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INTRACELLULAR FOLLOW UP AS AFECTED BY A NEW BACTERIAL SYSTEM ACTING AS ANTIBACTERIAL AND RELEASING SYSTEM OF FAVOURABLE BACTERIAL PRODUCTES IMPORTANT IN FOOD INDUSTRY

BY

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ABSTRACT

Bacterial cell lysis, initiated by induced expression of a protein destabilizing the basic bacterial cytoskeleton, allowed an electron microscopic study of intracellular structural features of *Escherichia coli* as a bacterial system. Densely packed parallel and twisted strands of a complex presumably consisting of chromosomal DNA and DNA binding proteins could be detected. In early states of lysis, polysome-like complexes could be visualized, arranged in parallel rows following a helical pattern, close to the inside of the cytoplasmic membrane. The basis of type I pili was seen to be not inserted in the cytoplasmic membrane; rather, these pili were found to be attached to the peripheral part of the basic cytoskeleton located close to the inner face of the cytoplasmic membrane. Implications for a better understanding of fundamental structure-function relationships in bacteria at the cellular and macromolecular levels are discussed. Therefore, our results of this lysis system confirmed its possible use in the production of fermented foods and some food additives used in food processing as well as in industrial fermentation to ease the downstream processing of many favourable bacterial products important in food technology and to allow better study of intracellular structures of bacteria.

INTRODUCTION

The final state of a bacterial culture, i.e. complete cell lysis, to make the desired recombinant products (proteins, plasmids, inclusion bodies) available for downstream processing, was in the center of interest. In recent years, experimental data accumulated that demonstrate that not only eukaryotic cells, but also prokaryotes possess cytoskeletons (Antranikian *et al.*, 1987; Mayer *et al.*, 1998; Jones *et al.*, 2001; Regula *et al.*, 2001; Van den Ent *et al.*, 2001; Mayer 2002; Kiessling *et al.*, 2000; Mayer 2003). Their building elements turned out to be proteins closely related to tubulin or actin of the eukaryotic cell, or proteins not closely related to tubulin or actin, but nevertheless belonging to the actin superfamily (summarized by Mayer 2002). In the meantime, several classes of

bacterial cytoskeletons were established (Mayer 2003): tubulin-related cytoskeletons (FtsZ-based; Erickson *et al.*, 1996), closely actin-related cytoskeletons (MreB/Mbl-based; Jones *et al.*, 2001), elongation factor Tu (EF-Tu) (member of the actin superfamily)-based cytoskeletons (this came as a surprise; Mayer 2002), and other kinds of cytoskeletons such as complexes made up of contractile proteins (Trachtenberg and Gilad 2001), or various kinds of specific proteins, occurring, for example, in the wall-less parasitic bacterium *Mycoplasma pneumoniae* (Regula *et al.*, 2001, Hegermann *et al.*, 2002). It was shown that *E. coli* contains genes coding for building elements of three kinds of cytoskeletons: proteins belonging to tubulin-based cytoskeletal elements, to an actin-based cytoskeleton, and at least one protein constituting the EF-Tu-based cytoskeleton. From thes forms, only the EF-Tu-based bacterial cytoskeleton appears to be ubiquitous (Mayer *et al.*, 1998; Mayer, 2003). Therefore, it was termed "basic" or "primary" bacterial cytoskeleton (Mayer 2002). A function of bacterial cytoskeletons consisting of actin-related proteins was shown to be that of a scaffold responsible for cell shape determination, stabilization, and extension, whereas tubulin-related bacterial cytoskeletal elements were found to be involved in septation of the daughter cells in the process of cell division.

Recent efforts regarding applications of these new data for biotechnological, pharmaceutical, and medical purposes resulted in approaches aimed at discovery or design of a new class of antibacterial drug (Mayer 2002), acting by destabilization of the bacterial cytoskeleton. In addition, an innovative system for induced lysis of bacteria, used for production of recombinant products, was developed (Mayer 2002, 2003).

