Instituto Juan March
de Estudios e Investigaciones

42 CENTRO DE REUNIONES INTERNACIONALES SOBRE BIOLOGÍA

Workshop on

Structure, Function and Controls in Microbial Division

Organized by

M. Vicente, L. Rothfield and J. A. Ayala

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FIRST SESSION: STRUCTURE AND FUNCTION OF FtsZ IN CELL DIVISION

Chairperson: Kurt Nordström

The structure of FtsZ polymers P protofilaments and protofilament sheets, with a 5 x 4 nm lattice like that of microtubules.

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FtsZ shows clear sequence homology to tubulin, and like tubulin it binds and hydrolyzes GTP, and it polymerizes (1-3). Previous studies of FtsZ polymers have reported tubular structures (1,2); although the lattice was not well resolved in these images it appeared to be quite different from that of microtubules. We have now used negative stain electron microscopy to examine the polymers formed by FtsZ in vitro. We do not find tubules, but rather protofilaments and protofilament sheets. The lattice of these sheets is remarkably similar to that of the microtubule wall, demonstrating a structural homology of FtsZ and microtubules that is much closer than previously known.

The primary polymer formed by purified FtsZ, at both pH 6.0 and 7.0, was a long, straight protofilament, about 5 nm in diameter. Many protofilaments were single and separated from neighbors, but they also assembled further into small sheets of 2-4 protofilaments. When the polycation DEAE dextran was added to the assembly mixture, the formation of sheets was greatly enhanced, and large, flat sheets containing 10-20 very straight and parallel protofilaments were commonly observed. Optical diffraction demonstrated a lattice that was ordered to about 30. The average spacing between protofilaments was 5.0 nm, and the subunit spacing along the protofilament was 4.1 nm. This is remarkably similar to the 5.0 x 4.0 lattice of the eukaryotic microtubule wall (4). Most recently, we have obtained assembly of very large and highly ordered sheets of FtsZ protofilaments by assembling the protein at pH 7, in the absence of DEAE dextran, but overlaid with a monolayer of positively charged lipid. This polycationic lipid layer apparently stabilizes the sheets similar to DEAE dextran, but the sheets are larger and more highly ordered. Computer reconstruction of the FtsZ subunits to about 20 resolution should be possible.

Tubular structures were never observed in assembly of purified FtsZ, but they were observed after assembly with DEAE dextran at pH 7-7.5. These tubules were quite different from microtubules, in which the protofilaments are parallel to the axis. In FtsZ-DEAE dextran tubules the protofilaments are tightly curved to form a shallow spiral. This spiral arrangement of protofilaments is very similar to the outer wall of Rdouble wall microtubulesS that are assembled from tubulin plus DEAE dextran (5). Remarkably, the curvature of the protofilaments demonstrates a second conformation of FtsZ, similar to that producing tubulin rings.

The striking structural similarity of the FtsZ protofilament sheets to the eukaryotic microtubule wall, especially the close match of the 5 x 4 nm lattice, extends substantially the homology of FtsZ to tubulin. The basic FtsZ polymer now appears to be the 5 nm diameter protofilament, but we have to ask whether this single protofilament is the functional unit in bacterial cell division. Probably not.

The ability of the protofilaments to form these sheets imposes remarkably stringent geometry on the FtsZ subunits, and we suggest that this geometry would not have evolved if it were not functionally important. Therefore we conclude that the functional polymer is a small sheet of protofilaments. The FtsZ sheets appear to be flat, in contrast to tubulin sheets which have an intrinsic curvature that allows them to form a microtubule (4). However, the evolution of a curved sheet that can form a microtubule would be straightforward once the lattice of the protofilament sheet was evolved.

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FURTHER CHARACTERIZATION OF FTSZ. Amit Mukherjee, Xunde Wang and Joe Lutkenhaus, Department of Microbiology, Molecular Genetics and Immunology, University of Kansas Medical Center, Kansas City, KS, USA, 66103

The ftsZ gene has been shown to be essential in both *E. coli* and *B. subtilis*. In both of these organisms FtsZ is localized to the leading edge of the septum in a ring pattern designated the FtsZ ring[1,2]. It has been suggested that the FtsZ ring functions to initiate septation by activating septal specific peptidoglycan biosynthesis[1]. We have found that *ftsZ* is present in organisms that lack peptidoglycan. We have cloned the *ftsZ* gene from a wall-less eubacterium, *Mycoplasma hyhorrinis*, and from an Archaebacterium, *Haloferax volcanii*. The *ftsZ* genes from these organisms encode typical FtsZs in which the amino-terminal 320 amino acids are highly conserved and the carboxy-terminal 60 amino acids are highly variable. Expression of the Mycoplasma *ftsZ* in *E. coli* resulted in a block to septation that could be suppressed by increasing the expression of the *E. coli ftsZ*. This dominant negative interference has also been observed with other distantly related FtsZs. It is thought that these FtsZs interact with the resident FtsZ but lack complete function. Such behavior is also observed with truncated E. coli FtsZs. One such FtsZ, FtsZ320, is truncated at the end of the conserved region. FtsZ320 has been purified and shown to possess wild type GTPase activity indicating the nonconserved carboxy tail is not required for this enzymatic activity. However, the failure of this truncated *ftsZ* to complement indicates an essential role for the carboxy tail.

One approach we have used to begin to analyze protein interactions with FtsZ is the yeast two hybrid system[4]. Utilizing this system we have found that heterologous FtsZs do interact and are in the process of delineating the region(s) required for interaction. We have also used this system to examine the interaction of FtsZ with the cell division inhibitor SulA, long thought to interact with FtsZ. Our results support a direct interaction between FtsZ and SulA. We have characterized a set of *sulA* missense mutations that fail to inhibit cell division. These mutations reduce the interaction between FtsZ and SulA as observed with the yeast two hybrid system. Furthermore, *ftsZ* mutations, isolated on the basis of resistance to high levels of SulA also show a reduced interaction in the test system. It may be possible to use this system to further define the regions of these proteins that interact as well as to determine other interacting proteins.

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The commitment of *E. coli* to cell division: the role of FtsZ

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Eubacteria such as *E. coli* and *B. subtilis* and the fission yeast *S. pombe* divide by forming a septum across the middle of the cell. The biochemical mechanisms governing the processes of division site selection and cytokinesis are poorly understood. Genetic evidence suggests that the bacterial *ftsZ* gene plays an essential role during the initiation of septation and immunoelectron microscopy has shown that an early event in the establishment of the division plane in *E. coli* is the localization of FtsZ as an annular ring to the equator of the cell[1].

We and two other laboratories have demonstrated that FtsZ is a GTP/GDP-binding protein with Mg^{2+}-dependent GTPase activity[2-4]. The thermosensitive *ftsZ84* and the lethal *ftsZ3* mutations, that impair the ability of FtsZ to bind and hydrolyze GTP *in vitro*, map to a short glycine-rich FtsZ segment. This region is conserved in eubacterial FtsZ homologs and is strikingly similar to the proposed GTP-binding motif in the eukaryotic cytoskeletal protein tubulin[2-5]. The mutant FtsZ84 protein has a glycine to a serine change at the first position of the tubulin signature motif. We recently found, quite unexpectedly, that the mutant FtsZ84, deficient in GTPase activity, displays a significant Mg^{2+}-dependent ATPase activity *in vitro*[6]. FtsZ84 ATPase activity as well as ATP binding could be specifically inhibited by sodium azide which did not antagonize FtsZ GTPase activity. This is a unique example of a missense mutation that converts a GTPase into an ATPase.

Nothing is known about how FtsZ ring is assembled at the equator of the cell under cell cycle control and the events that trigger contraction of the ring leading to the formation of daughter cells. We have started a genetic analysis to identify the protein partners that consort with FtsZ in the cell division pathway. To this end, we have constructed a genomic library from *ftsZ84* mutant chromosomal DNA in a lambda replacement vector and have mutagenized the entire library to search for suppressor clones that would allow growth of *ftsZ84* homoimmune lysogens at the restrictive temperature in a plaque complementation assay. We have obtained a number of candidate suppressor clones and have grouped them by restriction analysis and Southern hybridization. One of the suppressor isolates maps to *ftsZ* and the rest are extragenic. We have been able to subclone the intragenic suppressor on pBR322 only in *ftsZ84* (ts) background because it proved to be dominant-lethal in wild-type K12 strains. We are sequencing the intragenic suppressor to identify the presumed second-site mutation in *ftsZ84*. Recently,

we have been successful in subcloning one of the extragenic suppressors on a low-copy plasmid and we are now in the process of mapping and identifying the suppressor gene. These results together with the relatedness of FtsZ proteins and the tubulin superfamily to diverse ATP-binding proteins will be discussed.

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Specific interaction between FtsZ and ZipA, a newly identified membrane-associated protein.

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Immediately before the start of cell division, the essential division protein FtsZ becomes concentrated at the inner membrane into a ring-like structure at the prospective division site. During septation, the diameter of the FtsZ ring becomes smaller as it remains at the leading edge of the invaginating cell wall. Although the FtsZ-ring is thought to be crucial for cell wall invagination, its precise role is not known. In one attractive hypothesis, the ring is a contractile element which causes the cytoplasmic membrane to move inwards. This, in turn, could trigger the septum specific murein synthetase PBP3 (FtsI) to back up the inward moving membrane with a rigid murein layer.

In vitro experiments with purified FtsZ have shown that the protein is a GTPase with the ability to form large polymers in a nucleotide-dependent fashion. This is consistent with the idea that the FtsZ ring contains one large, or multiple smaller, polymer(s) consisting of GTP/GDP-bound FtsZ subunits.

Our current knowledge of FtsZ function raises several questions, i) What determines the site of assembly of the FtsZ ring, and what is the initiating step?, ii) How is the ring constricted during cell wall invagination?, and iii) How is the status of the ring communicated to the murein synthesis machinery in the periplasm and coordinated with murein ingrowth?

It can be predicted that FtsZ needs to interact with several different molecules that play specific roles in one or more of these processes. In principle, this might involve interactions between FtsZ and any of the other known division proteins. Although genetic studies have suggested possible interactions between FtsZ and several other proteins, physical evidence has, so far, only been obtained for an interaction between FtsZ and FtsA. In a biased search for proteins that interact with FtsZ, we have recently shown that FtsA can be co-purified with FtsZ and vice versa. Our results suggest that a large proportion of soluble FtsA is present in a complex with FtsZ. The function of this interaction is still unclear.

Alternatively, factors that interact with FtsZ may not have yet been identified. In order to address this possibility we have started an unbiased search for proteins with affinity to FtsZ. In one approach, we have made use of an FtsZ derivative (HHT-FtsZ) which carries at the N-terminus a histidine-tag as well as a substrate site for heart muscle kinase. The purified protein was radiolabeled in vitro by incubation with kinase and [γ-^{32}P]ATP and used as a probe on western blots. In this assay, HHT-FtsZ bound specifically to a minor protein band corresponding to a species with an apparent MW of 50 Kd. Binding of the protein was specific for the FtsZ portion of the HHT-FtsZ probe, since HHT-tagged derivatives of several other proteins failed to bind to the 50 Kd band. We named this protein ZipA for FtsZ interacting protein A. ZipA was exclusively present in the insoluble fraction of broken cells. Treatment of this fraction with urea did not release ZipA, whereas treatment with either Sarkosyl or Triton X-100 efficiently solubilized the protein. This suggests that ZipA is an integral inner-membrane protein.

The gene for ZipA (zipA) was isolated by expression cloning from a λgt11 library, and shown to be a previously unidentified gene at 52 min on the E.coli chromosome. Overexpression of the zipA ORF in a T7 RNA polymerase based system led to overproduction of a single protein which migrated as a 50 Kd species, and which readily bound FtsZ in the assay described above. The predicted primary structure of ZipA shows a hydrophobic N-terminus, and an abundance of proline residues (12.5%). The calculated MW is 36.4 Kd rather than 50 Kd indicating that the protein migrates abberantly in SDS-PAGE gels. Database searches did not reveal any known proteins with significant similarity to ZipA. DNA fragments carrying the complete zipA gene could not be readily cloned on high copynumber vectors, but could be maintained when carried on phage M13 or a low copy number plasmid. Cells carrying multiple copies of the zipA gene showed extensive filamentation. This phenotype was not dependent on the known division inhibitors SfiA or MinC but was, nevertheless, suppressible by increased expression of ftsZ. This indicates that the interaction between FtsZ and ZipA observed in vitro, also occurs in vivo. It is tempting to speculate that ZipA plays a role in FtsZ ring dynamics. Attempts to determine the zipA null phenotype, and to study the effect of ZipA on the in vitro behavior of FtsZ are in progress.

