6	$1.4\ e^{-6}$
Protein	**Group**	**No. of motifs**	***P* value**	**Group**	**No. of motifs**	***P* value**
bacterial B tubulin	*P. dejongeii* (Jenkins et al. 2002)			*P. debontii* operon 1		
	Tubulin	9 of 9	$1.9\ e^{-72}$	Tubulin	9 of 9	$1.6\ e^{-72}$
	beta tubulin	**8 of 13**	**$4.2\ e^{-26}$**	**beta tubulin**	**8 of 13**	**$4.2\ e^{-26}$**
	epsilon tubulin	6 of 10	$3.3\ e^{-19}$	epsilon tubulin	6 of 10	$1.6\ e^{-20}$
	alpha tubulin	5 of 13	$2.5\ e^{-14}$	gamma tubulin	5 of 8	$1.8\ e^{-13}$
	gamma tubulin	4 of 8	$1.4\ e^{-13}$	alpha tubulin	5 of 13	$9.3\ e^{-13}$
	FtsZ	3 of 6	$1.7\ e^{-10}$	delta tubulin	2 of 12	$9.6\ e^{-10}$
	delta tubulin	2 of 12	$5.6\ e^{-9}$	FtsZ	2 of 6	$4.1\ e^{-9}$
	P. debontii operon 2			*P. vanneervenii*		
	Tubulin	9 of 9	$2.2\ e^{-73}$	Tubulin	9 of 9	$1.2\ e^{-72}$
	beta tubulin	**7 of 13**	**$6.3\ e^{-24}$**	**beta tubulin**	**8 of 13**	**$3.9\ e^{-25}$**
	epsilon tubulin	6 of 10	$2.6\ e^{-20}$	epsilon tubulin	6 of 10	$3.4\ e^{-20}$
	gamma tubulin	5 of 8	$5.1\ e^{-14}$	gamma tubulin	5 of 8	$1.8\ e^{-14}$
	alpha tubulin	5 of 13	$2.3\ e^{-12}$	alpha tubulin	4 of 13	$2.2\ e^{-11}$
	FtsZ	3 of 6	$1.4\ e^{-10}$	delta tubulin	2 of 12	$5.6\ e^{-10}$
	delta tubulin	2 of 12	$4.1\ e^{-10}$	FtsZ	2 of 6	$1.5\ e^{-8}$

Note. Bacterial A and B tubulins analyzed with PRINTS (Attwood et al. 2003); P value, probability value; **bold lines**, tubulin subfamily with highest score after Tubulin superfamily

Figure 1

			Tubulin															*Bacilli*		archaeal FtsZ-like			FtsZ	
			BtubA				BtubB				Alpha		Beta		Gamma	Epsilon	Delta	TubZ	RepX					
			Pdb A2	Pdb A1	Pdj	Pva	Pdb B2	Pdb B1	Pdj	Pva	Pte	Ddi	Ddi	Pte	Ddi	Hsa	Hsa	Bth	Bce	Hal3	Hal4	Hal5	Pva	Pdb
Tubulin	BtubA	Pdb A2	100																					
		Pdb A1	82	100																				
		Pdj	81	94	100																			
		Pva	81	92	93	100																		
	BtubB	Pdb B2	35	37	35	37	100																	
		Pdb B1	37	37	36	38	88	100																
		Pdj	35	37	34	36	90	95	100															
		Pva	36	37	36	37	84	90	90	100														
	Alpha	Pte	39	40	41	40	44	43	43	42	100													
		Ddi	37	37	38	39	43	42	41	42	71	100												
	Beta	Ddi	38	37	37	38	41	41	41	41	44	44	100											
		Pte	37	37	38	38	41	41	41	40	45	43	79	100										
	Gamma	Ddi	29	31	30	31	38	38	38	38	37	37	37	36	100									
	Epsilon	Hsa	31	30	29	29	36	35	35	34	36	34	37	37	31	100								
	Delta	Hsa	22	21	23	22	23	23	22	23	24	24	24	24	23	27	100							
Bacilli	TubZ	Bth	14	13	13	13	15	13	14	14	12	13	14	13	11	11	11	100						
	RepX	Bce	10	11	12	12	11	12	12	11	12	11	11	11	11	14	12	13	100					
archaeal FtsZ-like	FtsZ3	Hal	17	18	19	18	16	16	16	16	16	16	17	18	14	15	13	17	12	100				
	FtsZ4	Hal	17	18	19	19	20	18	19	17	17	16	17	17	17	17	15	16	12	41	100			
	FtsZ5	Hal	15	16	16	16	14	14	13	14	12	11	13	13	12	14	10	13	16	31	25	100		
FtsZ		Pva	15	16	17	17	15	14	14	15	11	13	14	14	15	15	15	11	12	19	18	17	100	
		Pdb	16	17	17	18	15	15	15	14	12	13	15	17	17	15	15	14	11	19	19	15	80	100

