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A possible role for L24 of *Bacillus subtilis* in nucleoid organization and segregation

R. Exley[a]*, M. Zouine[a]*, J.-J. Pernelle[b], C. Beloin[a]**, F. Le Hégarat[a]***, A.M. Deneubourg[a]

[a]*Institut de Génétique et Microbiologie, Bâtiment 360, Université Paris-Sud, 91405 Orsay, France*
[b]*CEMAGREF, Institut de recherche pour l'ingénierie de l'agriculture et de l'environnement, Parc de Tourvoie, B.P. 44, 92163 Antony, France*

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Abstract — The condensation of DNA in bacterial nucleoids during cell cycle is a complex and dynamic process. Proteins displaying the physico-chemical properties of histones are known to contribute to this process. During a search for *B. subtilis* nucleoid associated proteins, HBsu and L24 were identified as the most abundant proteins in nucleoid containing fractions. Purified L24 binds and condenses DNA in vitro. In this paper we describe immunofluorescence studies that demonstrated that L24 is located at the poles of the nucleoids in exponentially growing cells. In contrast, the protein is dispersed in the cytoplasm during stationary phase. Moreover, overexpression of the *rplX* gene encoding L24 disrupts nucleoid segregation and positioning. © 2001 Société française de biochimie et biologie moléculaire / Éditions scientifiques et médicales Elsevier SAS

DNA condensation / L24 / nucleoid segregation

1. Introduction

Correct DNA packaging and condensation during the cell cycle is of paramount importance in all organisms. In eukaryotic organisms the chromosome is organized by histone proteins which compact the DNA [1]. The most dramatic alterations of chromosomal DNA are observed during mitosis, when the chromosomes are assembled and segregated into two daughter cells in a highly orchestrated fashion. DNA condensation plays a crucial role in this process and is necessary to ensure the correct partitioning of the chromosomes along the mitotic spindle. Specific proteins (other than histones) have been shown to be involved in these processes, such as the structural maintenance of chromosomes (SMC) family which are key regulators in both chromosome condensation [2] and sister chromatid cohesion during mitotic division [3].

In prokaryotes, DNA condensation and organization during the cell cycle is an equally crucial process, although the changes in DNA structure are less apparent than in eukaryotic organisms. The DNA must be correctly organized and condensed so as to be contained within the bacterial cell, whilst permitting vital cellular activities such as transcription-translation, repair-recombination and replication-segregation [4]. In order to allow these different processes to occur simultaneously the DNA must be maintained in a structure which is both fluid and dynamic.

A number of factors are believed to be involved in the organization of bacterial chromosomal DNA. Compaction and expansion forces, such as intracellular phase transitions and phase separation processes, result in compaction of the DNA within the nucleoid and formation of highly ordered assemblies which provide protection of vital macromolecules by structural sequestration [5, 6]. These compaction forces are believed to be counteracted by molecules of RNA polymerase which pull DNA via ribosomes and translocation complexes to the membrane [7]. Nucleoid associated proteins (NAPs) [8] also have a key role in nucleoid organization. These proteins possess similar physicochemical properties to eukaryotic and archaeal histones; they are small, generally basic, thermostable proteins which are abundant, have relatively non-specific DNA-binding properties, yet they do not package the DNA in the same manner as eukaryotic histones [9, 10]. In *E. coli* pleiotropic proteins such as HU, H-NS, IHF and Fis have important roles in maintaining nucleoid structure [11]. The genome of *Bacillus subtilis* lacks the genes for the abundant histone-like proteins H-NS, IHF and Fis found in *E. coli* [12]. In this organism, different proteins are implicated in chromosome structuring, such as HBsu [13], LrpC, recently shown to possess DNA architectural properties (C. Beloin, in preparation) and also the ribosomal protein L24. This latter 103 amino acid basic protein of 12kDa was originally identified in isolated exponential phase nucleoids [14]. It binds linear and

* These authors contributed equally to this work.