SECOND SESSION: CONTROLS OF WALL SYNTHESIS AND CELL DIVISION

Chairperson: Conrad Woldringh

MOLECULAR ANALYSIS OF THE GENE *mraW* AT THE DCW CLUSTER OF *Escherichia Coli.*

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Last steps of cell division in *Escherichia coli* take place by formation of a cross-wall at the center of the cell (the septum). A protein complex (called septosome, septalsome or septator) has been involved in septum formation and regulation of the process during cell division (1). This structure, although it has not been yet isolated, is defined at a functional level. The components of the septosome are not clearly defined, but some proteins have been shown (FtsZ, FtsA, PBP3) or predicted (FtsQ, MraR, PBP1b) to take part in the structure. Although there is no direct proof, some protein-protein interactions have been postulated (PBP1b-PBP1b, PBP3-PBP3, FtsZ-FtsZ, FtsA-PBP3 and FtsZ-PBP3). However, due to the complexity of the process, we think that some other components of the septosome are expected to be discovered. Immediately upstream of the gene *pbpB* at the dcw (division and cell wall) cluster of the 2 min. in *Escherichia coli* there is three open-reading-frames coding for three new proteins that we have termed *mraZ*, *mraW*, and *mraR*. These three genes define the 5' proximal end, been the *envA* gene the 3' proximal end, of the dcw cluster. We are currently studying these genes at molecular level and we have obtained information about localization, gene expression, and function of these three new proteins. Here, we will present the work done on *mraW* and its gene product.

MraW expression, transcription: Start point of transcription for *mraW* gene (Pw) was found by S1 nuclease analysis on the 5' proximal end of the dcw cluster and canonical sequences for the -10 and -35 region of the promoter were identified. As it is commonly found at the dcw cluster this promoter lies on the coding sequence of the previous *mraZ* gene. A 84-nucleotide-long untranslated 5' segment was identified in the mRNA encoding MraW. Chromosomal expression of the protein from its own regulatory sequences seems to be constitutive, i.e. no differences in the levelsof the protein were found in cell extracts of different phases of growth or under stress conditions. Two different *lexA* boxes have been found by nucleotide sequence homology search in the regulatory region of *mraW*, but both of them are far from the promoter and there is no evidence for functionality.

MraW expression, translation: The *mraW* gene codes for a 313 amino acid protein. The protein was efficiently translated both from the chromosome or plasmid-encoded gene. The NH_2 terminal was deduced by amino acid analysis as MMENYKHTTVLLDEAV, corresponding to the prediction by the sequence of the gene (2). Two molecular forms containing two or one methionine were found in a ratio 2:1 respectively. The presence of this two molecular forms may indicate some postranslational regulatory mechanism. Overexpression of the MraW protein did not produce relevant morphological effect when it was analyzed in different wild type *Escherichia coli* strains.

MraW localization: MraW was localized as a soluble protein. The protein was isolated from the cytoplasmic fraction, however a 10-20 per cent of the total amount of the protein was retained in the membrane fraction but it was recovered from this fraction by a 0.5 M ClNa wash. The analysis of gene fusions with the β-galactosidase reporter gene also confirmed the cytoplasmic location for the protein.

MraW function: The analysis of the chromosome insertion mutants of gene *mraW*, obtained by using three different methods, 1) a *polA* strain, 2) a *recBCD* strain, or 3) a

temperature sensitive replication plasmid, pMAK, indicate that *mraW* is an essential gene in *Escherichia coli*. Amino acid sequence homology search shows a high homology with a S-adenosyl-methionine-binding motif (3), indicating a possible methyl-transferase activity for the protein. Preliminary result indicates that a protein with a Mr of 60 Kdaltons is efficiently methylated in the membrane fraction of a MraW overproducing strain. As also a possible modification, in spite of the COOH terminal processing, has been predicted for PBP3 (4), we would like to hypothesize that this modification could be a methylation, due to the activity of MraW.

MraW during evolution: Overproduction of the MraW protein, by about 500-fold, was achieved in a stationary phase induction of a plasmid-containing strain. This high level of expression let us to purify the protein in a single step by a HPEC acrylamide system. Purified protein was used to produce polyclonal antibodies in rabbits. Immunoblot analysis of total cell extract from different Gram-negative and Gram-positive strains indicate that an analog of the MraW protein is present in all those examined strains and suggest a high degree of conservation during evolution and a critical role for the protein.

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THE *Escherichia coli dcw* CLUSTER: EXPRESSION AND FUNCTION

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Genetic evidence shows that the gene products of the *dcw* cluster (min 2.5 of the genetic map. Ayala *et al.*, 1994), mainly those of the *pbpB* (≡*ftsI*), *ftsW*, *ftsQ*, *ftsA*, and *ftsZ* genes, are essential for *Escherichia coli* septation.

Expression of cell division genes. *E. coli* directs the transcription apparatus toward specific sets of genes using basically the same device as that operating for *Bacillus* sporulation, namely association of core RNA polymerase with specific sigma factors. *E. coli* sigma factors can direct RNA polymerase to transcribe either housekeeper, heat-shock response, flagellar, or growth rate-sensitive genes (Vicente *et al*, 1991). Regions containing genes involved in essential processes, as the *dcw* cluster, present cases of transcriptional complexity that in some respects resemble the regulation of eukaryotic genes. This region contains no transcriptional terminator from *mraZ* to the end of *secA*, 22 kb downstream. Transcription of *ftsZ*, one of the distal genes which codes for a GTPase that forms a septal ring at the time of division, originates in at least five different promoters; in which transcription can be regulated by the cell cycle (Garrido *et al.*, 1993; Zhou and Helmstetter, 1994). These promoters include a gearbox upstream from *ftsQ*. Fusions to a reporter *lacZ* gene have been constructed in a system which generates an identical *lacZ* transcript independently of the particular fusion. Contrary to our interpretation of previous results, in which the real *ftsZ* transcripts were quantified by RT-PCR, we find that most of the transcription originates at promoters *ftsQ1p*, *2p* and *ftsZ3p*, *4p*. These observations could result from a cyclic transcription originated at, or upstream from these promoters, followed by an efficcient processing of the messenger at the *ftsA*, Z junction, provided that the upstream region of the transcript were unstable.

Transcription of *ftsZ*: temporal and quantitative regulation. Expression of *ftsZ* in strain VIP205 is dissociated from the natural promoters, and is under the control of an inducible *tac* promoter. This abolishes the natural oscillation in *ftsZ* transcription from its promoters, while it has no effect on the transcription of the downstream *envA* gene. A shift in IPTG from 30 μM, that supports division at wild type sizes, to lower (6 μM), or higher (100 μM) concentrations, indicates that cells can divide within a broad range of FtsZ concentrations. A detailed analysis of the cell morphological parameters during the transition from one IPTG concentration to another, shows that when the amount of FtsZ drops below certain levels, cell division is delayed and the cells become larger, suggesting that the correct timing of *ftsZ* expression, and the correct FtsZ concentration, are required for division to occur at normal sizes.

Structure and function of the FtsA protein. Among the components of the septator FtsA is a protein which is located partly in the cytoplasm and the remainder is associated to the cytoplasmic membrane facing the cytoplasm. FtsA is required during the last stages of cell division, and it may interact with PBP3. The structural model for FtsA, derived from sequence alignment with proteins of the eukaryotic actin, has been validated: both the ability to bind ATP, and the existence of different states of phosphorylation have been demonstrated (Sánchez *et al.*, 1994). A functional model for the action of FtsA considers that the phosphorylation state of the protein, and the presence of ATP may exert a regulatory role on the timing of FtsA action by preventing its premature association to the septator. One mutation, *ftsA106*, in which the 215 Thr residue, the phosphorylation site, has been replaced by a Glu residue, which mimics the phosphorylated form, may be lethal when introduced into *E. coli*. If this is confirmed it would support the regulatory role of phosphorylation, and would suggest the ability of FtsA molecules to interact among them.

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PBP2, ppGpp and Cell Division in *Escherichia coli*

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Penicillin-binding protein 2 of *Escherichia coli* is absolutely required for synthesis of the cylindrical portion of the peptidoglycan cell wall. Its inactivation, by mutation or by the specific ß-lactam mecillinam, results in loss of rod shape, formation of spherical cells, and cell death. One can easily select mecillinam resistant mutants which grow and divide as cocci in the presence of the antibiotic, showing that PBP2, although normally an essential protein, is not required for septum synthesis. Nevertheless, loss of PBP2 activity in wild type (mecillinam sensitive) cells results in a block in cell division[1]. Overproduction of the division proteins FtsQ, FtsA and FtsZ overcomes this division block and confers resistance to 10 µg/ml mecillinam[1]. Overproduction of FtsQ alone or of FtsQ and FtsA does not make cells resistant to mecillinam whereas overproduction of FtsZ alone confers partial resistance to 1 µg/ml mecillinam. This is compatible with our earlier suggestion[1] that spheres, with a threefold greater diameter than the corresponding rods, require more FtsZ to make a complete ring around midcell at septation. Mecillinam resistance can also be obtained by increasing the pool of ppGpp[2,3], effector of the stringent response. Many spontaneous mecillinam resistant mutants depend on induction of the stringent response, and indeed resistant mutants are 10- to 100-fold less frequent in cultures of *relA* strains, lacking (p)ppGpp synthetase I (the stringent factor)[2]. We previously suggested that ppGpp may stimulate transcription of the *ftsZ* gene[1]. However, mecillinam resistant cells with higher ppGpp levels than wild type cells do not have more FtsZ per mass, as evaluated by quantitative immunoblot. It is possible, however, that FtsZ activity is higher in these cells, perhaps accompanied by higher FtsQ and FtsA activity.

In the hope of identifying regulators of the *ftsQAZ* operon, we selected mecillinam resistant mutants after insertional mutagenesis in a *relA* strain, thereby avoiding alleles that induce the stringent response. One such mutant carries an insertion which confers resistance to 10 µg/ml mecillinam. In this mutant the amount of FtsZ per mass is higher than in the isogenic parent strain, suggesting that the insertion may result in loss of a negative regulator of the *ftsQAZ* operon. The insert is located at 99.2 min on the *E. coli* genetic map, in a gene not described previously.

A second mutant, resistant to 1 µg/ml mecillinam, carries an insertion in the *aroK* gene, at 75.7 min. The quantity of FtsZ per mass is not altered in this mutant. The *aroK* gene codes for shikimate kinase I. Other *aro* mutants, blocked before or after shikimate kinase or in *aroL* (which codes for shikimate kinase II), are not resistant to mecillinam but become so on loss of AroK. Furthermore, AroK is known to have an abnormally high K_m for shikimate, greater than 20 mM, over 100-fold higher than the isoenzyme AroL. We conclude that AroK acts on a second substrate, other than shikimate, to form a product which sensitizes the cells to mecillinam. We have not yet identified this alternative substrate. An interesting possibility is that AroK can act as a protein kinase, perhaps on one of the division proteins FtsQ, FtsA or FtsZ, decreasing their activity, or on the mecillinam target PBP2, making it more sensitive to mecillinam action.

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FtsH, a membrane-bound, ATP-dependent protease in *Escherichia coli*

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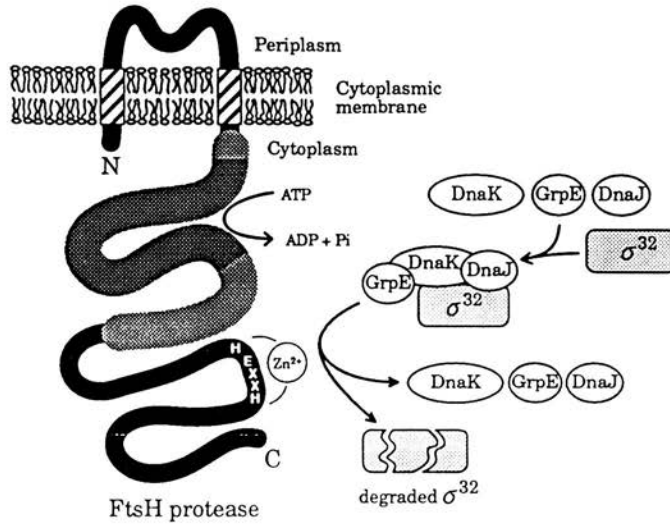
Gene *ftsH* is essential for cell viability in *E. coli*, and a thermosensitive *ftsH* mutation, *ftsH1*, causes a significant decrease in the amount of penicillin-binding protein 3 (PBP3), a septum-forming enzyme (1). The post-translational processing of PBP3 at the carboxy-terminal part, an event that occurs on the periplasmic side of the cytoplasmic membrane, is significantly retarded in the *ftsH1* mutant. Mutations in *ftsH* have been found to enhance translocation of normally anchored protein segments (stop-transfer defective) and to retard that of normally translocated proteins (export defective) (2). FtsH protein is an integral membrane protein of 70.7 kDa (644 aa) spanning the membrane twice and that it has a large cytoplasmic carboxy-terminal part with an ATPase domain (3, 4). FtsH belongs to a novel family of putative ATPases, "AAA-protein family", including some regulatory subunits of eukaryotic 26S proteasome.