Figure 2

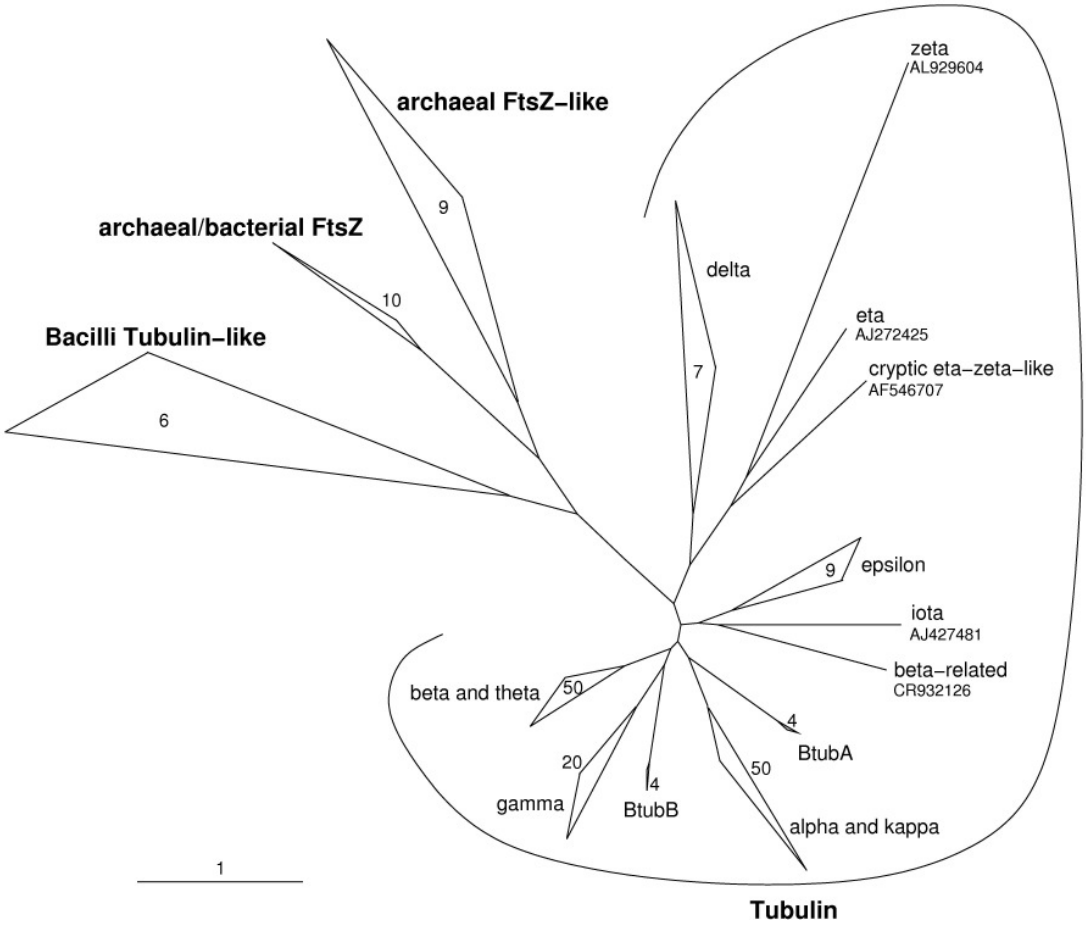

archaeal FtsZ–like
archaeal/bacterial FtsZ
Bacilli Tubulin–like
9
10
6
zeta
AL929604
delta
7
eta
AJ272425
cryptic eta–zeta–like
AF546707
9
epsilon
iota
AJ427481
beta–related
CR932126
beta and theta
50
4
BtubA
20
50
4
gamma
BtubB
alpha and kappa
1
Tubulin