** Present address: Department of Microbiology, Moyne Institute of Preventive Medecine, Trinity College, University of Dublin, Dublin 2, Ireland.

*** *Correspondence and reprints.*
E-mail address: lehegarat@igmors.u-psud.fr (F. Le Hégarat).

supercoiled DNA and condenses it in vitro and therefore is described as both a ribosomal protein and a nucleoid associated protein of *Bacillus subtilis* [15, 16].

Localization of proteins within the bacterial cell has contributed greatly to the understanding of protein function during the cell cycle. Cytological developments and novel image technology have provided new information concerning the bacterial chromosome and events during cell division. Nucleoid associated proteins such as HBsu have been localized to the nucleoid by GFP fusion [17]. The chromosome (of *Bacillus subtilis*) itself has been shown to have a specific orientation within the cell during the cell cycle [18]. The replication origin regions remain anchored in defined positions during cell elongation, then abruptly move to opposite ends of the nucleoids, in a precise manner, reminiscent of movement of eukaryotic chromosomes along the mitotic spindle [19]. Furthermore, proteins such as SpoOJ and SMC have been shown to have important roles in chromosome segregation during vegetative cell division in *Bacillus subtilis*, after they were co-localized with the origin regions towards the cell poles (for review see [20]).

Here we investigate the spatio-temporal localization of the L24 protein throughout the different growth phases, by immunofluorescence microscopy. We show that L24 has growth-phase dependent localization, capping the nucleoids in exponential phase and displaying a more uniform cellular distribution in stationary phase. In parallel, fluorescence in situ hybridisation (FISH) using a probe against ribosomal rRNA showed a colocalization of L24 with 23S rRNA. Overproduction of L24 in *Bacillus subtilis* affects nucleoid condensation and segregation. We discuss the correlation between L24 localization and its known functions.

2. Materials and methods

2.1. Bacterial strains and media

The strains of *Bacillus subtilis* used in this study are isogenic to *B. subtilis* 168. Strains were grown in LB (Luria-Bertani) rich medium supplemented with 50 μg/mL ampicillin and 10 μg/mL kanamycin where relevant.

2.2. Proteins and antibodies

L24 protein from *B. subtilis* was purified according to [16]. A rabbit polyclonal anti-L24 serum was affinity purified on HiTrap NHS-activated column from Pharmacia Biotech.

2.3. Immunofluorescence microscopy

Immunofluorescence staining was performed by a modification of methods described in [21]. Fixation and permeabilization of cells: 0.5 mL culture in LB medium was added to fixative solution giving a final concentration of 2.5% (v/v) paraformaldehyde and 30 mM sodium phosphate buffer, pH 7.5. Bacteria were fixed for 15 min at room temperature and 30 min on ice, washed three times in PBS, treated with lysozyme (2 mg/mL) for 2 min and affixed to poly-L-lysine treated multiwell slides (ICN Biochemicals). Immunofluorescence staining of cells: Cells were incubated with a 1:100 dilution in BSA-PBS of affinity purified rabbit polyclonal anti-L24 overnight at 4 °C. Secondary antibodies, goat Anti-IgG (H+L) rabbit-FITC (fluorescein isothiocyanate, BIO-SYS) at a dilution 1:50 were added in PBS containing 0.1 μg/mL DAPI (4',6-diaminidine-2' phenylindole dihydrochloride). Coverslips were mounted with Antifade Citifluor glycerol AF1.

2.4. Microscopy and photography

All photographs were taken using a Zeiss Axiophot microscope equipped with a 100x UPlanFluor objective. A 1.25 Optovar was used in all cases. Two filter sets were used, one for visualizing FITC(09-Zeiss) with a wide band-pass (490–525) and one for visualizing DAPI (01 Zeiss) with a wide band-pass (345–425). Fluorescent photographs were obtained with Fujicolor (ASA 1600).