It has also been found that *ftsH* is identical to *hflB*, a gene involved in the stability of the λ CII protein (5). The frequency of λ lysogenization is markedly increased in the *ftsH1* mutant at high temperatures, indicating that FtsH is involved in the proteolytic pathway of CII. The λ CIII protein stabilizes CII and its overproduction induces constitutive heat shock responses with stabilizing σ^{32} (6). These findings raise the possibility that one of cellular substrates of the FtsH-mediated proteolysis may be σ^{32}.

We report that FtsH is involved in degradation of σ^{32} (7) In the temperature-sensitive *ftsH1* mutant, the amount of σ^{32} at a nonpermissive temperature was higher than in the wild type under certain conditions due to reduced rate of degradation. In an *in vitro* system with purified components, FtsH catalyzed ATP-dependent degradation of biologically active histidine-tagged σ^{32}. Approximately 3 nmol of substrate was degraded per min per mg of FtsH at 42°C under the conditions where the substrate was present in 8-fold excess. The reaction at 42°C was ~2-fold faster than that at 37°C. In contrast, the major sigma factor, σ^{70}, was not degraded under the same conditions. Of the various nucleotides tested, ATP was specifically required; CTP could partially substitute for ATP. Nonhydrolyzable ATP analogs could not substitute for ATP, suggesting that the proteolysis requires hydrolysis of ATP and thus is energy-dependent. FtsH has a zinc-binding motif similar to the active site of zinc-metalloproteases. Protease activity of FtsH for histidine-tagged σ^{32} was stimulated by Zn^{2+} and strongly inhibited by the heavy metal chelating agent *o*-phenanthroline. Two particularly interesting features of FtsH protease, which distinguish it from the well-characterized ATP-dependent proteases of *E. coli* with active serine residues, Lon (La), ClpAP, and ClpXP, are its membrane anchoring and the metal-dependency of proteolytic activity. We conclude that FtsH is a novel membrane-bound, ATP-dependent metalloprotease with activity for σ^{32}. These findings indicate a novel mechanism of gene regulation in *E. coli*. We will also discuss possible functions of FtsH protease in the control of *E. coli* cell division.



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Control of Cell Wall Synthesis by Means of Multienzyme Complexes

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Cell septation is achieved by the timely controlled synthesis of murein forming a septum at the midpoint of the cell. Separation of the daughter cells that normally follows septation depends on the precise splitting of the septum by the action of murein hydrolases. Thus, cell division is the result of a coordinated action of murein synthases and murein hydrolases. In an attempt to understand the biochemical mechanisms that control the interplay of both enzyme systems affinity chromatography has been employed to study specific protein protein interactions.

The predominant murein hydrolysing activity in *Escherichia coli* is a lysozyme-like specificity that cleaves the ß-1.4-glycosidic bond between MurNAc and GlcNAc and concomitantly catalyzes an intramolecular transglycosylation that results in the formation of 1.6-anhydromuramic acid carrying reaction products (Höltje et al., 1975). Three different lytic transglycosylases have been identified and cloned up to now: A soluble enzyme, Slt70, and two membrane-bound lipoproteins, MltA and MltB (Betzner and Keck, 1989; Lommatzsch and Höltje, 1995; Ehlert et al., 1995). Construction of overproducing expression systems allowed purification of all three enzymes on a preparative scale.

The lytic transglycosylases were covalently linked to activated Sepharose and used as specific ligands for affinity chromatography in order to identify cellular proteins that bind to the lytic transglycosylases (Romeis and Höltje, 1994a). Among the few proteins that were retained by the affinity columns some turned out to be penicillin-binding proteins (PBPs). Besides PBP1b, known to be a bifunctional murein transpeptidase/transglycosylase and PBP3, a bifunctional synthase specifically involved in septum formation, also PBP7, recently shown to be a DD-endopeptidase (Romeis and Höltje, 1994b) was enriched by Slt70-Sepharose chromatography.

On the basis of the affinities demonstrated so far a multienzyme complex is proposed that combines murein synthases with murein

hydrolases. A complex consisting of PBP1b, PBP3, PBP7 and Slt70 would represent an ideal machinery to accomplish septation of the murein sacculus. Following the recently proposed three-for-one growth mechanism (Höltje, 1993) such a multienzyme complex could safely divide the stress-bearing sacculus without risking lysis of the cell. An analogous enzyme complex which instead of PBP3 includes PBP2, known to be responsible for cell elongation, could likewise be involved in longitudinal growth of the sacculus. As will be discussed, the postulated enzyme complexes would explain the specific effects of different ß-lactam antibiotics on *E. coli*: filamentation, spherical growth and bacteriolysis.

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Interaction Between FtsZ and Other Cell Division Proteins

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The essential *Escherichia coli* cell division protein FtsZ shares biochemical properties and amino acid similarities with tubulins, suggesting a cytoskeletal role for FtsZ Both have GTPase activity and undergo GTP-dependent polymerization to form structures comprised of protofilaments. To understand how a cytoplasmic FtsZ structure at the division site might direct cell wall synthesis in the periplasm, we searched for proteins that associate with FtsZ. Interaction between FtsZ and the septum-specific, transmembrane penicillin binding protein, PBP3 (the *ftsI* gene product), was detected by the yeast two-hybrid system. The domains of PBP3 that catalyze the synthesis of the cell wall peptidoglycan reside in the periplasm, across the membrane from the cytoplasmic domain. In vitro, the PBP3 cytoplasmic domain showed saturable binding to purified FtsZ and was displaced by a cognate peptide. Interaction between PBP3 and FtsZ provides a direct link between a bacterial cytoskeleton and cell wall synthesis.

Genetic Analysis of Formation of the Division Septum in *Escherichia coli*

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The long-term focus of our work is on cell division in the bacterium *Escherichia coli*, in particular how the division septum is formed and how its formation is coordinated with other events in the cell cycle such as DNA replication. We will approach this problem by studying the structure and function of several membrane proteins implicated in synthesis of the division septum. These proteins are FtsI (PBP3), FtsL, and FtsQ. One reason for choosing these proteins is that they have a similar overall structure--each consists of a small cytoplasmic domain, a single transmembrane helix, and a comparatively large periplasmic domain. Different possibilities can be imagined for the function of these domains. 1) *Localization* to the septum. 2) *Interaction* with other proteins to form a complex. 3) *Signalling* that coordinates septum formation with events in the cytoplasm. 4) *Anchoring* the large periplasmic domain in the membrane. 5) *Enzymatic activities* needed for peptidoglycan synthesis at the septum, as is known to be the case for FtsI.

To evaluate these possibilities we are introducing restriction enzyme recognition sites into the genes for the three cell division proteins and into the gene encoding MalF, a protein involved in maltose transport rather than cell division. These restriction sites will be used to make chimaeric genes which express proteins in which domains have been swapped. Hybrid proteins will be tested for their ability to complement temperature sensitive and null (depletion) mutations in the various *fts* genes. The results of the complementation analysis will be used to design further experiments aimed at understanding the structure and function of FtsI, FtsL, and FtsQ.

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On the identification of FtsW and its possible role in the initiation of division in *Escherichia coli*

The product of the *ftsW* gene has been identified as a polypeptide that, like the related RodA protein, shows anomalous mobility on sodium dodecyl sulphate-polyacrylamide gel electrophoresis. The estimated molecular size of FtsW is 46 kD whilst the calculated size from SDS-PAGE analysis is 32 kD. FtsW is produced at low levels that can be increased by altering the translation initiation region of the mRNA. Overproduction of FtsW strongly inhibits cell growth. A new mutant allele, *ftsW201*, causes a temperature-sensitive division block in the initiation stage of cell division which is similar to the division block in *ftsZ* mutants. The block in initiation of division in the *ftsW201* allele is shown to be independent of FtsZ or the FtsZ inhibitor, SulA. In addition, the *ftsW201* mutant is hypersensitive to overproduction of the division initiation protein FtsZ at the permissive temperature. The results suggest a role for FtsW in the initiation stage which may involve an interaction with FtsZ. Experiments being carried out are aimed at demonstrating this interaction between the two proteins using molecular, genetic and biochemical approaches as well as electron microscopy.

THIRD SESSION: THE TOPOLOGY OF SEGREGATION AND DIVISION

Chairperson: Richard D'Ari

The role of the Min proteins in ensuring the proper placement of the E. coli division septum

Lawrence Rothfield

The proper placement of the E.coli division septum requires the MinE protein. MinE accomplishes this by imparting topological specificity to a division inhibitor coded by the minC and minD genes. As a result, the division inhibitor prevents septation at potential division sites that exist at the cell poles but permits septation at the normal division site at midcell. In this paper we define two functions of MinE that are required for this effect and present evidence that different domains within the 88 amino acid MinE protein are responsible for each of these two functions. The first domain, responsible for the ability of MinE to counteract the activity of the MinCD division inhibitor, is located in a small region near the amino terminus of the protein ($MinE^{4-22}$). The second domain ($MinE^{23-88}$), is required for the topological specificity of MinE function. This domain affects the site-specificity of placement of the division septum even when separated from the domain responsible for suppression of the activity of the division inhibitor, as shown by the ability of $MinE^{4-88}$ to induce minicelling when expressed in wild type cells.

The results are consistent with two models for the topological specificity function of MinE, both based on the idea that a domain within MinE interacts with a topological target molecule that differentiates the proper division site at midcell from the residual division sites at the cell poles. In the first model, the recognition site for the topological target is located within the $MinE^{23-88}$ domain. When this domain is expressed in wild type cells, it induces minicelling, consistent with the view that $MinE^{23-88}$ competes with the wild type MinE protein for access to the topological target . In a second model, the $MinE^{23-88}$ domain is required only for oligomerization of MinE. In this view, oligomerization is required to create the binding site that interacts with the topological target, and formation of this binding site requires that both partners within the oligomer contain intact N-terminal domains.

According to our present view, the anti-MinCD domain of MinE ($MinE^{1-22}$) will interact directly or indirectly with MinD, thereby preventing MinD from activating the MinC division inhibitor. The topological specificity domain that is located either within $MinE^{23-88}$ (Model 1), or within the N-terminal $MinE^{1-22}$ domain in MinE oligomers (Model 2), will interact with the topological target that differentiates the proper division site at midcell from the residual division sites at the cell poles. As a result, MinE will prevent the division inhibitor from working at midcell but will not prevent it from blocking aberrant divisions at the cell poles.

Studies of the topological specificity factor of cell division.

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Accurate distribution of daughter chromosomes in newborn cells requires efficient mechanisms for coupling septation with partition, both with respect to the relative timing of these events and to the position of the septum relative to newly replicated nucleoids. We have previously shown that separation of the nucleoids and septation are tightly coupled and are under the common control of the rate of FtsZ synthesis (1). The *minCDE* operon, which prevents the re-use of polar division sites, is not involved in this temporal coupling process (2). However, we have become interested in the analysis one of the products of this operon, MinE, as this protein displays topological specificity (3), and therefore may have affinity for pre-septal structures, and more specifically to structures that serve as anchor points for nucleoid separation.

We have carried out a deletion analysis of MinE (88 aminoacids). Truncated derivatives were studied for i) their ability to neutralize the division inhibition capacity of *minCD* gene products, ii) their ability to confer topological specificity to the division process, iii) their ability to override the block exerted by wild-type MinE on cell division at the poles in a *minCDE*$^+$ context. The truncated derivatives were also fused to the GAL4 DNA binding and transcription activation domains, and their ability to interact with each other was studied by examining the activation of the GAL1 promoter in yeast cells.

This analysis has revealed the existence of three functional segments within MinE. The N-terminal segment alone suppresses the division inhibition capacity of MinCD, and does not dimerize. The central segment appears to be

mostly involved in MinE-MinE interactions, while the C-terminal segment is required, in addition to the two others, to confer topological specificity. Our results are best explained by supposing that MinE dimers recognize some component located at the poles, and conveys the MinCD inhibitor to the polar sites.