During the course of the development of this new lysis system, we realized that we could expect a wealth of new information on the cytology of bacteria. However, it turned out that, after induction of the intracellular synthesis of the lysis-inducing protein, but prior to complete cell lysis, states of the bacteria could be observed that exhibited various so far unknown structural cell features. New data could be gathered especially regarding the structural organization of the nucleoid, the mode of interaction of ribosomes/polysomes with elements of the basic bacterial cytoskeleton, and the insertion of pili. This was made possible by the fact that states of bacterial cells could be analyzed in which the cells had lost parts of the cell wall, or even the cytoplasmic membrane. An additional structural feature of the bacterial cell in general, not often considered to be of importance, was very helpful for these investigations: as in any other living cell, also in bacteria the cell cytoplasm can be considered to be in the state of a gel (Pollack 2001). In our case, the shape of the cells was transiently maintained even after complete loss of the cell envelope, due to the stiffness of the gel-like cytoplasm. This allowed electron microscopic imaging of the cell contents in the absence of the cell envelope, prior to cell disintegration.

On the other hand the lysis of bacteria has conciderable technological importance mainly in fermented foods and one of these is the dairy sector. Cheese ripening for instance, occure due to solublization of casein which is hydrolysed probably by the action of lysed lactic acid bacteria (bie and Sostrom, 1975;

Krishna and Dutta, 1976; Chapot-Chartier *et al.*, 1994; Crow *et al.*, 1995 and Zambonelli *et al.*, 1991). Cell lysis was also observedin *Propionibacterium freudenreichii* (Ostlie *et al.*, 1995). It is ascertained also that lysed lactobacilli release β -galactosidase which cause the hydrolysis of resdual lactose (Montanari *et al.*, 2000) and also other compounds which inhibit the growth of some molds (Chiavari *et al.*, 1998). In ripening of salami fermented sausage and similar fermented meat products, lysed lactic acid bacteria and micrococci contributes essentially in the protein and lipid hydrolysis (Coppola *et al.*, 1997). The biologically active compounds to the mediumincluding enzymes, pigments, hormones and other inclusion bodies, which are important for food industries and fermentation technology. Without effective lysis system, the cells must be mechanically or biologically opened during the downstream processing of these compounds, which negatively influenced the quality and production costs. The presence of a lysis (act also as antimicrobial) system, make revolutionary evolution in the food technology. Such a system makes it possible to decrease the costs and increase the quality and safety of the microbial products. Therefore, the aim of this study is to follow up the intracellular structures and stages of lysis while the system is working to confirm the effectiveness of this lysis system.

MATERIALS AND METHODS

Bacterial strains, cultivation, induced lysis, and cell harvest:

Bacterial strains *Thermoanaerobacterium thermosaccharolyticum* (DSM 571) (Antranikian *et al.*, 1987, Mayer *et al.*, 1998), *Mycoplasma pneumoniae* M129 (ATCC 29342) (Hegermann *et al.*, 2002), *Escherichia coli* XL1-Blue, obtained from Stratagene (La Jolla, USA) (Schwienhorst *et al.*, submitted), and *Desulfovibrio vulgaris* were grown and harvested as described by Rohde *et al.* (1990). Induced lysis of *E.coli* by destabilization of the "cytoskeletal web" was performed as described (Mayer, 2002, Mayer, 2003; Schwienhorst *et al.*, submitted).

Electron microscopic preparation techniques:

Conventional and cryo sectioning, negative staining, and imaging were done as described (Hegermann *et al.*, 2002, Regula *et al.*, 2001).