2.5. Fluorescence in situ hybridisation (FISH)

Cells were grown in LB medium at 37 °C. At optical density (600 nm) of 0.5 and 1.5, 10 mL samples were fixed and permeabilized as described for immunofluorescence procedures. Ten microlitres of a suspension of approximately 10^8 bacteria/mL were affixed to gelatine-treated, multiwell slides (Polylabo). Slides were placed at 46 °C for 10 min then dehydrated in successive ethanol baths: 50%, 80%, 96% for 3 min each. After air-drying, the slides were hybridized at 46 °C for 90 min in a humid atmosphere with the fluorescent probe in hybridization buffer containing 20% formamide, 900 mM NaCl, 20 mM Tris-HCl, pH 7.2, SDS 0.01%. The 16S and 23S probes (Genset) are described in [22] and [23] respectively and were used at a final concentration of 50 ng/μL. After hybridization, the slides were washed with 2 mL of wash buffer containing 180 mM NaCl, 20 mM Tris, pH 7.2, 5 mM EDTA, 0.01% SDS, pre-warmed to 48 °C, then incubated in 50 mL of the same wash buffer for 10 min at 48 °C. Slides were rinsed with distilled water and air-dried. Coverslips were mounted with Antifade Citifluor glycerol AF1. Photographs were taken using a Zeiss Axiophot microscope equipped with a 100x UPlanFluor objective. A 1.25 Optovar and filter (Zeiss) with a wide band pass (540–580) were used for visualizing Cy-3. Fluorescent photographs were obtained with Fujicolor (ASA 1600).

2.6. Construction of the BMZ301 plasmid

The *rplX* gene encoding L24 of *B. subtilis* was amplified by PCR using oligonucleotides containing artificial *Hin*dIII cloning sites (in bold). The sequences of these primers were as follows: N-terminal oligonucleotide: AAAT**AAGCTT**CTCAAGGAGGTGCGATCAGG (RBS underlined); and a C-terminal oligonucleotide: CCTT**AAGCTT**CTATTTATCTAGAACTTGCC. The product of amplification was purified and digested by *Hin*dIII before cloning into pDG1832, linearized with the same enzyme. The resulting plasmid, pMZ101 (pDG1832*rplX*), containing the *rplX* gene under the control of the *xylA* promoter was linearized by *Eco*RI and ligated with the replicative *B. subtilis* pUB110 plasmid, linearized by *Eco*RI. The resulting shuttle plasmid pMZ301 was selected by the ability to confer resistance to kanamycin (from pUB110 plasmid) and ampicillin (from pDG1832 plasmid).

3. Results

3.1. Localization of L24 is growth phase-dependent

At different stages of growth in rich medium, *B. subtilis* cells were examined by epifluorescence microscopy for the in situ localization of L24. In situ immunodetection was performed using affinity purified primary polyclonal antibodies against L24 and secondary antibodies coupled to fluorophore FITC, as described in *2.Materials and methods*. DAPI staining was used to observe nucleoids. The results are presented in *figure 1A*. During exponential phase of growth *(figure A1)*, fluorescence indicating the presence of L24, was observed mainly at the poles of the cells as intensely labeled regions (*figure A1*, a, d) which appear to cap the nucleoids stained with DAPI (*figure A1*, b, e) as shown in *figure A1*, (overlay, c, f). In cells reaching the stationary phase, fluorescence was more uniformly dispersed (*figure A2*, a-c) and appeared weaker in agreement with Western blot analysis of the quantity of L24 throughout the cell cycle (data not shown) which indicated a substantial decrease in the quantity of L24 upon transition into stationary phase.

3.2. L24 colocalizes with the ribosomal RNAs

Fluorescence in situ hybridisation was performed using a fluorescent probe against the 23S and 16S rRNA in order to compare the cellular localization of the ribosomal RNA with that of L24. Cells were observed in both exponential and stationary phase and are shown in *figure 1B*. In exponential phase *(figure 1, B1)*, the labeling is heterogeneous, with maximum fluorescence observed at the cell poles, implying that the majority of the 23S rRNA molecules are localized within this region. The localization of L24, which binds nascent 23S rRNA, is coherent with these results suggesting that, in exponential phase, the ribosomal 50S subunits localize at the cell poles, around the polar nucleoid origin regions. In stationary phase *(figure 1, B2)* the bipolar localization was less evident and fluorescence was more uniformly distributed throughout the cell although some regions of more intense polar fluorescence could be observed.