A protein consisting of MinE fused to a cytoplasmic form of the maltose-binding protein MalE inhibits nucleoid partitioning. This inhibition does not depend upon the expression of the chromosomal *minCDE* operon. Under strong inhibition conditions, septation is also abolished, and cell survival is severely reduced. Extragenic multicopy suppressors allowing growth in the presence of the fusion protein were isolated. Some of these suppressors affect*ftsA* activity, suggesting that MinE may interact with *ftsA* gene product.

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Cell Division and Chromosome Partitioning during Growth and Sporulation of *Bacillus subtilis*

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B. subtilis is a rod-shaped gram-positive bacterium. Its growth and division are superficially similar to those of other rod-shaped bacteria, such as *E. coli*. Many, if not all of the genes needed for cell division in *E. coli* are conserved in *B. subtilis*[1,13]. On starvation, *B. subtilis* initiates an intricate adaptive response in which two sister cells cooperate in the formation of a highly resistant, dormant endospore. The two cells involved in sporulation arise by a modified asymmetric cell division. After septation, the small prespore and much larger mother cell differentiate, by the execution of different programmes of gene expression[5]. Comparative studies of the vegetative (central) and sporulation (polar) division processes are beginning to provide new insights into some of the basic problems of cell division in bacteria.

Systematic DNA sequence analysis has revealed that *B. subtilis* has a cluster of genes that is closely related to the 2 min cluster in *E. coli* (e.g.[1,8,13]). Most of the genes in the *B. subtilis* cluster exhibit 30 to 40% identity to their *E. coli* counterparts, and the order of genes within the cluster is conserved. At the beginning of the *B. subtilis* cluster, we have found homologues of the *mraZ, mraY, mraR (ftsL)* and *pbpB* genes of *E. coli*. Gene disruption studies have shown that the first two genes in the *B. subtilis* cluster are dispensible, but both *ftsL* and *pbpB* are required for septation during growth and sporulation[2].

Cell cycle studies in *B. subtilis* are hampered by the tendency of *B. subtilis* cells to grow in chains. To overcome this problem, we have developed a method based on digital imaging microscopy and have applied this to analyse the cell cycle events that accompany the onset of sporulation[7]. Using a nutritional shift-down procedure to initiate sporulation, Mandelstam and co-workers previously showed that there is a "sensitive point" in the cell cycle[3,4]. When the starvation stimulus is applied, cells that have not reached this point can alter their cell cycle and switch to asymmetric division immediately. Cells that have passed the sensitive point must reinitiate a round of DNA replication and traverse another cell cycle before dividing asymmetrically. Our recent results indicate that the sensitive stage probably corresponds to a size or mass at which the cell can not avoid attaining the initiation mass for DNA replication, even at the reduced growth rate imposed by the starvation medium[7].

Chromosome partitioning during sporulation differs morphologically from that of vegetative cells in that the prespore chromosome must move to the extreme pole of the cell to achieve its final location in the prespore compartment. We previously showed that the *spoIIIE* gene of *B. subtilis* is specifically required for prespore-chromosome partitioning[11]. In the absence of *spoIIIE*, about 30% of the prespore chromosome is trapped in the polar compartment, the remainder being inappropriately left in the mother cell. The segment of DNA trapped in the prespore compartment in *spoIIIE* mutant cells is a specific one, suggesting the existence of a centromere-like sequence. The origin of chromosome replication is centrally located within the sequence that is trapped, suggesting that it may be involved in centromere function.

More recently, we have shown that in wild type sporulating cells, the sporulation septum closes before the prespore chromosome moves to the pole[12]. Initially, about 30% of the chromosome is trapped, as in *spoIIIE* mutants. The remainder of the chromosome is then translocated through the nearly complete septum, by a mechanism that is reminiscent of the

conjugative transfer of DNA. Interestingly, the SpoIIIE protein shows significant sequence similarity to a group of Tra proteins from conjugative plasmids of *Streptomyces*[12].

Although *spoIIIE* mutations have no overt effect on vegetatively growing cells, at least two lines of evidence suggest that its function may not be specific to sporulation: firstly, genes closely related to *spoIIIE* have been found in diverse bacterial species (e.g.[9]); secondly, the *spoIIIE* gene is expressed constitutively in *B. subtilis*[6]. In support of the idea of a more general role, we have found that *spoIIIE* is required by non-sporulating cells of *B. subtilis*, under conditions in which the primary chromosome partitioning machinery fails[10]

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A new *E. coli* cell division gene, *ftsK*.

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A conditional block in cell division in the mutant strain, TOE44, is due to a single base change in a new gene, *ftsK*, located at 21 min. on the *E. coli* map. The block appears to be at a very late stage in division and is specifically suppressed by deletion of *dacA*, coding for the D-ala:D-ala carboxypeptidase, PBP5. (Deletion of *dacB* or *dacC* does not suppress the *ftsK44* mutation.) The *ftsK* gene is located immediately downstream of *lrp* [1](coding for the "leucine-responsive protein") and *dinH* [2](an SOS-inducible promoter) and is probably cotranscribed with *lrp*.

The *ftsK* gene consists of an ORF of 3912 bp. The deduced amino acid sequence of FtsK predicts an N-terminal domain consisting of a number of membrane-spanning helices, possible other membrane-spanning regions elsewhere, and a large cytoplasmic domain. The putative cytoplasmic domain contains an ATP/GTP-binding motif. There is also a striking set of 6, 12 amino acid repeats. The *ftsK44* mutation is a Gly_{55} -> Ala_{55} substitution, and the cell division defect can be complemented by a chromosomal fragment consisting of the region immediately upstream of *lrp* and the first 1094 bp of *ftsK* (the *lrp* gene itself is not required). Cloning this region is possible only in vectors with low copy-number, and the entire *ftsK* gene has not yet been successfully subcloned except in the original Kohara λ-phage.

The deduced FtsK polypeptide has extensive sequence similarity to at least 3 other proteins from unrelated bacterial species. The closest similarity is to a deduced polypeptide of unknown function from *Coxiella burnetii*, but the regions of similarity or identity, especially in the large presumptive cytoplasmic domain, are also mostly shared with proteins from *Campylobacter jejuni* and *Bacillus subtilis*. In particular, the sequence around the nucleotide-binding motif is almost identical in all 4 proteins. This region and other sections of the C-terminal cytoplasmic domain are also shared with plasmid-encoded proteins from 4 *Streptomyces* species and a transposon-encoded protein from *Enterococcus faecalis*. FtsK also contains a long sequence without close similarity to any other recorded polypeptide, so that the *E. coli* protein is much larger than any of these other proteins.

The plasmid encoded *Streptomyces* proteins are required specifically for intermycelial transfer of plasmid DNA, and also cause inhibition of growth and sporulation in the host [3,4,5,6,7,8]. The *B. subtilis* protein, SpoIIIE, is required both for a stage in the transfer of a chromosome from the mother cell into the prespore compartment, and for final closure of the spore septum [9]. The *ftsK44* mutant of *E. coli* appears to be defective only in cell division; cell growth, DNA replication and chromosome segregation being normal.

The stage in division at which the *ftsK44* mutant is blocked is later than in any other *fts* mutant that we have yet encountered and this defect is corrected completely in cells that also lack PBP5 (the *dacA* product). We therefore propose that an increased proportion of pentapeptide side chains in murein facilitates the activity of the defective FtsK44 protein in completing septal closure. FtsK may itself be an enzyme which is required for the final "topological transition" that converts a single sacculus into two. The fact that the *ftsK44* mutation alters a possible periplasmic domain is consistent with this idea. Even if this proves to be a correct interpretation however, a function for the large, highly-conserved cytoplasmic domain with its nucleotide-binding site remains to be discovered. The role of Lrp, if any, and of the *dinH* promoter, and the nature of any downstream members of this operon are also unknown as yet.

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FOURTH SESSION: THE TOPOLOGY OF GENE EXPRESSION, SEGREGATION AND DIVISION

Chairperson: Philippe J. Sansonetti

CHROMOSOME REPLICATION, NUCLEOID PROCESSING AND CELL DIVISION IN *intR1* STRAINS

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During the bacterial cell cycle, there is an exponential growth in cell masss and in the amount of most of the components of the cells. In addition, there are a number of key events that occur only once during each doubling in cell mass: chromosome replication, nucleoid processing, and cell division. These processes have to be controlled such that they match the increase in cell mass and coordinated to each other such that one process does not occur until a previous one has been completed. The general aim of our studies is to determine the key control points in the cell cycle, the molecular basis for the control(s), and the consequences of changed timing of one process on the execution of the other processes.

In order to achieve the aims of our studies, we uncoupled chromosome replication in *Escherichia coli* from its normal control. This was achieved by deletion of 16 bp at the very left end of *oriC* - this removes one of the 13-mers in the AT rich region and inactivates *oriC* - and insertion of the basic replicon of plasmid R1 into the deletion. The resulting strains are denoted *intR1*. Since plasmids replicate independently of the cell cycle and since plasmids control their own replication, chromosome replication in *intR1* strains is uncoupled from its normal control and from the cell cycle and is placed under R1 control (1). Plasmid R1 replicates unidirectionally and was, therefore, inserted in both orientations giving rise to two classes of strains, $intR1_{CW}$ and $intR1_{CC}$ in which R1 initiates its replication clockwise and counterclockwise, respectively, with respect to the genetic map of *E. coli*. The DNA content and cell size distribution of the $intR1_{CW}$ strains are similar to those of normal strains and replication is bidirectional but random in time.

Replication of R1 is determined by the rate of transcription of the *repA* (coding for the rate-limiting initiator protein) and of the *copA* genes (coding for an antisense RNA that regulates translation of the *repA* mRNA) (2). By using a runaway-replication derivative of R1, the rate of *repA*-transcription can be increased resulting in overreplication (3), whereas conditional *copA* expression results in underreplicaiton. Hence, the DNA/mass ratio can be varied continuously from very low to very high values compared to that of the wild type. Under- as well as over-replication disturb cell division (4). Changing the DNA/mass ratio is equivalent of moving chromosome replication to later and earlier times, respectively, in the cell cycle compared to normal strains. Moderate overreplication did not change the cell size distribution, indicating that cell division has its own control (5). We are at present using the same approach to study if nucleoid processing is independently controlled or is part of the replication cycle.

By using various degrees of moderate underreplication, we are determining the latest time at which replication can occur and still allow normal cell division. The preliminary data indicate that cell division only takes place at discrete cell sizes, either the normal one or a size that is twice as big. Preliminary data also suggest that cell division is omitted in some cells at severe underreplication; this leads to the appearance of a large fraction of cells with three, four or more nicely separated nucleoids. Uncoupling of cell division from chromosome replication also occurs during the stationary phase; stationary cells often contain more than one genome and cells with only one genome appear only slowly during many days in the stationary phase, particularly in rich media.

The nucleoid morphology of *intR1* strains has a tendency to be abberrant and resembles that of *minB* mutants suggesting that the MinB system is involved in nucleoid processing rather than in the choice of cell division sites (6). Some of the cells in *minB* mutants and *intR1* strains are branched cells with a changed plane of division between the nucleoids (7).

Conditional *copA* expression makes it possible to reversibly inhibit initiation of replication and to follow the kinetics and localisation of cell divisions after resumption of replication (8). Inhibition of initiation of replication leads to runout replication and to the formation of cells with only one nucleoid. This is centrally located and the cells continue to grow. Induction of replication results in cell division about one hour later. Cell division either occurs in the middle of the cells giving two daughter cells of normal baby size or such that one daughter cell is of normal baby size and the other three times as big. Some cell divisions result in one of the daughters lacking DNA; these cells vary in size from that of minicells to that of normal baby cells. Induction of only one round of replication in most of the cells gives synchronous replication and a burst of cell divisions about one hour later. However, only cells of normal size divide, whereas the larger ones don't. Some of the cell divisions lead to the formation of one daughter cell without DNA; in this case, the DNA-less daughter cell is of normal baby size. Hence, completed replication is required but not enough for cell division to take place and cell division only occurs at distinct cell sizes.

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Chromosome Partitioning in *E. coli*

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We previously described *mukB* mutants, which produce anucleate cells with normal cell-size at significant frequency during cell divisions, suggesting a defect in chromosome partitioning (1). A *mukB* null mutant can form colonies at 22°C, but not at higher temperatures than 30°C. The null mutant produces anucleate cells normal in cell-size at 22°C during cell divisions. After transfer to 37°C from 22°C, nucleate cells of the null mutant elongate and nucleoids distribute irregularly in the elongate cells. The MukB protein is the first candidate of motor protein involving in bacterial chromosome partition (2,3,4).