RESULTS AND DISCUSSION

Untreated *Escherichia coli* cells, and cells after induced lysis:

Figures 1a and 1 b depict *E.coli* cells without treatment, and shortly after induced lysis, respectively. Without treatment, the cells exhibit the usual properties (shape, size, type I pili). After onset of synthesis of a factor destabilizing the "cytoskeletal web" (Mayer 2002, 2003, Schwienhorst *et al.*, submitted) by insertion into the protofilaments and meshes of the web, different states of the cells could be seen: seemingly unaffected cells (U in Fig.1b), cells that did appear to be rendered translucent (T), and cells that had lost their cytoplasmic membrane and their wall CB); nevertheless, these cells did maintain their basic shape and size, and they still carried type I pili. Two reasons may be responsible for preservation of shape even after loss of the cell envelope. One is

that such cells may still contain parts of their cytoskeleton in undisturbed state (s. Fig.2d), one is that living cells are, in fact, gel-like masses (Pollack, 2001) that loose their shape only after prolonged exposition to the surrounding liquid medium and to mechanical (shearing) forces. In addition, cell contents set free by the loss of cell integrity could be seen (M).

Structural organization of the bacterial nucleoid:

Fig.1c illustrates the structural organization of the DNA-containing partial volume (nucleoid) in a typical eubacterium *(D. vulgaris)* in an ultrathin section prepared after cryo fixation. Fibrils and bundles thereof can be seen. A clear order cannot be detected. In Fig.1d I, structures similar to "beads on a string" are seen (Griffith, 1976). Griffith's data had been obtained when *E.coli* cells were disrupted on the electron microscopic support film by treatment with solvents partially dissolving the layers of the cell envelope. In experiments aimed at visualization of the structural organization of the nucleoid in the thiobacterium *Thiosphaera pantotropha* (Mayer and Friedrich, 1986), disruption of the cells was obtained by bacteriophage-induced lysis on the electron microscopic support film. This was a methode that was believed to be the most gentle one for experiments in which structural preservation of the cell contents was the goal. It turned out that the nucleoid is organized into complex, ordered bundles of twisted "beads on a string" substructures, resembling the "solenoid" structure, i.e. a higher complex of eukaryotic chromosome organization. There, histones are known to interact with the DNA. Histone-like proteins in prokaryotes are best known from archaea (Sandman, 1990). Though electron micrographs such as that in Figs. 1d II and d III exhibit considerable details, they do not reveal the intracellular organization of these solenoid-like structures in the bacterium. A better impression on this organization could now be obtained after induced lysis (Fig. 1e). Complexes very similar to those seen in Fig. 1d II *(Th.pantotropha)* are obviously also present in *E.coli.* In addition, it can be seen that these complexes are densely packed within the nucleoid region of the cell, forming parallel bundles consisting of "beads on a string" substructures. Hence, it may be concluded that prokaryotes, in general, have their chromosome organized this way. Even more difficult is it to understand how such a densely packed structured complex can manage to be replicated and transcribed. It has to be concluded that all these structures that can be depicted by electron microscopy are very highly dynamic, and that the states depicted in a sample will have changed within the next few seconds. Application of cryo electron microscopy may allow further analyses.

A "cytoskeletal web":

Indications have been obtained for the existence, in bacterial cells, not only of MreB- (actin-) based (Jones *et al.*, 2001) and FtsZ- (tubulin-)based (Erickson *et al.*, 1996) cytoskeletal elements, but also of an ubiquitously occurring bacterial cytoskeleton based on bacterial elongation factor Tu (EF-Tu) (Mayer *et al.*, 1998; Mayer, 2002; Mayer, 2003; Schwienhorst *et al.*, submitted). In *Mycoplasma pneumoniae*, a highly differentiated cytoskeleton has been described (Hegermann *et al.*, 2002) that cannot be assumed to be based on MreB-related proteins; genes coding for such kinds of proteins are missing in this parasitic wall-less bacterium (Regula *et al.*, 2000, Regula *et al.*, 2001). In coccoid