3.3. Overexpression of L24 affects cell division and nucleoid segregation

Several attempts to interrupt the *rplX* gene encoding L24 were unsuccessful. We therefore investigated the effect of overexpression of this gene on *B. subtilis* cells and particularly on nucleoids. The *rplX* gene was cloned in a multicopy plasmid under the control of the P*xyl* promoter which is induced by xylose and repressed by glucose. The resulting recombinant plasmid, pMZ301, was introduced into the wild type *B. subtilis* strain 168. Growth of this L24-overproducing strain (BMZ301) was followed in non-induced and xylose-induced conditions. We noted that the induced cells reached a maximum optical density of 1.2 absorbance at 600 nm whereas the non-induced culture reached around 2.4 *(figure 2A)*. Western blot verification of the L24 overproduction, using affinity purified antibody against this protein, indicated that after 2 h of xylose induction, the quantity of the recombinant protein increases approximately five times (data not shown). Light microscopy studies were performed to investigate whether high levels of the *B. subtilis* L24 protein could alter cell morphology and viability of the overproducing strain. Cells overproducing L24 were also examined by fluorescence microscopy after staining with DAPI. The micrographs in *figure 2B* revealed that 20% of overproducing cells examined formed filaments characteristic of defects in cell division. Very long filaments containing many nucleoids could be observed soon after induction of L24 overexpression (30 min after addition of xylose). As L24 overexpression increases (1 and 2 h after xylose induction) the cells form filaments with some regions rich in DNA whilst in other regions DNA is not detected. In contrast, in both controls, (i.e., the wild type strain in the presence of xylose and the overproducing strain BMZ301, in the absence of xylose) where the quantity of L24, verified by Western blot, was the same as in the wild type, cells did not filament and their nucleoids had wild type appearance (data not shown).

4. Discussion

The present study was carried out to investigate the cellular localization of the bifunctional protein L24 from *Bacillus subtilis* in exponential and stationary phase. This