We have recently found that a disruptant of the *orf50* gene (named newly *mukF*), which is a member of the *mukB* operon, shows the same phenotypes as the *mukB* null mutant; temperature-sensitive colony formation and anucleate cell production. Both the phenotypes of the *mukF* null mutant are complemented by a plasmid carrying the wild-type *mukF* gene, but not by a plasmid carrying the wild-type *mukB* gene. This indicates that the *mukF* gene has an essential role, which is different from that of the *mukB* gene, for chromosome partitioning. Interestingly, overproduction of MukF protein (10 to 20 fold of a wild-type strain) causes anucleate cell production. In contrast, overproduction of MukB protein does not cause anucleate cell production. Homologous recombination, mutation frequency, UV sensitivity, and initiation of chromosome replication are normal at 22°C in the *mukB* and *mukF* null mutants as well as those of the wild-type strain. A mini-F plasmid, which has its specific centromere (*sopC*), a centromere-recognition protein (SopB) and an ATPase (SopA) (3), is stably maintained in the *mukB* (5) and *mukF* null mutants.

To isolate the similar type of temperature-sensitive mutants in other genes, we first isolated 90,000 tetracycline-resistant mutants at 22°C by random insertion of mini-Tn*10* into the *E. coli* chromosome, using λ Pam mini-

Tn*10* (λNK1323: ref. 6). Among them, we found 11 temperature-sensitive (at 37°C) mutants which showed the similar phenotypes as the *mukB* and *mukF* null mutants. Though the 11 mutants were found to have a defect in the *mukB* gene, no mutant of any other genes which showed the similar phenotypes were isolated.

We isolated temperature-resistant extragenic suppressor mutants of the *mukB106* mutation, which caused an amino acid substitution ($Ser_{33} \rightarrow Phe$) near the ATP-binding consensus sequence of MukB protein (7). A linkage group of the suppressor mutations was recently mapped in the *ams*(*rneE*) gene coding for RNaseE.

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Compartmentalization of gene expression during sporulation of *Bacillus subtilis*.

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In *Bacilli* sporulation involves an asymmetric division yielding two distinct cell types, the mother cell and the prespore. Different genes are transcribed in the two cell types. Transcription in the prespore is effected by RNA polymerase containing σ^F ($E\text{-}\sigma^F$), and in the mother cell by $E\text{-}\sigma^E$. The structural genes for these σ factors are transcribed and translated before the asymmetric division. Compartmentalized activity of σ^F does not require σ^E activity. Further, activation of σ^E requires the activity of σ^F. Thus activation of σ^F is potentially pivotal for the compartmentalization of gene expression during spore formation.

To study the determinants of σ^F compartmentalization, we have employed a strain in which σ^F synthesis is under the control of the IPTG inducible P_{spac} promoter. When expression was induced during sporulation by IPTG addition, activity of σ^F was compartmentalized. Disruption of *spoIIAA*, ordinarily required for σ^F activation, did not prevent compartmentalization. Disruption of *spoIIAB*, which encodes an inhibitor of σ^F, prevented the asymmetric sporulation division - probably as a result of σ^F hyperactivity; consequently we were not able to test for a specific role of SpoIIAB in compartmentalization.

We have identified an $E\text{-}\sigma^F$-transcribed gene, *spoIIR*, that is required for the activation of σ^E (Karow et al.1995. PNAS 92:2012-2016). By placing *spoIIR* under the control of the *spoIIE* promoter, which is activated before septation, we have been able to obtain σ^E activation in strains where σ^F is not active. Amongst the organisms displaying σ^E activity, about 50% had the activity restricted to the mother cell. Thus σ^F is not necessary for the compartmentalization of σ^E activity. We have also found that *spoIIAA* is not necessary for this compartmentalization, and are now testing the effects of *spoIIAB* disruption. A reasonable conclusion is that the σ factors F and E may enforce cell-specific gene expression, but are not essential for it. The determinants of the cell specificity remain open to speculation, as do the determinants of the asymmetry of the sporulation division.

Nucleoid partitioning and cell division in *Escherichia coli*: the concepts of segregation axes and nucleoid occlusion

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Under steady state conditions the *E. coli* cell grows by elongation, keeping its diameter virtually constant. When growth rate is shifted-up or when nucleoids contain more DNA (step-down), cell diameter increases by an unknown regulation mechanism, even allowing the cell to grow as a sphere. How does the cell under such widely different conditions of size and shape, partition its replicated nucleoids and determine the site of division?

Measurement of the distance between nucleoid border and cell pole showed that the nucleoid expands gradually and continuously along with the elongating cell (1). To test the idea that the increase in cell diameter following nutritional shift-up is caused by the increased amount of DNA in the nucleoid, cells were subjected to DNA-synthesis inhibition. In the absence of DNA replication, the nucleoids continued to move in the growing filaments and were pulled apart into small domains along the length of the cell. When these cells were then transferred to a richer medium, their diameters increased, especially in the region enclosing the nucleoid. It would thus appear that the nucleoid motive force does not depend on DNA synthesis and that cell diameter is not determined by the amount of DNA per chromosome but rather by the synthetic activity surrounding the nucleoid (2). Measurement of the sites of constriction in SOS filaments during division recovery, indicated that after a round of replication and termination, constrictions were placed close to the nucleoid border. In contrast, during continuing division in non-replicating filaments, constriction sites appeared randomly placed along the DNA-less regions (3). This suggests that termination of DNA replication induces division in the vicinity of the nucleoid. That constrictions do not occur at the site of a synthetically active nucleoid, may be related to the observation (4) that the rate of peptidoglycan synthesis as measured by autoradiography, is inhibited in the nucleoid-containing region. The presence of a nucleoid, actively involved in

transcription/translation thus inhibits the surrounding cell wall synthesis and, in addition, is able to modify cell shape and the division plane (2).

To explain these observations we propose that "Nucleoid Segregation Axes" (NSA) play a role in the mechanism relating direction of nucleoid movement to positioning of the division plane and cell shape. We envisage the formation of two microcompartments around the duplicated *oriC* by expression of four nearby *rrn* operons (A, B, C, E) (5). Expansion of these "Ribosomal Assembly Centers" (RAC's) is the primary force separating the newly-replicated *oriC*'s; these nucleolus-like compartments would thus play a centromeric role. The transient attachment of DNA loops to the membrane through cotranslational transport of membrane proteins, subsequently pulls newly replicated DNA loops into their respective cell halves. An axis for segregation (NSA) is assumed to develop between the two RAC's, which elongates at a rate proportional to the rate of protein synthesis but independent of DNA synthesis. In rod-shaped cells, the NSA is forced to lie eventually along the long axis; it then induces a perpendicular division plane. In spherical cells, consecutive division planes occur at perpendicular or tilted angles, because their NSAs are free to float in the cytoplasm.

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Importance of the membrane anchor for in vivo functioning of E. coli PBP3

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A water-soluble form of PBP3 has been produced in the periplasm of E. coli by replacing the membrane anchor (residues 1 to 36) by the OmpA signal peptide. The soluble PBP3 F37-V577 shows the same properties as the membrane-bound enzyme (thermostability affinity for ß-lactams) (1). The protein recognized thiolester substrates (2). In vivo activity of PBP3 F37-V577 has been analysed by complementation experiments in different E. coli strains containing mutated ftsI encoding thermosensitive PBP3. Modifications of the amino end of the N-terminal module were also analysed by complementation. The results suggest that the membrane anchor is important for positioning of the protein in the septator.

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DivIB in growth and sporulation of *Bacillus subtilis*

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DivIB is thought to be a structural homologue of *E. coli* FtsQ (1), the concentration of which has been estimated at ~ 20 molecules/cell (2). The two proteins have very similar hydrophobicity profiles (3), and are both necessary for the initiation of cell division (4,5). In addition, both proteins are membrane bound, with a large C-terminus directed towards the outside, and a small N-terminus on the cytoplasmic side of the membrane (2,6). The possibility has been raised that the N-terminal portion of *E. coli* FtsQ plays a role in transmitting information from the cytoplasm of the cell to the periplasm, where the C-terminal segment acts directly in the septation process (2). In the first part of the work presented here we have investigated the cellular level of DivIB and the effect of altering this level on growth and sporulation in *B. subtilis*. In the second part we have examined the effect on growth and sporulation of substituting the DivIB N-terminus with that of another membrane-bound cell division protein, DivIC (7).

The number of DivIB molecules in *B. sustilis* was estimated in Western transfer experiments to be~3500 per nucleoid during exponential growth. This is noticeably higher than for *E. coli* FtsQ. A strain of *B. subtilis* (SU300) in which DivIB was under the control of the IPTG inducible *spac* promoter was constructed. Surprisingly, this strain produced a non-detectable level of DivIB in the presence and absence of IPTG. Control experiments established that it was indeed producing some DivIB and it appears likely that the poor expression is due to a problem with translation. The low level of expression of DivIB in SU300 resulted in a temperature sensitive phenotype in both the presence and absence of IPTG. Cells appeared normal at 34 °C, but became progressively longer as the temperature was increased growing as long septationless filaments at 48 °C. When tested at 34 °C for the ability to sporulate it was found that IPTG had a significant effect. It its presence the level of sporulation was somewhat reduced (40% of wild type). In its absence sporulation was lowered to 0.5% of wild type. Interestingly, in both cases, minicell-like structures containing DNA formed at the ends of cells to a significant extent. Experiments are currently underway to find out if such minicell-like structures are related to the induction of sporulation. From the foregoing results it appears that *B. subtilis* can grow vegetatively and normally with very low levels of DivIB, comparable to that of FtsQ in *E. coli*, although they do become temperature sensitive. On the other hand, sporulation appears to be more severely affected by the low level of DivIB.

The cytoplasmic N-terminus of DivIB was replaced by the cytoplasmic N-terminal segment of DivIC in a construct (SU301) that placed the hybrid DivIB gene under spac control. SU301 grew normally at 34 °C and 48 °C in both the presence and absence of IPTG. Western transfer experiments established that in the absence of IPTG the hybrid DivIB was barely detectable. In the presence of IPTG the level of hydrid DivIB production was comparable to that of DivIB in the wildtype. Surprisingly, in both situations (±IPTG) the level of sporulation was significantly reduced (3-5% of wildtype). The implications of this finding with respect to a possible special role of the N-terminal segment of DivIB in sporulation are currently being investigated.

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ABSTRACT

Intracellular proliferation of *Salmonella typhimurium* within epithelial cells.
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Salmonella typhimurium is an intracellular bacterial pathogen able to invade (penetrate) non phagocytic cells. This property correlates with the pathogenesis of *Salmonella* infection. *S. typhimurium* resides in membrane bound vacuoles during all the intracellular stages and, after a lag period of approximately 4 hours, initiates bacterial proliferation within the vacuolar compartment. To date, the mechanisms that controls *Salmonella* proliferation inside epithelial cells are totally unknown.

With the aim of determine the intracellular environment that permits *Salmonella* to grow and divide within eucaryotic cells, we have carried out an extensive analysis of the traffic of *Salmonella*-containing vacuoles within epithelial cells. Our data shows that *S. typhimurium* reside in a vacuole which fuses with a subpopulation of lysosomes devoid of certain lysosomal enzymes. This fusion process bypasses the interaction with the late endosome, an intermediate organelle in lysosome biogenesis. In a later stage, intracellular *S. typhimurium* is able to induce the formation of filamentous structures containing lysosomal membrane glycoproteins (lgp). These so called Sif (*Salmonella* induced filaments) are apparently required for *Salmonella* to grow inside epithelial cells. At present, we are infecting different types of epithelial cell lines (of different species or tissue origin) for the appearance of Sif and capacity of *Salmonella* to grow intracellularly. Our preliminary results suggest that some cell lines are not competent for *Salmonella* to grow within vacuoles. In one of these cell lines, intracellular wild type bacteria is able to grow but not divide, with the subsequent formation of filamented bacteria. This phenotype indicates that putative host eucaryotic factors may control division of intracellular *Salmonella*.