bacteria, MreB-coding genes are also absent (Jones *et al.*, 2001). Surprisingly, in low-temperature resin-embedded *E.coli* cells (Figs.2a I, II, III), repetitive substructures could be detected in close vicinity to the inner face of the cytoplasmic membrane, and all around the inner circumference of the cell. These kinds of structures would be expected when "stalks", as they have been described for *M. pneumoniae* (Hegermann *et al.*, 2002), also exist in *E.coli*. In *M.pneumoniae*, they were interpreted to function as "supports" for the cytoplasmic membrane, with their proximal ends inserted into the peripheral part of the cytoskeletal web. In older conventional ultrathin sections (e.g., Fig. 2a IV), these structures could not be detected. Nevertheless, also in Fig. 2 IV, the peripheral ribosomes can be seen in a certain distance from the inner face of the cytoplasmic membrane. As these stalks could be seen in Fig. 2 a all around the inner circumference of the cell, most probably they were not part of the MreB-based bacterial actin cytoskeleton (Jones *et al.*, 2001) which exhibits a shape of a helical band. These observations further support our view that the cytoplasm of bacteria is enclosed by a peripheral cytoskeletal web, organized similar to Fig. 2 b I to VI. In Fig. 2 c (I –VI), results are presented that were obtained when ultrathin sections of *Th.thermosaccharolyticum* cells were immuno labelled with the immuno gold system, with application of anti-EF-Tu antibodies (Mayer 2003). Distribution of the colloidal gold particles does not appear to be statistical as claimed earlier (Schilstra *et al.*, 1996); this is not only concluded from simple visual inspection, but also on the basis of respective calculations (Schwienhorst *et al.*, submitted). It should be noted that the huge majority of the EF-Tu proteins (as indicated by the positions of the gold particles) is, in fact, inserted into rows (shown in Figs. 2 c IV, V, and VI). This is remarkable because the number of copies of EF-Tu in an average *E.coli* cell is about 4 to 5 times higher than that needed to fulfil the function as an elongation factor. Up to now, no data are available explaining this huge surplus. It may be concluded that EF-Tu, in fact, also acts as the structural element of the cytoskeletal web as described above. This implies that EF-Tu may be an example for a bacterial protein with a double function.

Interactions between ribosomes/polysomes and the cytoskeletal web:

In Fig. 2 d I, an area depicted in Fig. 1 b is shown at higher magnification. The groups of arrows point to rows of particles of the size of a ribosome. These rows can be seen attached to the cell remnants marked "I" in Fig. 1b, and to cell remnants obviously devoid of cytoplasmic membrane and wall layers (marked "CB" in Fig. 1 b). I may be noted that these rows follow a basic helical pattern very similar to the structural organization of the peripheral cytoskeleton in *M. pneumoniae* (Figs. 2 b II, V, and VI). A conclusion could be that polysomes are attached to protofilaments of the cytoskeletal web. This would make sense because, according to our observations, these protofilaments contain EF-Tu. Hence, ribosomes/polysomes in the state of performing translation, may have to approach the EF-Tu "immobilized" in the protofilaments of the cytoskeletal web; the conventional view is that the – monomeric - EF-Tu approaches the ribosome. As shown above, most probably EF-Tu is not present in a monomeric state in the cell (s. Figs. 2 c I – VI). Therefore, the "grazing" ribosome might be a model worth to be further investigated (Mayer, 2003).