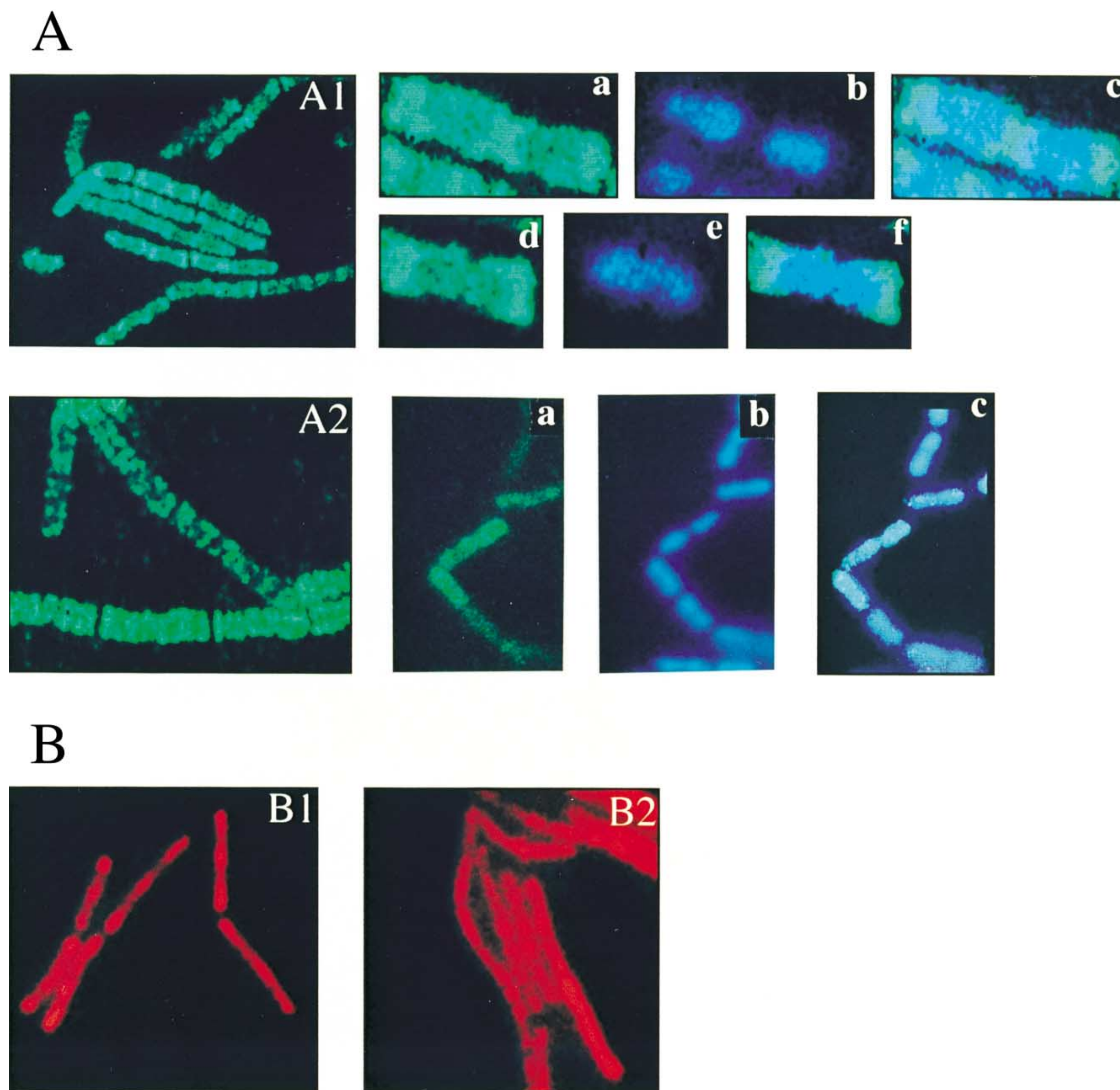
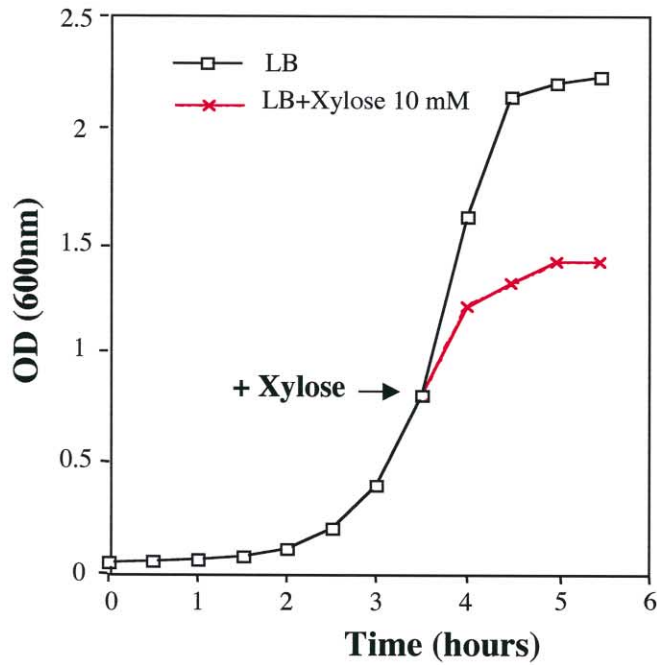


