Histol Histopathol (2013) 28: 1351-1360
DOI: 10.14670/HH-28.1351

http://www.hh.um.es

Histology and Histopathology
Cellular and Molecular Biology

Histological approach to *Bacillus subtilis* colony-biofilm: evolving internal architecture and sporulation dynamics

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Summary. *Bacillus subtilis* has been used as a classic model to study biofilm formation and sporulation process. Colonies of wild-type strains usually have a complex external morphology, but the details of their internal architecture are still undisclosed. Since bacterial biofilms fulfill the criteria to be considered tissues, the aim of this work was to analyse *B. subtilis* colony-biofilm internal architecture evolution and sporulation dynamics using histological techniques. Transversal sections of colony-biofilms incubated from 24 hours up to 20 days were stained using histochemical techniques to analyse the internal structure by light and electron microscopy. A morphometric study of the different structural biofilm components was performed by image analysis, and an application to quantify spores was developed. Internal biofilm architecture was characterised by a stratified pattern, which evolved from 3 strata at 24 hours, up to 5 strata at 20 days. At 48 hours, strata at the central area of the biofilm was folded, resulting in elevated structures (vein-like structures) that could reach up to 465 μm in height. Sporulation started at 48 hours, at the top of the vein-like structures, at the interface between the two uppermost strata. At 20 days spores formed a continuous central layer, representing 7.5% of the total biofilm. In summary, our results demonstrate that *B. subtilis* colony-biofilm has a complex and organized internal architecture, evolving over time, and taking place in different cell subpopulations with different functionalities. Furthermore, *in situ* spore quantification described in this work could be a good alternative to the classical chamber counting.

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Key words: *Bacillus subtilis*, Biofilm, Image analysis, Light microscopy, Electron microscopy

Introduction

Bacterial cells in their natural environments are not asocial but can exist as communities with complex organisation and exhibit sophisticated and highly regulated collective behaviour, using advanced communication (Shapiro, 1998; Cho et al., 2007; Ben-Jacob, 2008; Dubey and Ben-Yehuda, 2011). Biofilms are one kind of these multicellular communities that have been involved in a large number of ecological and biotechnological processes (Davey and O'Toole, 2000; Watnick and Kolter, 2000; Kolter and Greenberg, 2006; Veening et al., 2006) and also in human infections (Parsek and Singh, 2003; Hall-Stoodley et al., 2004; López et al., 2010). Therefore, it is important to search for a deeper understanding on the formation, development and evolution of these structures.

Bacillus subtilis is perhaps the best characterised Gram-positive sporulated rod, important for the environment, medicine and industry, as its biochemistry, physiology and genetics have been studied intensely for more than forty years (Kunst et al., 1997). It has also been used as a classic model for molecular study of biofilm formation (Lemon et al., 2008; Bridier et al., 2011) and sporulation dynamics (Yudkin and Clarkson, 2005).

Classically, colony-biofilms from *B. subtilis* wild-type strains cultured on solid agar show a complex external architecture, characterised by a central area with elevated structures which have been named by different authors as fruiting bodies, beanstalk-like, vein-like, or tongue-like structures (Branda et al., 2001; Veening et

al., 2006; Bridier et al., 2011), surrounded by an homogeneous ring. However, biofilm internal architecture has not yet been completely revealed, despite having been described in three-dimensional models by means of confocal microscopy (Bridier et al., 2011) and various cellular subpopulations with different functions and the corresponding gene expression having been deciphered (Vlamakis et al., 2008).

Recently, our group explored the use of histological methods to study morphologically simple bacterial colonies (circular, convex, and smooth surface), particularly those from *Staphylococcus aureus* and *Escherichia coli* species, permitting the observation of their internal structure (Gómez-Aguado et al., 2011). The aim of this work was the application of such methodology supplemented with image analysis, to study a more complex *B. subtilis* colony-biofilm, in order to explain its internal architecture, its evolution over time, and the spatial-temporal dynamics of sporulation.