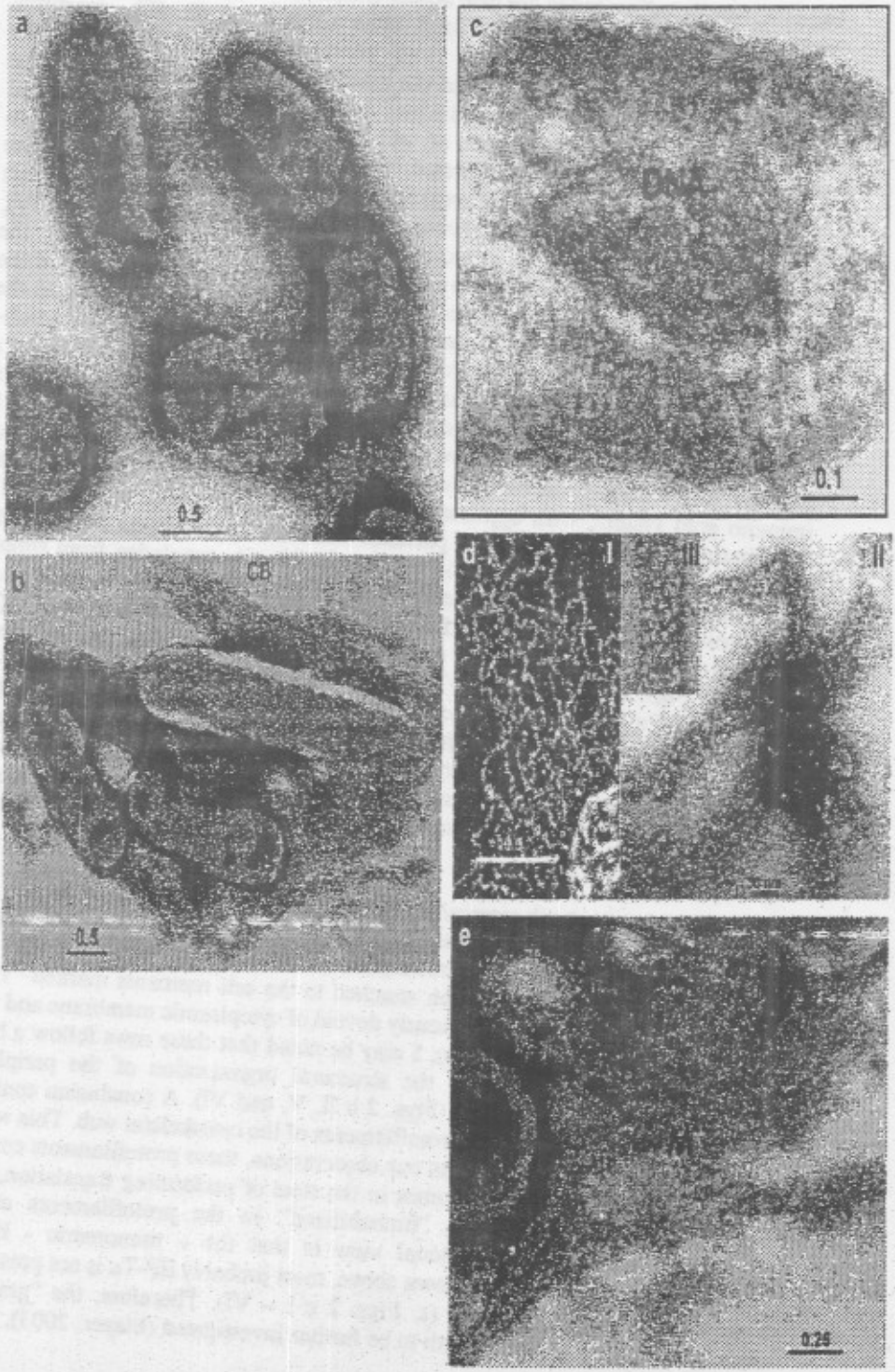
a
0.5
b
CB
0.5
c
DNA
0.1
d
I
III
II
e
M
0.25

Fig. (1): Bacterial cell integrity, and organization of the nucleoid:

a, *Escherichia coli* cells, without induction of cell lysis, exhibit typical structural features such as size, shape, and type I pili. The cell surface is artificially deformed by air drying. Negative staining. Dimension given in μm

b, *E.coli* cells, after induction of lysis (early state of lysis; compare with Fig.2f). Abbreviations: CB, cell remnant lacking wall and cytoplasmic membrane; nevertheless, it exhibits an elongated shape similar to a native cell; M, fibrillar cell contents set free by lysis; T, cell prior to loss of the wall; U, seemingly unaffected cells. Negative staining. Dimension given in μm

c to **e,** structural architecture of the bacterial nucleoid

c, nucleoid as it can be seen in a typical eubacterial cell (*Desulfovibrio vulgaris*) prepared for ultrathin sectioning by chemical fixation and low temperature embedding (Rohde *et al* 1990). Note the fibrillar substructure, interpreted to be caused by projections of DNA-containing fibers (the chromosome); no indications for the existence of DNA-protein complexes. Dimension given in μm.