The role of *ftsZ* and *ftsA* in *Caulobacter* cell division and differentiation

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One of the fundamental problems in Biology is how one cell differentiates to give rise to different cell types. In an attempt to address this problem we are trying to establish an understanding of the global controls responsible for the generation of asymmetry during cellular differentiation in *Caulobacter crescentus*. In this organism, cell division gives rise to two morphologically and functionally distinct progeny cells: a motile swarmer cell, with a single polar flagellum and a sessile cell which has a stalk at one pole. This stalk is a thin cylindrical extension of the cell surface, composed of internal and external membranes and a peptidoglycan layer. A major advantage of Caulobacter as an experimental system is the ease with which cell populations can be synchronised without perturbing the normal physiology of the cell. This allows the examination of changes in morphology, gene expression protein levels, protein modification and protein localisation at defined stages of the life cycle.

The process of reorienting the cell membranes in stalk formation is very similar to that of cell division and may involve some of the same proteins. It has been shown that FtsZ, a GTP-binding protein, forms an annular ring at the site of cell division in *E.coli*, *Bacillus* and *Caulobacter* . In an effort to understand how such asymmetric cell division and stalk biosynthesis are controlled, the role of FtsZ is being investigated. Western blot analysis using anti-*E. coli* FtsZ antibodies has shown that FtsZ in *C. crescentus* is under cell cycle control; FtsZ is not found in swarmer cells, but first appears at the initiation of stalk biosynthesis, increases in the pre divisional cell and decreases upon cell division. Constitutive synthesis of FtsZ during the cell cycle causes defects in stalk synthesis, suggesting a role for FtsZ in this process in addition to its role in cell division. Gene disruption experiments indicate that it is essential for cell viability in *Caulobacter*, as is the case in *E. coli* and *Bacillus*. *Caulobacter* FtsZ has an additional 130 amino acids at the C-terminal domain compared to FtsZ in *E. coli* and *Bacillus*. A *Caulobacter* FtsZ Histidine tagged fusion protein was purified and antibodies made to enable us to determine if FtsZ can be seen at the base of the stalk. We have also overproduced a truncated FtsZ lacking these extra amino acids in order to study the role of this domain in the function of FtsZ. One mechanism by which the levels of FtsZ at different stages of the *Caulobacter* cell cycle can be controlled is by specific degradation of the protein. Initial results suggest that the protein has a short half-life, and is likely to be targeted by proteases.

The *ftsZ* promoter has been localised to a 200 bp region. Within this region, we found two perfect matches to a 9-mer sequence motif found in the *Caulobacter* origin of DNA replication that has been shown to be essential for replication suggesting that *ftsZ* transcription and DNA replication could be coordinately regulated by a protein binding to this sequence. In support of this hypothesis, we have shown that transcription of *ftsZ* parallels DNA replication.

We have identified and characterized a homologue of the actin-like cell division protein FtsA in *C. crescentus*. Sequence analysis of *C. crescentus* FtsA indicates a high degree of sequence similarity with FtsA from *E. coli*, *Bacillus subtilis*, and *Rhizobium melliloti*. Results of a mutagenic analysis of *ftsA* indicate that *ftsA* is essential and suggest that it too is involved in stalk biosynthesis.

REGULATION OF CELL DIVISION DURING GROWTH AND SPORULATION IN *STREPTOMYCES COELICOLOR*

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In this study we aim to understand the mechanisms involved in septation in *Streptomyces coelicolor*. *Streptomyces* colonies grow as a branching mycelium and growth occurs by cell wall extension at hyphal tips. Septa are infrequent and hyphal compartments contain many copies of the genome. After 1-2 days white aerial hyphae emerge. When extension growth of aerial hyphae ceases septa are formed synchronously, sub-dividing the hyphal tips into many unigenomic compartments which will become chains of grey-pigmented spores.

Many mutants blocked in spore formation have been isolated in this lab. These mutants were isolated due to their white colour (*whi* mutants). They produce a white aerial mycelium and do not develop the grey colour characteristic of colonies undergoing sporulation. Much progress has been made in the lab towards understanding the complex interplay between regulatory genes which switch on sporulation in *S. coelicolor* (*see* Chater, 1993 *Annu. Rev. Microbiol.* 47, 685-713 for review). The regulatory genes *whi A,B,G* and *H* have been cloned and are being studied in some detail. Mutations in any of these four genes prevent sporulation septation.

To complement this ongoing work on sporulation we are attempting to isolate genes involved in the positioning of the septum, both in vegetative cells and during sporulation. In *E. coli* the genes contained in the *minB* operon are involved in positioning the division site. Two of these genes encode a division inhibitor, MinCD, and the third encodes a topological specificity factor, MinE, the mode of action of which is currently unknown. The locus *divIVB* contains homologues of *minCD* in *Bacilius subtilis* but to date no *minE* gene has been isolated. On the basis of homology between the *B. Subtilis* and *E. coli minD* genes we have designed "guessmer" oligonucleotide probes to isolate any *minD* homologue(s) in *S. coelicolor*. However, this approach has not proved fruitful.

Cytological evidence implicates *whiI* in playing a role in septum placement and we have recently isolated this gene. In *whiI* mutants sporulation septa can form which are unusually widely spaced in tightly curled aerial hyphae. Factors determining the positioning of septa and the timing of septation during both vegetative growth and sporulation are major questions which we hope to address. Proteins more directly involved in septum formation are being studied in other groups.

FIFTH SESSION: CELL DIVISION IN AN EUKARYOTIC WORLD

Chairperson: Joachim-Volker Höltje

Molecular and cellular cross-talks between bacteria, cells and tissues : bases for microbial division in the host.

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In the presence of a mucosal surface, pathogenic bacteria can affect either of two characteristic phenotypes. They can bind to the apical surface of cells and colonize this epithelial surface, or invade cells, thus proceeding to epithelial and tissue invasion/colonization. The nature of the ligand-receptor interaction that lead to either of these two different behaviours is now better known at the molecular and cellular level. For invasive pathogens, two different strategies lead to bacterial internalization into epithelial cells which are non professional phagocytes. The "zipper" mechanisms involves a bacterial outer membrane binding with high affinity to a cellular receptor most likely to be a cell adhesion molecule. In the case of invasion of *Yersinia pseudotuberculosis*, this protein binds with high affinity to β1 integrins. In the case of *Salmonella* and *Shigella*, the bacteria secrete proteins which induce localized membrane ruffling that achieve bacterial internalization. In *Shigella flexneri*, the Ipa proteins (IpaB and IpaC) are secreted by a type III specialized translocon upon contact with epithelial cells. These two proteins form a complex able to achieve major cytoskeletal rearrangement at the level of the target cell and to cause membrane ruffling. The Ipa complex interacts with the membrane, induces formation or recruitement of actin nucleation foci which then extend in long filaments of actin in a rho-dependent process.

These are subsequently tightly bundled by plastin which stabilizes the actin projections, *Shigella* then lyses its phagocytic vacuole, grows freely within the host cell cytoplasm (i.e. one generation every 40 min.). It also expresses IcsA, a 120 kDa outer membrane protein which induces actin nucleation at the bacterial surface. This protein is essentially exposed at one pole of the bacterium, the one opposite to the septation furrow. Movement occurs at the speed of actin polymerization, leaving a trail of dense actin filaments behind the bacterial body. Bacteria then reach the actin network present in the area of intermediate junctions. They engage the components of this junction (i.e. cadherins, catenins, vinculin), using them as a transporter to reach the intracellular compartment of adjacent cells.

Bacteria then lyse the two membranes and reach the cytoplasm again. This allows very efficient intracellular and intercellular colonization. *S. flexneri* therefore appears to be extremely well adapted to invading epithelial cells by using key components of its cytoskeleton.

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NEGATIVE AND POSITIVE ELEMENTS REGULATING PASSAGE THROUGH START IN FISSION YEAST.

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In the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* the process of commitment to cell division, termed Start, defines a period in late G1 beyond which cells can no longer undergo other developmental fates, such as sexual differentiation[1-2]. At Start, yeast cells monitor the extracellular microenviroment (presence or absence of nutrients, sexual pheromones, etc.) and either make a commitment to progression through the mitotic cell cycle, appropiate for cells growing in rich medium, or undergo cell cycle arrest in G1 as a prelude to conjugation and meiosis, appropiate for nutritionally starved cells. Before cells can pass Start, they must grow sufficiently to attain a critical cell mass, and small cells in G1 cannot undergo S-phase until they reach this mass.

We will describe the identification and characterization of the gene *rum1*$^+$, encoding a 25 KD protein important for determining the duration of the G1 phase[3]. When *rum1*$^+$ is deleted, the critical cell mass required at Start is reduced and the pre-Start G1 interval is eliminated, indicating that *rum1*$^+$ is a major element determining the timing of Start. *rum1*$^+$ is also important in restraining mitosis until G1 is finished: when *rum1*$^+$ is deleted in a mutant that normally blocks at Start, cells proceed to undergo mitosis and cell division. Overexpression of *rum1*$^+$ initially causes cell cycle delay in G1, since the critical mass required for Start is increased relative to wild type cells. These cells undergo multiple rounds of S-phase in the absence of mitosis, leading to very long cells with increased ploidy. Therefore *rum1*$^+$ is important for defining the G1 status of a fission yeast cell. We will show that rum1 functions as a cell cycle inhibitor of p34^{cdc2} kinase activity both *in vitro* and *in vivo* and that it counteracts the positive effects of fission yeast G1 cyclins.

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Subcellular Organization in Yeast

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Yeast cells can divide in two spatially programmed patterns of cell division: axial (**a** and α cells) and bipolar (**a**/α cells) (see below). These patterns are produced by budding, a mode of cell division requiring a highly polarized cellular organization. During budding, a new daughter grows from the surface of a ellipsoidal mother cell and then a nucleus is mitotically deposited in the bud. A group of genes (typified by the *BUD* genes) is important for producing these patterns; when mutant for these genes, cells no longer produce the proper patterns for budding and cell division but they are otherwise normal. A second group of genes (typified by *CDC24*, *CDC42* and *BEM1*) is important for polarizing the cell to build the bud. Here we present progress in understanding how cells choose sites of their cell surface for producing ordered patterns of budding and how this spatial information is communicated to the cytoskeleton.

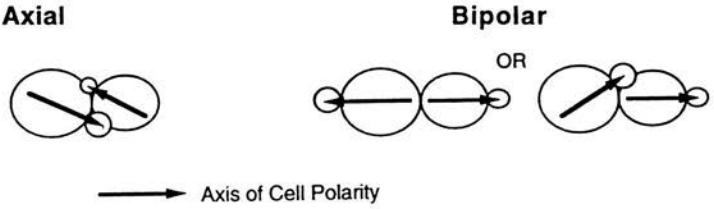
Physiological experiments suggest that, in the axial pattern, the mother-daughter neck region is marked by a structure that provides a spatial memory from one cell cycle to the next. Genetics suggested that this structure might include BUD3 and BUD4 proteins, since they were encoded by the only axial specific *BUD* genes of the original five identified. Consistent with this prediction, both BUD3 and BUD4 proteins localize to the neck region as a double ring structure encircling the mother bud neck. This structure appears at approximately the G2->M transition point of the cell cycle and persists until cytokinesis. It splits into two single rings, one on each progeny cell. Each remains for part of the unbudded G1 phase of the cell cycle and then disappears. Thus, both genetically and in terms of localization BUD3 behaves as a spatial memory marking the neck region in one cell cycle for bud initiation in the next cell cycle.

Two important objectives are to identify the template for BUD3 assembly and to uncover the temporal basis of the appearance and disappearance of the BUD3 rings. Towards the first objective, several lines of evidence suggest that BUD3 and BUD4 use the neck filaments which also encircle the neck as templates. Interestingly, preliminary evidence suggests that BUD3 and BUD4 are codependent for assembly, as if they copolymerize. With regards to the second objective, the *BUD3* message is cell cycle regulated in its abundance; therefore, BUD3 assembly may be a direct result of the rise in mRNA abundance. The mechanism of disassembly is not yet well understood. (BUD4 data from Sylvia Sanders of Ira Herskowitz's laboratory)

Less is known about how the poles of the cell are recognized for bipolar budding. Physiological experiments suggest that structures, which persist for many generations, exist at the poles of the cell. Identification of genes specifically involved in the bipolar pattern is ongoing as a route to understanding the mechanism of pole marking.

An important question concerns the mechanism by which the axial or bipolar spatial information is communicated to ultimately polarize the cytoskeleton. Although the mechanism is not understood, it appears to involve a cascade of at least two GTPase cycles, the BUD1 (RSR1) GTPase cycle and

the CDC42 GTPase cycle. Work is ongoing in my laboratory to understand how exactly these GTPases control polarization of the cytoskeleton within the cell. The mechanisms by which these GTPases function should have broad importance as it appears, from our work and that of others, that CDC42 is required for the formation of axes of polarization in animal cells as it is in yeast.