Figure 1. **A.** Subcellular localization of L24 and DAPI staining of nucleoids in *B. subtilis* 168 at two different stages of growth. Cells harvested at various times of growth were fixed, stained and photographed as described in *2. Materials and methods*. **(A1).** Cells in mid-exponential growth phase (OD of 0.6) showing the distribution of L24 at the cell poles **(a, d)** and between nucleoids stained with DAPI (**b, e**). The FITC and DAPI images are overlaid in (**c, f**). (**A2**). Cells in stationary phase (OD of 2.4) showing the L24 protein dispersed throughout the cell (**a**), DAPI stained nucleoids **(b)** and overlaid image **(c)** (magnification is approximately 1600). **B.** Fluorescence in situ hybridisation. Probes hybridising to the 23S and 16S rRNAs were used to detect the localization of ribosomal RNA in **(B1)** exponential and **(B2)** stationary phase cells of *B. subtilis.* Bacteria were fixed and hybridised as described in *2. Materials and methods*.

A



B

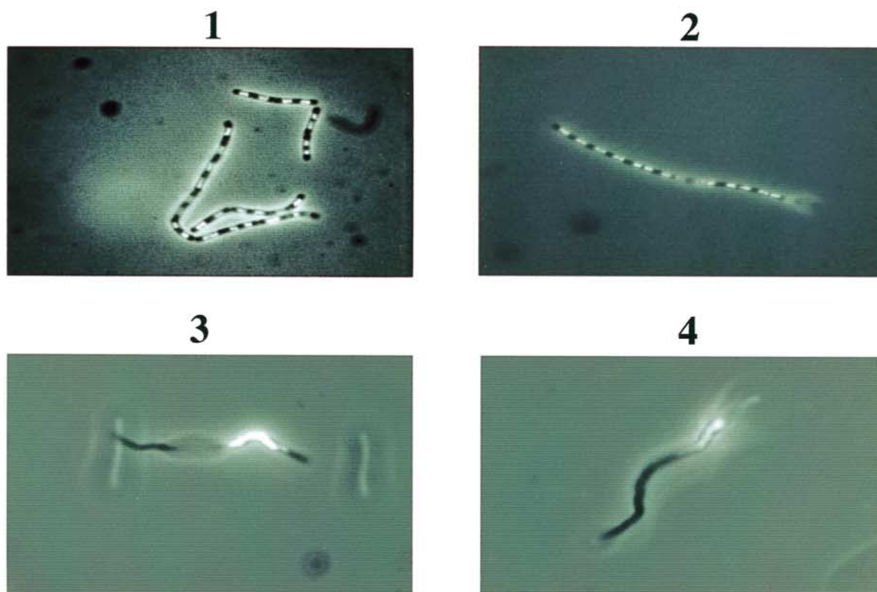


Figure 2. Effect of overproduction of L24 on *B. subtilis* cells. An overnight preculture of the BMZ301 strain was diluted in LB medium to OD (600 nm) of 0.05. Growth at 37 °C was monitored by measurement of OD 600 nm **(A)**. The overexpression was induced at an optical density of 0.5 by addition of 10 mM xylose (final concentration). As a control, a second BMZ301 culture was grown without induction by xylose. **B1-4.** Light micrographs of *B. subtilis* overproducing L24 stained by DAPI. Cells were harvested before xylose induction (1) and 0.5 h (2), 1 h (3) and 2 h (4) after induction.

protein has been previously described as both a ribosomal protein and nucleoid associated protein [16]. We found that, in vivo, during the exponential phase of growth, L24 is located predominantly at the poles of the nucleoids, whereas in stationary phase the protein is more evenly distributed throughout the cell. Several interpretations are possible considering the bifunctional nature of the protein.

L24 is implicated in ribosomal assembly by analogy to L24 of *E. coli* which binds to the 23S rRNA resulting in formation of a distinct nucleus on the filamentous rRNA, modifying its conformation and stimulating assembly of the 50S subunit [24]. Once the ribosome assembly is complete, the L24 protein is not involved in translation per se. Recent studies in *B. subtilis*, using an S2-GFP fusion protein, have shown that the ribosomes are localized predominantly at the cell poles during exponential phase and distributed throughout the cell in stationary phase [25]. FISH experiments performed in this study showed that L24 colocalizes with ribosomal RNA in *B. subtilis*. This is consistent with the idea that L24 of *B. subtilis* is analogous to L24 of *E. coli* and binds to the 23S rRNA during ribosome biogenesis. These data therefore suggest that the observed localization of L24 is a result of the ribosomal function of the protein.