d I, "beads on a string" fibrils set free from an *E.coli* cell, treated, on the electron microscopic support film, with solvents destabilizing the cell envelope. These structures were interpreted to be regularly-condensed chromatin-like fibers. Metal shadowing. Dimension given in μm.

d II, d III, "solenoid"-like complexes set free from a *Thiosphaera pantotropha* cell lyzed, on the electron microscopic support film, by lytic bacteriophages. These structures were interpreted to be complexes formed by "beads on a string"-like fibrils depicted in Fig. 1d I. Negative staining.

e, higher magnification of the area "M" in Fig.1b. Note that the solenoid-like complexes visualized in Fig.1 d II and III appear to have been densely packed in the interior of the cell prior to loss of the cell envelope. Dimension given in μm.

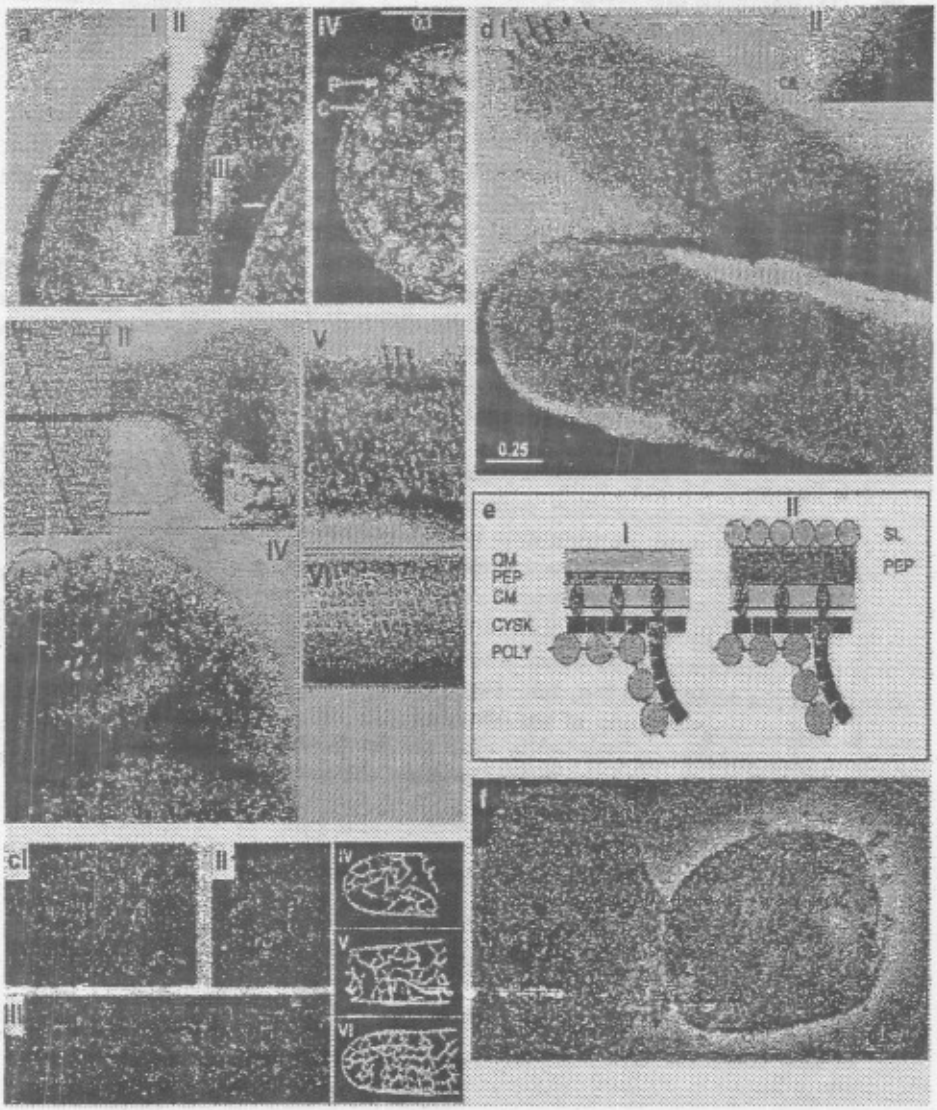

Fig. (2): The bacterial cytoskeletal web, and ribosome – cytoskeleton interactions:

a I to a III, ultrastructural details of the cell periphery of an ultrathin-sectioned *E.coli* cell, prepared by low temperature embedding and depicted at increasing magnifications. The arrows point to regularly spaced short fibrillar structures seemingly extending from the cytoplasmic membrane towards the interior of the cell. They are interpreted to be "stalks"; they are assumed to be attached to the peripheral part of the cytoskeletal web located, as a lining, close to the cytoplasmic face of the cytoplasmic membrane, extending outward into the cytoplasmic membrane, thus supporting and stabilizing the cytoplasmic membrane (Hegermann *et al.*, 2002; Mayer, 2003). Dimension given in μm.

a IV, ultrathin section of a *Thermoanaerobacterium thermosaccharolyticum* cell harvested from a continuous cell culture grown for production of amylase/pullulanase (s. Antranikian *et al.*,.1987). Conventional preparation for ultrathin sectioning. No indications for the presence of "stalks" as depicted in Fig.2a I-III. Arrow, ribosome; C, cytoplasmic membrane; P, peptidoglycan. Printed in reversed contrast. Dimension given in μm.