Figure 3

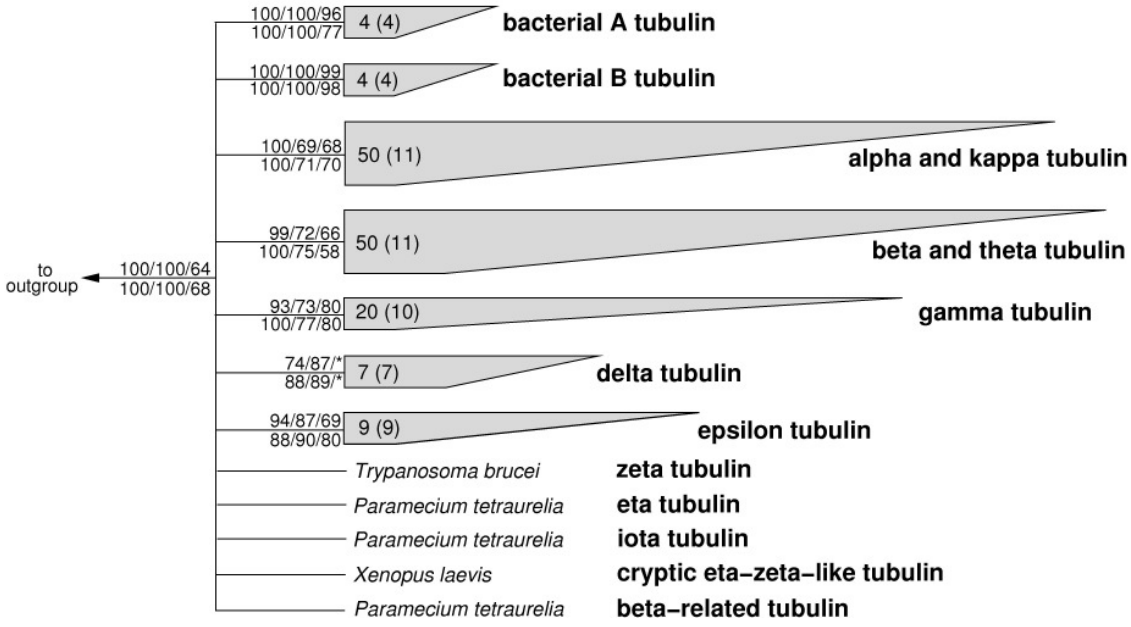
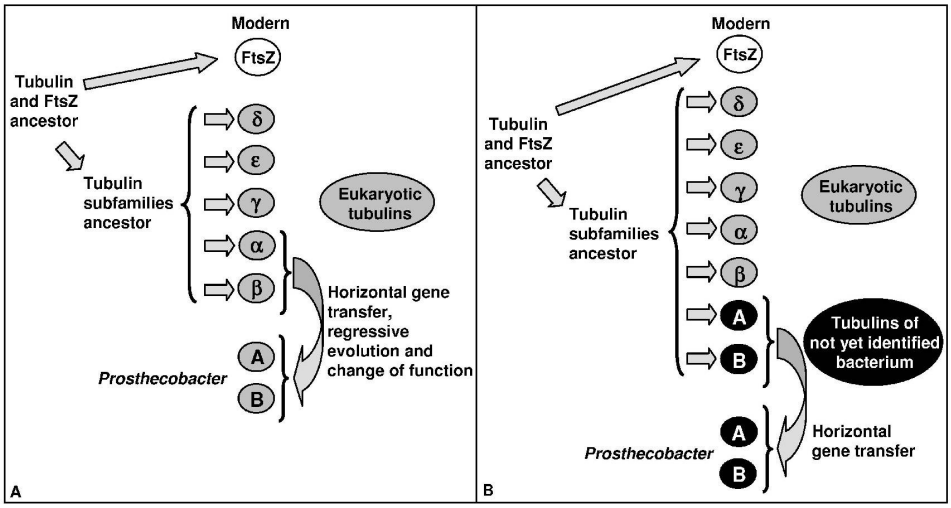

100/100/96
100/100/77
4 (4)
bacterial A tubulin
100/100/99
100/100/98
4 (4)
bacterial B tubulin
100/69/68
100/71/70
50 (11)
alpha and kappa tubulin
99/72/66
100/75/58
50 (11)
beta and theta tubulin
to outgroup
100/100/64
100/100/68
93/73/80
100/77/80
20 (10)
gamma tubulin
74/87/*
88/89/*
7 (7)
delta tubulin
94/87/69
88/90/80
9 (9)
epsilon tubulin
Trypanosoma brucei
zeta tubulin
Paramecium tetraurelia
eta tubulin
Paramecium tetraurelia
iota tubulin
Xenopus laevis
cryptic eta–zeta–like tubulin
Paramecium tetraurelia
beta–related tubulin

Figure 4


Modern
FtsZ
Tubulin and FtsZ ancestor
Tubulin subfamilies ancestor
δ
ε
γ
α
β
Eukaryotic tubulins
Horizontal gene transfer, regressive evolution and change of function
Prosthecobacter
A
B
A
Modern
FtsZ
Tubulin and FtsZ ancestor
Tubulin subfamilies ancestor
δ
ε
γ
α
β
A
B
Eukaryotic tubulins
Tubulins of not yet identified bacterium
Prosthecobacter
A
B
Horizontal gene transfer
B

Appendix E

Additional Materials and Methods

Materials and methods which were used in this study and which were not described in detail or referenced in Appendix A-D are specified in this section.