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POSTERS

CHARACTERIZATION OF A NEW GTPase OF *E. coli* POSSIBLY IMPLICATED IN CELLULAR DIVISION.

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We have dilucidated the transcriptional organization of the *rpmH-rnpA-60K ORF-50K ORF* gene cluster of E. coli. They seem to constitute a transcriptional unit, although we have detected and localized multiple regulatory elements, including transcription promoters and terminators, which could possibly account for differential expresion of each gene. *rpmH* and *rnpA* code for the ribosomal protein L34 and for C5, the protein component of ribonuclease P, respectively. The last two genes of the cluster code for two proteins of aproximately 60 and 50 kD whose functions are not konwn. Analysis of the sequence of 50K revealed a consensus for GTP-binding. By making use of UV-crosslinking experiments, we have demonstrated that 50K binds GTP/GDP. As this is a typical binding pattern of GTPases, we tried to determine whether 50K had this enzimatic activity. We have proved that 50K cleaves GTP to produce GDP. Studies on distribution of this protein, by using western blots on subcellular fractions and immuno-electronmicroscopy, have proved that a fraction of the total amount of 50K present in the cell is associated with the citoplasmic membrane. Preliminary results indicate that 50K might be involved in cellular division.

On the control and coordination of chromosome replication, nucleoid processing and cell division.

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The processes which take part in the bacterial cell cycle have to be controlled and coordinated in order to avoid that cell division takes place before chromosome replication and nucleoid processing have been completed (5, 7). The *Escherichia coli intR1* strains can be used as an alternative approach to study the bacterial cell cycle (6). The *intR1* strains carry a deletion that inactivates the normal replication origin in *E. coli*, *oriC*, and the insertion of various derivatives of the R1 plasmid replicon in this place. Chromosome replication in the constructed *intR1* strains retains the plasmid replication characteristics. In this way chromosome replication can be adjusted to give both normal and altered chromosome copy number. This allows studies of how different events in the cell cycle respond to the new control situation, and how chromosome replication, nucleoid processing and cell division are interconnected.

The replication frequency of plasmid R1 is regulated by an antisense RNA, CopA, which inhibits the synthesis of the rate-limiting initiation protein RepA. By using a temperature-dependent replication derivative of R1, the rate of *repA* transcription can be increased resulting in over-replication, whereas conditional *copA* expression results in under-replication (2, 4). An increased copy number of the chromosome corresponds to transfer the initiation of replication to earlier times in the cell cycle and a decreased of it corresponds to a delay of the initiation time. Under-replication causes inhibition of cell division and over-replication causes the formation of both elongated cells and DNA-less minicells (1, 3).

By varying the level of replication, the control of nucleoid processing and the coordination between nucleoid processing and cell division are studied. By using different levels of under-replication, the time window during which chromosome replication and nucleoid separation have to be completed to allow normal cell division is determined. These studies are carried out using flow cytometry and computer-aided image-processing of microscope pictures from bacteria in which nucleoids have been stained. The results seem to indicate that nucleoid processing is dependent on the time of initiation of chromosome replication and that it is necessary to reach a minimal distance between the nucleoids and a discrete cell length to allow cell division.

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MAPPING REGIONS OF THE CELL DIVISION PROTEIN FTSZ INVOLVED IN PROTEIN-PROTEIN INTERACTIONS. Paul Casaz, Jean-Pierre Bouché, Laboratoire de Microbiologie et Génétique Moléculaire du CNRS, 118, route de Narbonne, 31062 Toulouse, France

FtsZ is an essential cell division proteln required at the earliest stages of septum formation in E. coli. It is found throughout the cytoplasm of nondividing cells, but it organizes into a ring-like structure located at the future division site just prior to the start of septation. This dynamic ring constricts such that it remains associated with the leading edge of the inward growing septum until cytokinesis is complete. Purified FtsZ is a GTPase, and it was recently demonstrated that wild type FtsZ, but not mutants lacking GTP binding activity, can form cylindrical filaments *in vitro* in the presence of GTP. In addition to forming homopolymers, FtsZ is also likely to interact with other components of the cell division machinery, such as the products of the *min* operon, which governs the position of the division septum. The goal of this work is to identify regions of FtsZ required for polymerization using a combination of *in vivo* and *in vitro* approaches. Such techniques may also be useful in the future to study the interactions of FtsZ with other cell divison proteins.

As an *in vivo* approach to study FtsZ-FtsZ interactions, we are using the "two-hybrid system", developped originally in Saccharomyces cerevisiae to study protein-protein interactions. Our results indicate that FtsZ-FtsZ interactions can be readily detected by this assay, which will permit the mapping of the sequences required for this interaction using a series of deletions from the N-terminal and C-terminal of FtsZ. Preliminary experiments indicate that a deletion mutant of FtsZ consisting of residues 1-227 retains the ability to interact with wild type FtsZ in this assay.

To complement the results of the two hybrid system, polymerization is also being studied *in vitro* using purified proteins. In agreement with published results, purified wild type FtsZ is efficiently precipitated by centrifugation in the presence of GTP but not in the presence of ATP. Mutant FtsZ proteins are currently being purified to determine whether they can polymerize alone, and if they can form mixed polymers with wild type FtsZ.

THE STRUCTURAL MODEL FOR FtsA PROTEIN OF *Escherichia coli*

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The prediction of FtsA being a member of the family of actin and other ATP binding proteins (Bork *et al.*, 1992) and the alignment of its sequence with the other members of the family made it possible to construct a putative structural model of the protein. We have designed a series of point mutations to test the correlation between the structure and the function of FtsA.

A first mutation was design to confirm the ability to bind ATP, the main characteristic of proteins that belong to this family. The change of the 336 Glycine in the putative ATP binding pocket of the wild type protein by an aspartic yields the FtsA102 mutant protein which contrary to FtsA$^+$ was not retained by an ATP-agarose column (Sánchez *et al.*, 1994). The possibility that the protein had at least two differents phosphorylation states as DnaK, was also proven. The candidate for this phosphorylation was the 215 Threonine, conserved in almost every protein of the family and which is the phosphorylation substrate in DnaK. The absence of the lower pI form in a 2D electrophoresis western blot of a mutant protein, FtsA104, in which an Alanine had replaced the Threonine, confirmed the predictions (Sánchez *et al.*, 1994).

The intracellular localization of FtsA and the ability to bind ATP are related with the phosphorylation state, thus, the phosphorylated form is in the cytoplasm and is able to bind ATP, while the unphosphorylated one is located in the membrane and is unable to bind ATP (Sánchez *et al.*, 1994). FtsA104 cannot be phosphorylated and does not bind ATP, but is able to rescue the reversible *ftsA2*, the irreversible *ftsA3* and, almost with the same efficiency the *ftsA16* amber alleles. This seems to indicate that phosphorylation and ATP binding are not essential for the activity of the molecule and perhaps they have a regulatory role on the action of FtsA (Sánchez *et al.*, 1994).

A mutation in the phosphorylation site, *ftsA106*, has been obtained changing Threonine by Glutamic. The rationale being that the FtsA106 molecule would mimic a constitutive phosphorylation. The new mutant protein seems to be lethal for the cell.

A possible mechanism for the action of FtsA is postulated; the protein in the cytoplasm would be phosphorylated and would bind ATP, here the ATP would be hydrolised. The unphosphorylated form unable to bind ATP would bind to the membrane where the protein exercises its activity. FtsA$^+$ protein derived from the chromosal gene could be sequestered by FtsA106, preventing its action on septation.

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Growth rate and cell cycle dependent control of cell division genes *ftsQAZ*

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The essential cell division genes *ftsQ*, *ftsA*, and *ftsZ* reside in the *dcw* gene cluster at 2.5 min on the *Escherichia coli* chromosome. Their levels of expression are tightly controlled with respect to the cellular growth rate and the stage in the cell cycle, and this is required for cell division to occur at the correct time and to proceed normally. The genetic control of these genes is complex and not totally understood. They are closely linked and controlled from several promoters within the coding regions of upstream genes. In order to identify regulatory mechanisms that are responsible for the cell cycle-dependent and growth rate-dependent control of these genes, we have constructed a large set of transcriptional fusions of this region to the *lacZ* gene. By making deletions from both ends of the regulatory region, the quantitative contribution from each of the promoters to the expression of *ftsZ* has been assayed. Although the region proximal to *ftsZ* is required for full expression of the gene, the proximal promoter *ftsZ2p* has very low intrinsic activity. The majority of the transcripts reaching *ftsZ* appears to originate from promoters upstream of the *ftsZ2p* promoter. Since it is known that not more than 21% of the *ftsZ* transcripts in the cell have their 5´-ends upstream of this promoter (Garrido, et al.,1993), a large fraction the transcripts appears to be cut by RNase E at the processing sites proximal to *ftsZ* (Cam, et al.,1994). The regulatory significance of this intercistronic mRNA processing is addressed. In addition to the growth rate-dependent control of the gearbox promoter *ftsQ1p*, also *ftsZ3p4p* and *ftsZ2p* display inverse growth rate-dependence when fused to *lacZ*, and the effect of the regulatory nucleotide ppGpp on the activity of these promoters will assessed. Furthermore, the set of transcriptional fusions provides the tools to localize the regulatory regions that are subject to cell cycle-dependent control and to set up genetic screens to find *cis*-acting regulators of the different promoters.

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Sporulation Transcription Factor Spo0A Causes the Site of FtsZ Nucleation to Switch from Midcell to Potential Division Sites at Both Poles

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Sporulation in the Gram positive soil bacterium *Bacillus subtilis* is characterized by a switch from binary fission, which is characteristic of vegetative growth, to polar septation. The polar septum divides the cell into two compartments, the smaller forespore and the larger mother cell, and is critical to establishing the two cell types each with their own, independent lines of gene expression. In a wild type cell the septum forms at a single pole, although genetic experiments indicate that both poles are potential division sites. While numerous sporulation genes have been identified, no single gene has been found that is responsible for mediating this switch. It is known that two transcription factors, SpoOA and σ^H, are required for polar septation. However, mutations in any of the known targets of these factors block sporulation following formation of the polar septum.

As a means of identifying at which point in the sporulation cycle the switch between medial and polar septation is made, we used immunofluorescence microscopy to localize FtsZ within developing *B. subtilis* cells. As expected, in vegetatively growing cells FtsZ localizes to the medial position. Surprisingly, early in sporulation, FtsZ localizes to not one, but both poles of the predivisional cell, reinforcing the idea that both poles are potential division sites although the septum is formed at only one pole. The switch from a medial to bipolar pattern was not dependent on σ^H but was prevented in cells lacking Spo0A. FtsZ localization in *spo0A* mutant cells continued to be medial well after the onset of sporulation. The addition of a *minD* mutation to a *spo0A* null mutant strain did not restore bipolar localization of FtsZ indicating that the switch is *minD* independent.

HALOARCHAEAL TANDEM REPEATS ARE INVOLVED IN REPLICON PARTITIONING

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We report the presence of long stretches of tandem repeats in the genome of the halophilic *Archaea Haloferax mediterranei* and *Haloferax volcanii*. A 30 bp sequence with dyad symmetry (including 5 bp inverted repeats) was repeated in tandem, interspaced by 33-39 bp unique sequences. This structure extends for long stretches - 1.4 Kb at one location in *H. mediterranei* chromosome and about 3 Kb in *H. volcanii* chromosome. The tandem repeats (designated TREPs) show a similar distribution in both organisms, appearing once or twice in the *H. volcanii* and *H. mediterranei* chromosomes, and once in the largest, probably essential megaplasmid of each organism but not in the smaller replicons. Sequencing of the structures in both *H. volcanii* replicons revealed an extremely high sequence conservation in both replicons within the species, as well as in the different organisms. Homologous sequences have also been found in other more distantly related halophilic *Archaea*. Transformation of *H. volcanii* with a recombinant plasmid containing a 1.1 Kb fragment of the TREPs produced significant alterations in the host cells, particularly in cell viability. Introduction of extra copies of TREPs within the vector significantly alters the distribution of the genome among the daughter cells, as observed by DAPI staining. Although the precise biological role cannot be completely ascertained, all the data conform with the tandem repeats being involved in replicon partitioning in halobacteria.