b I to b VI, cytoskeletal elements in *Mycoplasma pneumoniae*.

b I, diagrammatic view of the lattice forming the peripheral part of the cytoskeletal web.

b II, spherical part of a cell; the group of arrows points to a helical substructure (s. also Fig.2b V and VI). Negative staining. Magnification bar 0.1 μm.

b III, meshes of the peripheral part of the cytoskeletal web. Negative staining. Printed in reversed contrast. Magnification bar 10 nm.

b IV, meshes (circles, arrows, rectangle) and fibils (arrows and arrowheads) as cytoskeletal elements in cells; after chemical fixation with aldehyde, the cytoplasmic membrane had been removed by Triton X-100 treatment; thus, negative staining solution could easily penetrate into the cell interior, rendering internal cell structures visible (1, cytoskeletal web; 2, "rod"). Negative staining.

b V, higher magnification of a part of Fig.2b II.

b VI, simplified diagrammatic presentation of the tube-like helical lattice of the peripheral cytoskeletal web.

c I to c VI, results obtained by electron microscopic immuno labelling of ultrathin sections of *Thermoanaerobacterium thermosaccharolyticum* cells with anti-EF-Tu antibodies using the immunogold technique.

c I to c III, three ultrathin sections; the colloidal gold particles appear to be aligned in rows. Printed in reversed contrast.

c IV to c VI, drawings based on the sections depicted in I to III, illustrating the network formed by the rows ("cytoskeletal web").

d I, higher magnification of *E.coli* cell remnants "T" and "CB" depicted in Fig.1b. The groups of arrows point to rows of particles with a size similar to that of ribosomes. These rows follow a basic helical pattern with an angle similar to that seen in Fig.1b. Dimension given in μm.

d II, the basis of a type I pilus appears to be attached to the exposed peripheral part of the cytoskeletal web of cell remnant "CB" (lacking wall and the cytoplasmic membrane).

e, diagrammatic views of the cell periphery of a Gram-negative (I) and a Gram-positive (II) eubacterial cell.