Heterologous gene expression, Western hybridization and antibody production

Production of recombinant expression vectors

Expression constructs were produced by ligating pHIS17 (Daniel Schlieper, MCRI, Oxted, UK; personal communication) vector DNA with PCR-amplified open reading frames of the genes of interest. Primer design allowed to produce recombinant protein having a C-terminal 6xHIS-tag. The used primers are listed below.

Pdj_btubA_F_NdeI	TGA CAG TCA TAT GAA GGT CAA CAA CAC CAT TG
Pdj_bklc_R_BamHI	TAG CTA GGA TCC TTA TCA CAG CGA TTT ATC AAT CAA CAG
Pdj_bklc_R_EcoRI	TAG CTA GAA TTC TTA TCA CAG CGA TTT ATC AAT CAA CAG
Pdj_bklc_R_BamHI_His	TAG CTA GGA TCC CAG CGA TTT ATC AAT CAA CAG AG
Pdj_bklc_F_NdeI	TGA CAG TCA TAT GTC GCG GTT TAG CCT ATT C
Pdj_btubA_F_NdeI_v2	TGA CAG TCA TAT GAA GGT CAA CAA CAC CAT TGT CGT T
Pdj_btubA_R_EcoRI	TAG CTA GAA TTC TTA GCG GCG GCG ATC CAC C
Pdj_btubB_F_NdeI	TGA CAG TCA TAT GAG AGA GAT TTT AAG CAT TCA CG
Pdj_btubB_R_EcoRI	TAG CTA GAA TTC TTA ACT CGC GTC GCG GTA AG
Pv_btubA_F_NdeI	TGA CAG TCA TAT GAA GGT CAA CAA CAC AAT CG
Pv_bklc_R_EcoRI	TAG CTA GAA TTC TTA CAA GCT CTT GTC GAT CAA AAG

Agarose pieces containing the DNA fragment of interest were cut from the gel and purified using the Perfectprep Kit (Eppendorf, Germany). Restriction digestion of vector and insert DNA was performed in two steps. Firstly, the DNA was digested with NdeI and purified using the Perfectprep Kit (Eppendorf, Germany). Secondly, the DNA was digested with EcoRI or BamHI (depending on the construct) and purified using the Perfectprep Kit (Eppendorf, Germany). Digestions were performed according to the instructions of the manufacturer. The sticky ends of the restricted vector and insert DNA were ligated using T4-DNA ligase (Roche, Germany) according to the instructions of the manufacturer. Produced constructs and corresponding primers used for insert amplification are listed below.

pHIS17-*btubA*(Pdj)	Pdj_btubA_F_NdeI_v2	Pdj_btubA_R_EcoRI
pHIS17-*btubB*(Pdj)	Pdj_btubB_F_NdeI	Pdj_btubB_R_EcoRI
pHIS17-*bklc*(Pdj)	Pdj_bklc_F_NdeI	Pdj_bklc_R_EcoRI
pHIS17-*bklc*(Pdj)-6xHis	Pdj_bklc_F_NdeI	Pdj_bklc_R_BamHI_His
pHIS17-*btubA-btubB-bklc*(Pdj)	Pdj_btubA_F_NdeI	Pdj_bklc_R_EcoRI
pHIS17-*btubA-btubB-bklc*(Pva)	Pv_btubA_F_NdeI	Pv_bklc_R_EcoRI

Preparation of electrocompetent cells

200 ml culture medium was inoculated with 1 ml of an over night culture of the strains *E. coli* C41 (DE3) (Dumont-Seignovert et al. 2004) or *E. coli* BL21 (DE3)pLysS (Paisley, UK). Cells were grown to an OD_{600} of 0.6-0.8 and cooled to 4 °C. The culture was centrifuged (4,300 rpm, 30 min, 4 °C) and the pellet was suspended in 100 ml ultrapure H_20 (4 °C). This washing step was repeated three times. Subsequently, the pellet was resuspended in 50 ml 10% (v/v) glycerol (4 °C), centrifuged and finally resuspended in an appropriate volume of 10% glycerol. The competent cells were stored in aliquots at -80 °C.