Alternatively, the specific localization of L24 could be simply a result of gene positioning as opposed to specific recruitment of the protein to a particular site within the cell. The genes of the translational apparatus, including *rplX* which encodes L24 and the ribosomal RNA operons, are close to the *oriC* region [12]. Consequently the L24 protein would be synthesized within this region and during rapid growth, where transcription and translation are at maximal rates, an accumulation of L24 at its site of production could occur. Moreover, we observed regions of intense fluorescence at the cell poles using a probe against ribosomal RNA. This probably corresponds to both nascent and ribosome-incorporated rRNA. Therefore this region, containing DNA, RNA and proteins undergoing replication, transcription and translation, would be a locus of intense activity and could be compared to the nucleolus of eukaryotes [25]. In slower growth, or stationary phase the metabolic activity of the cell is reduced so the intense fluorescent foci corresponding to high, local concentrations of L24 or rRNA are no longer observed.

L24 could also localize at the cell poles as a function of its DNA organizing properties. This protein has previously been identified as a nucleoid associated protein by virtue of its DNA-binding and organizing properties [15]. Most proteins implicated in DNA condensation and chromosome organization, such as H-NS and HU of *E. coli* or HBsu of *B. subtilis* are localized within the nucleoid however the majority of L24 is found at the nucleoid poles. This type of localization has been observed primarily for proteins which are involved specifically in chromosome segregation and partitioning, such as the centromere-like protein SpoOJ which binds to a large (> 150 kb) chromosome region situated > 100 kbp from the origin *oriC* [26, 27], Topoisomerase IV, an enzyme responsible for decatenation of replicating chromosomes [28] and the motor protein SMC which is required to maintain correct layout of the chromosome in the cell and for chromosome condensation and segregation [29, 30]. Therefore L24 could have a role in organization of DNA during the segregation process. The real boundary between nucleoid and cytoplasm is indefinable as these proteins sequester the origin region, possibly in a type of condensation complex. In such a situation, with a low resolution light microscope, it is impossible to distinguish the precise localization of L24 which could have multiple functions within a confined region.

In support of this latter hypothesis, our results show that overproduction of L24 in *B. subtilis* leads to abnormalities in nucleoid segregation, supporting the idea that L24 could be involved in DNA organization during chromosome segregation. We propose that the RNA nucleation property of L24 could be exploited to condense and ensure the correct folding of the nucleic acids around origin regions in exponential phase of growth. Overexpression of L24 would result in an excess of condensation of DNA around the origin, and therefore prevent appropriate segregation, as indeed was observed. When cells stop dividing and reach stationary phase, L24 is no longer required at cell poles as there is a decrease in chromosome replication. L24 may dissociate from the origins, by decrease of its DNA and RNA-binding capacity, perhaps due to maturations such as phosphorylation, proteolysis etc. In this situation L24 is dispersed throughout the cells, primarily as a component of ribosomes which, during stationary phase, are uniformly distributed throughout the cell [25]. The depletion in L24 in the transition between exponential growth phase and stationary phase, as seen by immunofluorescence images and confirmed by immunodetection (data not shown), could be explained by a decrease in the number of ribosomes at this time, degradation of L24 or a change in accessibility of antibodies to the L24 protein.

Condensation appears to be a primary event in order to actively segregate chromosomes. This is well known for eukaryotic cells and more recently appears to be true of prokaryotic systems. We hypothesize that the physiological role of L24 as a ribosomal nucleoid associated DNA-binding protein in *B. subtilis* could be to make a link between the DNA segregation apparatus and the cytoplasmic translational apparatus, in other words between active chromosome separation and cytodieresis.

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