Abbreviations: CM, cytoplasmic membrane; CYSK, cytoskeletal web; L, linker proteins; OM, outer membrane; PEP, peptidoglycan layer; POLY, polysomes; S, "stalks"; SL, surface layer.

f, *E.coli* cells in a late state of induced lysis. The interior organization appears to be completely lost; the cell at the right is still surrounded by an envelope however, indications for decomposition of the envelope are clearly visible; the cell at the left has lost its wall layers and probably is only enclosed by the cytoplasmic membrane; however, this membrane appears to be in a state of decomposition. Negative staining. Dimension given in μm.

Interactions between pilus insertion complexes and the cytoskeletal web:

In Figs. 2 d I and II, pilus structures can be seen even after loss of the cell envelope (compare with Fig.1 a). From Fig. 2 d II, it is obvious that the pilus insertion complex is still present though the cytoplasmic membrane has disappeared. This observation indicates that insertion of pili is not in the cytoplasmic membrane proper as assumed earlier (Mayer 1986). It may be tempting to speculate that also insertion of the complex at the basis of the bacterial flagellum might somehow be connected to the peripheral part of the cytoskeletal web.

Conclusions and perspectives:

New insights into intracellular organization of bacteria have been obtained by electron microscopic investigations of bacterial cells treated in the uncommon way of induced lysis. The final states of induced lysis (Fig. 2 f) were found to be preceded by states that allowed analysis of nearly native cytological situations inside the cell. These new observations may stimulate discussions on very basic aspects of cell biology of prokaryotes such as organization of the nucleoid, existence of cytoskeletons, especially of a cytoskeletal web, interactions of ribosomes with elements of the bacterial cytoskeleton, and implications for a better understanding of bacterial cell functions.

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متابعه التركيب الخلوي الداخلى المتأثر بنظام بكتيرى جديد يعمل كنظام مضاد للبكتيريا أو كنظام لإفراد المنتجات البكتيريه الهامه والمرغوبه فى الصناعات الغذائيه

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بدأ تحلل الخلية البكتيرية الذي يحفز بواسطة تكوين بروتين يعيق ثبات الهيكل الدعامى البكتيري (يعمل كمضاد للبكتريا وكنظام لافراد المنتجات الهامة للبكتريا) يسمح بالدراسة والتتبع بواسطة الميكروسكوب الإلكتروني للتركيب الخلوي الداخلي للـ *Escherichia coli* كنظام بكتيري. أمكن اكتشاف وجود جدائل ملتوية Twisted متوازية كثيفة التعبئة غالبا من معقد من الـ DNA الكروموزومي والبروتينات الرابطة للـ DNA . في المراحل الأولي للتحلل شوهدت معقدات مشابهة للبوليزوم Polysomes مرتبة في صفوف متوازية تتبع نظام حلزوني قريبة من الغشاء السيتوبلازمى. وجد ان قاعدة base زوائد الـ I pili غيرمنغمسة not inserted فى الغشاء السيتوبلازمى . كما ان هذه الزوائد وجد انها تتصل بالجزء المحيطى من الهيكل الدعامى الموجود بالقرب من الوجه الداخلي للغشاء السيتوبلازمى. كما تم مناقشة العلاقات المتداخله للفهم الافضل لاسس العلاقة بين التركيب والوظيفة فى البكتريا على المستوى الخلوى والجزيئات الكبرى macromolecules ولذلك فان نتائجنا لنظام التحلل تؤكد إمكانية استخدامه فى مجال تكنولجيا الأغذيه الحيويه والتخمرات الصناعيه لتسهيل عملية أل downstream processing للمنتجات البكتيرية الهامة فى الأغذيه كما يسمح بدراسة افضل للتركيب الخلوي الداخلي للبكتريا.