Transformation

After ligation, the constructs were transformed into chemically competent *E. coli* TOP10 cells (Invitrogen, Germany) according to the instructions of the manufacturer. Clones were screened for the presence of the insert using PCR and vector specific primers. Plasmid inserts of selected clones were resequenced.

Error-free constructs were transformed in electrocompetent expression strains *E. coli* C41 (DE3) (Dumont-Seignovert et al. 2004) or *E. coli* BL21 (DE3)pLysS (Paisley, UK) by electroporation. Vector-DNA was dialyzed for 20 min on a nitrocellulose membrane (0,025 µm, Millipore, Germany) against ultrapure H_2O. For transformation, 90 µl of competent cells were thawed at 4 °C. Ca. 0.5 µg of plasmid DNA were added to the cells and incubated at 4 °C for 30 min. The mixture was applied into an electroporation cuvette (1 mm electrode distance) and electroporated using a Gene pulser II transfection apparatus (Bio-Rad, Germany) at the following settings: 2.5 kV, 25 µF, and 200 Ω. Immediately after electroporation, 1 ml of prewarmed SOC medium (Invitrogen, Germany) was added to the cuvette, the mixture was transferred into a new tube and incubated shaking at 37 °C for 1 h. Subsequently, the cells were plated on selective agar plates.

Expression of recombinant proteins

Expression cultures were grown at 37 °C to an OD_{600} of 0.6 prior to induction with IPTG (1 mM final concentration). Protein synthesis was carried out for 3 h.

Denaturing polyacrylamide gel electrophoresis (SDS-PAGE)

For the preparation of crude cell extracts, bacterial cultures were pelleted and resuspended in 1x LEW buffer (Macherey-Nagel, Germany). The samples were loaded onto the SDS-gel after heating for 10 min at 100 °C in 1 × loading dye.

4 x SDS-loading dye
0.25 M Tris-HCl (pH 6.8)
0.4 M ß-mercaptoethanol
8% (w/v) SDS
40% (v/v) glycerol
0.4% bromophenol blue

Protein samples were separated and analyzed in denaturing polyacrylamide gels (12% polyacrylamide) as described by Laemmli (1970). The size of the gels was 10 × 7 × 0.75 cm, and electrophoresis was performed at room temperature at 200 V per gel. To visualize proteins in polyacrylamide gels, gels were incubated for 15 min in Coomassie Blue staining solution. Protein bands were visualized after removing unbound Coomassie dye by incubating the gel in destaining solution for 30 min, followed by incubation in ultrapure H_2O.

Coomassie Blue staining solution
0.125% Coomassie Brilliant Blue R-250
50% methanol
10% acetic acid

Destaining solution
7% (v/v) acetic acid
40% (v/v) methanol

Western blotting and hybridization

Separated proteins from SDS-PAGE gels were transferred to PVDF membranes (0.45 µ; Pall, USA) as described by Towbin et al. (1979). The transfer was carried out in the Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad, Germany) as recommended by the manufacturer. After transfer of the proteins, the membrane was blocked at 4 °C for 24 h in PBS-T solution [pH 7.4; 0.14 M NaCl, 2.7 mM KCl, 10.1 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 0.1% (v/v) Tween 20] supplemented with 5% fat-free dry milk. Subsequently, the membrane was incubated with the primary antibody (diluted in PBS-T) for 1-2 h at 20 °C. For dilution factors of the primary antibody see B.5.3.2. After washing the membrane once with PBS-T for 15 min and three times for 5 min, a secondary peroxidase-conjugated antibody (diluted 1:100,000 in PBS-T; Pierce, USA) was added and incubated for 1 h at 20 °C. After washing (see washing after primary antibody), the signal was detected using ECL Western Blotting Substrate (Pierce, USA) and X-ray films according to the manufacturer's instruction.