Cloning and characterization of *MKC1*, a *Candida albicans* Map kinase related to cell integrity and cell growth

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Mitogen-activated (MAP) kinases represent a group of serine/threonine protein kinases playing a central role in signal transduction processes in eukaryotic cells and so are a key mechanism in the integration of external stimuli with the internal cell cycle machinery. As part of a line of investigation towards the analysis of this signal transduction mechanisms in pathogenic fungi, we have isolated a *Candida albicans* functional homolog of *Saccharomyces cerevisiae* MAP kinase gene *SLT2* (*MPK1*) - involved in the recently outlined *PKC1*-controlled signalling pathway- by a strategy based on the complementation of the thermosensitive autolytic phenotype of *slt2* null mutants. The isolated gene, named *MKC1* (MAP Kinase from *Candida*), coded for a putative protein, Mkc1p, of 58,320 Da that displayed all the characteristic domains of MAP kinases and was 55% identical to *S. cerevisiae* Slt2p (Mpk1p). The role of *MKC1* gene in *Candida albicans* was investigated creating single and double deletion strains Deletion of the two alleles of *MKC1* gene gave rise to viable cells that grew at 28°C and 37°C but, nevertheless, displayed phenotypic traits that were lethal under more stringent conditions. These included, among others, a low growth yield and a loss of viability in cultures grown at 42°C, a high sensitivity to thermal shocks at 55°C for 20-60 min, an enhanced susceptibility to caffeine that was osmotic remedial and the formation of a weak cell wall with a very low resistance to complex lytic enzyme preparations. The *MKC1* gene presumably belongs to the *PKC1*-mediated pathway, representing the first experimental evidence of a similar transduction pathway in *Candida* spp. These strains, finally, will allow us to explore the connection between cell growth, cell wall formation and the relationship with the cell cycle progression in this clinically important fungus.

Molecular Mechanisms that Determine the Timing of Cell Division in *Escherichia coli* K12.

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To elucidate molecular mechanisms of the timing of cell division in *E. coli* K12, we isolated novel mutants, *cfcA, B, C, D, E, and F,* (A. Nishimura, 1989, Mol. Gen. Genet., 215:286-293) in which the DNA replication and cell division are uncoupled. These mutations partially suppressed both division arrest and lethality induced by treatments that inhibit DNA replication. Under permissive conditions for DNA replication, the *cfc* mutants divide 1.3 to 1.5 times more frequently per round of DNA replication. Complementation tests and sequencing analyses showed that *cfcA* gene encods the α subunit of glycine tRNA synthetase. From genetic and physiologic analyses of *cfcA1* mutant, *cfcA* gene was found to be specifically involved in the regulation of cell cycle by a pathway different from stringent response or SOS induction. The locus of *cfcB1* mutation was found in *apaH* gene mapped at 1 minute of the *E. coli* genetic map. *ApaH* gene encodes diadenosine tetraphosphate (AppppA) hydrolase, and *apaH*$^-$ mutants showed $\geqq$ 16 fold increase in cellular level of AppppA. AppppA may be a pleiotropic signal for cell growth and DNA replication (review: P. Zamecnik, 1983, Anal. Biochem. 134:1-10). AppppA is formed *in vitro* by aminoacyl tRNA synthetases in a reaction in which an enzyme-bound aminoacyladenylate intermediate donates AMP to ATP (Goerlich, O. et. al., 1982, Eur. J. Biochem. 126:135-142). However, so far, neither aminoacyl tRNA synthetase mutants, inhibitory treatments of amino-acylation, nor structural mutants of tRNA has been known to overproduce AppppA *in vivo*. The *cfcA1/glyS* mutation was suppressed by *a high copy number plasmid carrying apaH*$^+$ gene. HPLC analyses showed that the intracellular level of AppppA in *cfcA1* and *cfcB1* cells was about 100-times higher than in *cfc*$^+$ cell. This suggests that *cfcA1*, the mutant of glycine tRNA synthetase, could synthesize AppppA *in vivo* and that the AppppA nucleotide is involved in the determination of the timing of cell division in a manner that couples cell division to DNA replication. We are currently analyzing other *cfc* mutants to search for targets of AppppA.

Fail-safe post-septational mechanism of DNA segregation in *Bacillus subtilis*.

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During sporulation *Bacillus subtilis* undergoes a modified cell division in which the septum forms at an asymmetric location. This modified division is related to vegetative division morphologically and the two forms of division require common gene products (1). Mutations in *spoIIIE* block partitioning of one chromosome into the developing spore during sporulation but have no overt effect on partitioning in vegetatively dividing cells. However, the expression of *spoIIIE* in vegetative cells (2,3) and the finding of genes closely related to *spoIIIE* in a range of eubacteria (4,5) suggested a more general function for the protein.

We reasoned that SpoIIIE might come into play in vegetative cells if the chromosomes failed to separate before sepatation. The septum should then close around the nucleoid creating a structure similar to the one on which SpoIIIE acts during sporulation. To test this hypothesis we transiently treated isogenic wildtype and *spoIIIE* mutant cells with HPUra a specific inhibitor of DNA replication. Inhibition for 1 hr ensured that the completion of DNA replication would be delayed until well after septation in a large portion of cells. As expected, the treatment resulted in the formation of anucleate cells. There were also some nucleoids that appeared to have been bisected by a septa. Although some cells of this type were detected in the wild-type strain, they were far more abundent in the *spoIIIE* mutant cultures. The relative infrequency of these cells in the wild type culture was consistent with the predicted action of SpoIIIE in allowing the partitioning of chromosomes to occur after septation.

As an independent way of testing for a vegetative role for SpoIIIE we tried introducing a *minD* mutation, since such mutations are thought to affect partitioning in *E. coli* (6). We found little evidence for a partitioning defect in the *minD1* mutant of *B. subtilis*. However, when the cells entered stationary phase, in the presence of the *spoIIIE* mutation a substantial proportion of minicells now contained DNA. Again the observations are compatible with the notion that the SpoIIIE protein provides a post-septational chromosome partitioning system.

In the natural enviroment, partitioning might be expected to fail when DNA replication is interrupted by DNA damage or antibiotic inhibitors of replication. We found that the *spoIIIE* deletion mutant was more sensitive to nalidixic acid and mitomycin C than the parent strain.

In conclusion, we have shown that SpoIIIE protein provides a post-septational mechanism of chromosome partitioning in *B. subtilis*. The mechanism comes into operation when the normal coordination between septation and nucleoid partitioning is perturbed or septum positioning is altered. In addition, SpoIIIE is also required for maximal resitance to antibiotics that interfere with DNA metabolism. The results have important implications for our understanding of the functions of the genes involved in the primary partitioning machinary and of how septum placement is controlled.

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Escherichia coli mutant requiring high concentration of Ca^{2+} for growth.

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In eukaryotes, calcium plays a fundamental role in the regulation of the cell cycle, but its role in the prokaryotic cell cycle has yet to be elucidated. In order to approach this problem genetically, we have isolated *E. coli* mutant SH3450 with its temperature sensitive growth suppressed by the presence of the high concentration of $CaCl_2$ in the medium.

SH3450 mutant was isolated as a *muk* mutant which produced anucleate cells at 42°C by the method of Hiraga *et al.* (J. Bacteriol, 171:1496-1505, 1989). Temperature sensitivity of the growth was suppressed by the presence of 50-100mM $CaCl_2$. Cosmid clone λL3 which suppressed temperature sensitivity of the mutant was isolated. Following subcloning revealed that the *rpoB* gene (90 min) encoding the β subunit of RNA polymerase was responsible for the suppression. P1 transduction revealed that SH3450 mutant had two mutations which were co-transducible with *argH* (90 min). One, which we named *cal88*, caused Ca^{2+}-dependent growth even at 30°C as well as at 42°C. The other is a *rpoB* mutation, named *rpoB88*, which suppressed Ca^{2+}-dependency of the *cal88* mutant at 30°C but not at 42°C. The *cal88* mutant showed typical *par* phenotypes at 30°C and 42°C, i.e., formation of elongated cells with large nucleoids and production of anucleate cells from the ends of the elongated cells. The *rpoB88* mutation suppressed the *par* phenotypes of the *cal88* mutant at 30°C but its suppression at 42°C was partial, resulting in formation of elongated cells with un-evenly segregated nucleoids. In the *cal88 rpoB88* double mutant cells, superhelicity of a reporter plasmid was decreased at 42°C.

Further analysis of these mutants and cloning of the *cal* gene will reveal the role of calcium in the regulation of the *E. coli* cell cycle.

CONCLUSIONS

L. Rothfield, J. Ayala and M. Vicente

The Workshop on Structure, Function and Controls in Microbial Cell Division brought together scientists who study cell division in both procaryotic and simple eucaryotic organisms. This bringing together of investigators who are approaching a similar biological question in a group of disparate organisms was one of the strengths of the meeting. The following exciting and new topics were discussed.

(1) The structure and function of FtsZ in cell division in bacteria-
This produced some of the most exciting presentations and discussion of the three day meeting. Work from seven laboratories demonstrated that the initial step in the bacterial division process is now accessible to biochemical analysis. This work was an outgrowth of studies reported over the past few years showing that septation appears to be initiated by formation of a ring, composed of the cell division protein FtsZ, that is formed at the site of septum formation at the time of onset of septal ingrowth. Amongst the major new insights that emerged from the workshop were the demonstration that FtsZ forms structures in the test tube that closely resemble the structure of microtubules in eucaryotic cells, the finding from several laboratories that FtsZ binds specifically to several division-related proteins as shown by both biochemical and genetic methods, the identification of a new FtsZ-binding protein, ZipA, that may prove to be the membrane receptor for initiation of formation of the FtsZ ring, and evidence that FtsZ plays a role in differentiation of the bacterium *Caulobacter* that had previously been unsuspected. A general consensus appeared to develop that FtsZ probably acts by forming a contractile ring that is responsible for invagination of the new septum. This led to a fruitful discussion of three possible molecular mechanisms that could be reponsible for the hypothetical contractile mechanism. This, hopefully, will provide a framework for further advances in this rapidly moving area.

(2) Controls of cell wall synthesis and cell division-
A number of essential cell division genes, *ftsZ* among them, are grouped in a small region of the bacterial chromosome, the *dcw* cluster. This was shown to be true in several unrelated bacterial species. Several new division-related genes in this gene cluster were described, in which the same general pattern of genetic organization, as well as the general structure of the division proteins, was conserved. Several participants described studies designed to define the regulation and physiological role of the essential cell division genes and their protein products. Indirect evidence was presented that expression of one or more of the essential division genes might be regulated as part of the general cellular mechanism that coordinates RNA and protein synthesis in the bacterial cell. It appeared from the discussion that important regulatory mechanisms remain to be discovered to explain the timing of septum formation during the cell cycle and the regulation of the division process in response to physiological and environmental perturbations.

(3) The topology of segregation, gene expression and division-
One focus of the Workshop was the mechanisms used by cells to place the division septum at the proper location in the cell and to segregate daughter chromosomes to their desired positions in the predivision cell. Genetic, biochemical and cell biological studies have identified genes and gene products that participate in the process of division site selection and chromosome segregation. There was a fruitful conjunction of information from several different organisms in which the septum is placed at different locations in the cell and these included two bacterial species-- *Escherichia coli*,where the septum is placed at the midpoint of the rod-shaped cell but can be placed at the cell poles in certain mutants, *Bacillus subtilis* , where the sepum is placed near the cell pole when sporulation takes place; and the yeast *Saccharomyces*

cerevesiae, where the septum is placed eccentrically at the bud site. Work from the bacterial and yeast systems supported the view that division can occur at one of several predetermined sites and that cellular mechanisms exist to select the desired site. However, this remained a controversial point and the contrary view, that the site of division in bacteria is determined by the positions of the nucleoids, was also expressed. Work from two laboratories described studies that began to dissect the functions of a protein, MinE, that is responsible for the specificity of septal placement in *E.coli* . Studies of formation of the asymmetrically placed spore septum in *B.subtilis* and the mechanism whereby the spore chromosome is moved to the end of the cell were also discussed. Elegant work in the yeast system identified several skeletal elements that appear to mark the division site and that presumably play a role in initiating formation of the bud septum.

(4) Cell division in an eukaryotic world

The regulation of passage through the key checkpoint in the eucaryotic cell cycle (START) was the subject of discussion, provoking interesting comparisons of this aspect of the cell cycle in bacteria and in fission yeast. A further focus on interactions between bacterium and eucaryotic cell came from discussion of the mechanism used by the pathogenic organism *Shigella* to invade and spread through its eucaryotic host cells. This provided an impressive demonstration of the ability of microorganisms to use elements of the host cell machinery to facilitate its entry and spread, both being key elements in pathogenesis.

The success of this workshop suggests that future meetings that bring together workers from procaryotic and eucaryotic research arenas have the potential to provide new insights on important biological problems.

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