Bklc purification and antibody production

An induced *E. coli* C41/pHIS17-*bklc*(Pdj)-6xHis culture was used for protein purification of Bklc-6xHIS. The purification had to be performed at denaturing conditions (using urea), as the protein was almost exclusively expressed into inclusion bodies. 200 ml culture were pelleted by centrifugation, resuspended in 20 ml LEW buffer (Macherey-Nagel, Germany) and treated with 1 mg/ml lysozyme for 30 min at 4 °C. After 20 min of sonification at 4 °C, insoluble constituents (including inclusion bodies) were pelleted (10,000 x g, 30 min, 4 °C). The pellet was washed once in 8 ml LEW buffer and resuspended in 2 ml DS buffer (LEW buffer supplemented with 8 M urea). After 2 min of sonification, the mixture was incubated agitating for 60 min at 4 °C. The sample was centrifuged for 30 min at 10,000 x g at 20 °C. For purification, the clear supernatant was applied to a Ni-TED 2000 column (Macherey-Nagel, Germany). Purification was based on the binding of the 6xHis-tag of the recombinant protein to a silica-based resin precharged with Ni^{2+}-ions. Washing and elution of the protein was performed according to the instructions of the manufacturer. The protein was concentrated by precipitation using ammoniumsulfate (4 M) and redissolving it in DS buffer containing 8 M urea. 3000 µg (4.4 µg/ml) of protein were sent to Sigma-Genosys (UK) for polyclonal antibody production (New Zealand White Rabbits, 77 days immunization protocol).

Specific antibodies were purified from the serum. 375 mg Bklc-protein were loaded and run on an SDS-PAGE gel and blotted to a PVDF membrane. The membrane was stained for 1 min using Ponceau S and washed 3 min in ultrapure H_2O. The region with Bklc was cut out and the membrane was destained for 1 min using 0.1 M NaOH and for 5 min using ultrapure H_2O. The membrane was blocked at 20 °C in PBS-T supplemented with 5 % fat-free dry milk for 1 h. 5 ml serum were 1:1 diluted using PBS-T and incubated at 4 °C with the membrane for 24 h. Subsequently, the membrane was washed three times in PBS-T for 5 min. Specific antibodies were eluted using incubation with 1.5 ml glycine (100 mM, pH 2.6) for 10 min with occasional vortexing. The supernatant was immediately neutralized with 150 µl Tris-HCl (1 M, pH 8), supplemented with 0.1 % Na-azide and stored at 4 °C. After a second elution and two washings with PBS-T, the membrane was stored at -20 °C for reuse.

Immunofluorescence staining

Cells were directly fixed in the culture medium as described by Sontag et al. (2005) or by addition of 2% formaldehyde and incubation at 20 °C for 30 min. Cells were washed three times in PBS and stored at 4 °C. 4 µl cell resuspension were applied to each well of an epoxy resin coated glass slide (Marienfeld, Germany) and dried at 37 °C. Cells were permeabilized by incubation with 5 mg/ml lysozyme at 4-37 °C for 10 s to 1h (see results). Slides were washed three times in PBS-T and air-dried. 10 µl PBS supplemented with 2% BSA were applied to each well and incubated for 15 min at 20 °C. Blocking solution was replaced by the primary antibody (diluted in PBS-BSA; for dilution factors see results). Slides were incubated in a humid (PBS) chamber for 16 h at 4 °C. Slides were washed 10 times in PBS-T (30 s each) and air dried. 10 µl secondary antibody (anti-rabbit-IgG antibody, FITC-conjugated or AlexaFluor488-conjugated; diluted 1:50 to 1:100 in PBS-BSA; Sigma, Germany; Invitrogen, Germany) were applied to each well. Slides were incubated at 20 °C for 3 h in the dark, washed 10 times in PBS-T (30 s each) and air-dried. The slides were mounted with Citifluor AF1 (Citifluor, UK) prior to microscopical analyses using a confocal laser scanning microscope (Zeiss, Germany).

Electron microscopy

For negative staining 10 µl of culture were mixed with 10 µl phosphotungstic acid (2 %, pH 7.0), transferred to a grid and examined using a Jeol 100 SX (Japan) electron microscope.

For transmission electron microscopy (TEM) cells were harvested by centrifugation at 4 °C. The supernatant was reduced to 0.5 ml and the pellet was resuspended. 1 ml glutaraldehyde [5% in cacodylate buffer (1 M, pH 7.2)] was added to the resuspension and incubated at 20 °C for 1.5 h. Cells were pelleted, and washed twice in cacodylate buffer (0.05 M, 7.2 M). Cells were pelleted, resuspended in 100 µl osmium tetroxyde [2% in cacodylate buffer (0.05 M, 7.2 M)] and incubated at 20 °C for 30 min. Cells were pelleted and washed in cacodylate buffer (0.05 M, 7.2 M). Cells were dehydrated using graded ethanols (10 min each 30%, 50%, 70%, 90%; twice 15 min 100%). Cells were resuspended in acetone, incubated at 20 °C for 30 min and pelleted. Pellet was resuspended in 0.5 ml acetone / epoxy resin mixture (1:1), incubated at 20 °C for 24 h and cells were again pelleted. Supernatant was removed and cap was incubated opened at 20 °C for 5 h. 0.5 ml epoxy resin were added and incubated at 20 °C for 5 h. The opened cap was incubated at 60 °C for 72 h. The samples were cut into thin sections and placed onto copper grids. The sections were counter stained by incubation in a saturated aqueous uranyl acetate solution for 15 min and in 2.6% lead citrate for 15 min. Sections were examined using a Jeol 100 SX (Japan) electron microscope.
Epoxy resin was prepared by mixing Durcupan (4 ml), Epon812 (5 ml), DDSA (12 ml) and DMP30 (0.4 ml) for 30 min using a glass rod. Resin was stored at -20 °C.

Electron Microscopic Immunocytochemistry

For electron microscopic immunocytochemistry cells were directly fixed in growth medium by addition of 2% glutaraldehyde (25 %) and incubated at 20 °C for 45 min. Cells were pelleted, and washed in PBS. Cells were dehydrated using graded ethanols (10 min - 30%, 10 min - 50%, 5 min - 70%, 5 min - 80%, 5 min - 90%, 5 min - 100%, 10 min - 100%). Cells were resuspended in LR White Resin (Fluka, Germany) and incubated shaking at 20 °C for 24 h. Cells were pelleted, again resuspended in LR White Resin and incubated at 55 °C for 24 h. The samples were cut into thin sections and placed onto nickel grids.
Grids were incubated Tris-HCl (0.2 M, pH 7.6) for 5 min and blocked for 15 min at 20 °C using 50 mM Tris-HCl-0.15 M NaCl-1% BSA (TSB-buffer). Grids were incubated with the

first antibody (in TSB buffer; for dilution factors see results) at 4 °C for 24 h. Grids were washed ten times by dipping into TSB buffer and were incubated with the second antibody (anti-rabbit-IgG antibody, gold-conjugated, 10 nm particle size, 1/10 dilution; Sigma, Germany) for 1.5 h at 20 °C. Grids were washed ten times by dipping into TSB buffer and ten times by dipping into ultrapure water. Thin sections were counter stained by incubation in a saturated aqueous uranyl acetate solution for 15 min and in 2.6% lead citrate for 15 min. Thin-sections were examined using a Jeol 100 SX (Japan) electron microscope.

Epoxy resin embedded samples were deosmificated prior to hybridization by incubation of the grids in saturated $NaIO_4$ solution for 30 min at 20 °C.

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CURRICULUM VITAE

Martin Pilhofer

Hiltenspergerstraße 32
80796 Munich, Germany
+49 (0) 89 28 77 81 63
+49 (0) 171 24 765 75
martin-pilhofer@web.de

Date of birth: 05 April 1980
Nationality: German

Professional Experience

Nov. 2004 - Present	**PhD thesis** at the Department of Microbiology, Technical University Munich (TUM), Germany Advisor Karl-Heinz Schleifer Title: Elucidation of the cell division mechanism and characterization of tubulins in the bacterial phylum *Verrucomicrobia*

Education

Nov. – Dec. 2004	**Studentship** of the Bayerische Forschungsstiftung, Munich
Sep. 2004	**MSc** (german university diploma) **in Biology** Grade 1.1 (on a scale of 1 to 4, with 1 being the highest) "with distinction" *Major Microbiology, minor subjects Genetics and Biochemistry*
Nov. 2003 – Sep. 2004	**Diploma thesis** at the Department of Microbiology, TUM, Munich Grade 1.0 (on a scale of 1 to 4, with 1 being the highest) Advisor Giulio Petroni Title: Characterization and phylogeny of bacterial tubulin genes and their genomic environment in two *Prosthecobacter* species and search for tubulin genes in *Verrucomicrobia*
Oct. 2001 – Sep. 2004	**Biology studies** at the TUM, Munich Practical trainings in Microbiology (6 month), Genetics (1 month) and Biochemistry (1 month) Undergraduate studentship (3 month) in the group of W. Ludwig, Department of Microbiology, TUM, Munich
Oct. 1999 – Oct. 2001	**Biology studies** at the University of Bayreuth, Germany Intermediate diploma
Sep. 1990 – Jun. 1999	**A-levels** (German Abitur), Sulzbach-Rosenberg, Germany

Teaching activity

Supervision of two diploma students
Supervision of practical trainings for undergraduate and graduate students

Languages

German (native)
English (fluent, written and spoken)
French (basic knowledge)

PC skills

MS-Office (Word, Excel, Power Point)
Basic knowledge in Linux
ARB software package

Hobbies

Skiing and snowboarding instructor
Tour guide

Publications

Articles

Pilhofer M, Rosati G, Ludwig W, Schleifer KH, Petroni G.
Coexistence of Tubulins and *ftsZ* in Different *Prosthecobacter* Species.
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Prepared for submission.

Weber SD, Hofmann A, Wanner G, **Pilhofer M**, Agerer R, Ludwig W, Schleifer KH, Fried J.
The diversity of fungi in aerobic sewage granules assessed by 18S rDNA and ITS sequence analyses
Submitted.

Grünwald S, **Pilhofer M**, Höll W.
Transient, stable and obligate symbiotes in gut systems of wood inhabiting long-horned beetle larvae (*Col. cerambicide*)
In preparation.

Oral presentations

Pilhofer M, Rosati G, Ludwig W, Schleifer KH, Petroni G.
Search for tubulin genes in free-living bacteria and symbionts of ciliates. Characterization of the genomic environment of tubulin genes from *Prosthecobacter*.
Italian Section Society of Protozoologists 24th Annual Meeting, 1-2 October 2004, Rapallo, Italy

Pilhofer M, Rosati G, Ludwig W, Schleifer KH, Petroni G
Studies of *Verrucomicrobia* shed new light on the origin of bacterial tubulins.
5th International Symbiosis Society Congress, 4 - 10 August 2006, Vienna, Austria

Poster presentations

Pilhofer M, Rosati G, Ludwig W, Schleifer KH, Petroni G
Cell division gene *ftsZ* in bacteria also possessing tubulin genes: hypothesis on evolution of bacterial tubulin.
Annual conference of the VAAM (society for general and applied microbiology) 25 – 28 September 2005, Göttingen, Germany

Pilhofer M, Ludwig W, Schleifer KH
In situ detection of genes for dinitrogenase reductase (*nifH*) in bacterial cells using fluorescent dye-labeled polynucleotide probes.
Development and control of functional biodiversity at micro- and macro-scales, 5 - 7 October 2005, Munich, Germany

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Molecular characterization of genes coding for cell division in *Verrucomicrobia*.
Annual conference of the VAAM (society for general and applied microbiology) 19 - 22 March 2006, Jena, Germany

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Transcriptional analyses of tubulin and cell division genes in different *Prosthecobacter* species
Annual conference of the VAAM (society for general and applied microbiology) 09 – 11 March 2008, Frankfurt, Germany

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The novel, highly efficient Two-Step Gene Walking method revealed the genomic organization of peculiar bacterial tubulin genes and cell division genes in the phylum *Verrucomicrobia*
Annual conference of the VAAM (society for general and applied microbiology) 09 – 11 March 2008, Frankfurt, Germany

References

Prof. Dr. Karl-Heinz Schleifer, PhD advisor, head of department
Department of Microbiology, Technical University Munich
+49 (0) 89 310 1875
+49 (0) 8161 7154-41
schleiferkh@web.de

Dr. Wolfgang Ludwig, group leader
Department of Microbiology, Technical University Munich
+49 (0) 8161 7154-51
ludwig@mikro.biologie.tu-muenchen.de

Dr. Giulio Petroni, diploma thesis advisor
Department of Biology, University of Pisa
+39 050 2211 384
gpetroni@biologia.unipi.it

Skills

Molecular Biology Techniques

- Diverse DNA / RNA isolation and purification techniques
- Reverse transcription
- Pulsed-Field-Gel-Elektrophoresis (PFGE)
- PCR techniques
  - Design of specific and degenerate primers targeting rRNA and protein coding genes
  - Various gene walking techniques
  - Development of the novel Two-Step Gene Walking technique
  - Quantitative PCR
- Cloning techniques
- DNA Sequencing
- Microplate Subtractive Hybridization (MASH)
- Heterologous gene expression in *E. coli*
- Protein purification
- Southern / Western blot and hybridization
- Immunofluorescence
- Immunoelectron microscopy
- Fluorescence *In Situ* Hybridization (FISH)
  - Catalyzed Reporter Deposition (CARD) FISH
  - Recognition of Individual Genes (RING) FISH
- Use of robotic systems

Microbiological techniques

- Cultivation of very diverse aerobic and anaerobic growing microorganisms
- Physiological characterization and phenotypic investigations of microorganisms
- Laserscanning microscopy / Epifluorescence microscopy
- Transmission Electron Microscopy

Bioinformatic skills

- Construction of DNA / protein sequence databases using the ARB software package
- Phylogenetic sequence analyses using various treeing methods
- Sequence analyses using BLAST, PRINTS, MEME