Materials and methods

Strains, media and culture conditions

For biofilm study, the *Bacillus subtilis* collection strain ATCC 6633 was used. Five μL spots from a 0.2 OD suspension were inoculated in Columbia Blood Agar (BioMérieux, France) and incubated at 35°C during 24, 48 and 72 hours at constant humidity conditions to avoid desiccation of culture medium. Additionally, a group of the 72 h incubation plates were kept at room humidity and temperature for up to 20 days, to facilitate sporulation.

Morphological study

After each incubation period, 10 colony-biofilm were fixed with 4% formaldehyde for 24 h and embedded in paraffin using an automated vacuum tissue processor LEICA TP 1050, following the histological conventional protocol for biopsy specimens. Transverse thin biofilm sections (4 μm) were processed with histochemical staining procedures such as Hematoxylin-eosin, Giemsa, PAS-alcian blue, and Gram (Luna, 1968), and Wirtz-Conklin stain (Hamouda et al., 2002) modified for spore visualisation. Specifically, this staining procedure was done as follows: the sections were introduced in a pre-heated at 95°C 5% brilliant green aqueous solution (Panreac Química S.A.U., Spain) for 5 minutes, followed by cooling down at room temperature and posterior washing under running tap water. Later, sections were introduced in 0.25% safranine O solution (Panreac Química S.A.U., Spain), for 2 minutes at room temperature, then washed with running tap water for 2 minutes, dried in an oven at 40°C and mounted with Eukit (Thermo Shandon Ltd., UK) for light microscopy observation (Leica DM5000B).

From each batch, another 10 colony-biofilm were embedded in epoxy resin blocks following a recently described methodology (Gómez-Aguado et al., 2011). In brief, agar blocks containing colony-biofilm were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4), dehydrated in increasing gradation alcohols and embedded in epoxy resin (Eponate 12, Ted Pella Inc., CA., USA).

Transverse semithin biofilm sections (0.5 μm) were stained with 1% toluidine blue and with the modified Wirtz-Conklin stain for spores, described above.
Transverse ultrathin sections (50 nm) were stained with 1% lead citrate and observed by transmission electron microscopy (Hitachi 7000).

Image analysis

Morphometric biofilm analysis and spore quantification were done by use of Leica QWin image analysis software. Macroscopic images to determine diameter and area of colony-biofilm on culture plates were taken with a conventional digital camera. Biofilm height and thickness of the different components of their internal structure were quantified by means of digitized images of semithin sections coloured with toluidine blue taken with a digital camera, Leica DC 300, coupled to a light microscope Leica DM 5000B, provided with a motorised plate. Sections were representative of the central biofilm area from specimens embedded in epoxy resin, to avoid measurement errors due to possible artefacts when handling specimens embedded in paraffin. Specifically, on each section 10 different measures of the thickness of the different components of the biofilm internal structure were performed, on areas nearer to the growth medium which corresponded to the valleys of the vein-like structures. Similarly, 10 measurements were taken for the distance between the agar line and the outermost point of the biofilm surface, which in those biofilm of 48-72 h coincided with the crests of the vein-like structures. For each incubation period 10 sections where analysed, corresponding to the same number of colony-biofilms, and the mean value for those 10 measurements was considered the thickness for each architectural component and the biofilm height. Statistical package SPSS 11 was used for statistical analyses of biofilm height. The one-way analysis of variance (ANOVA) test was used for multiple comparisons. Significance was taken as $p<0.05$.

Sporulation was quantified on semithin transverse sections, representative of the central biofilm area, stained with Wirtz-Conklin modified technique. The complete length of each section was digitized by means of sequential images taken with objective 20 X. For each image, in the first instance, green structures corresponding to spores were segmented by using the image analysis software function "colour detection". The second step corresponded to the measurement of the area occupied by the green structures, using the function "measure field" in μm^2 (S_i). Finally, the whole colony

area was segmented and measured (C_i). For each colony-biofilm the relative area occupied by spores was calculated by the following formula:

$$A_R = \frac{\sum_{i=1}^{n} S_i}{\sum_{i=1}^{n} C_i} \times 100$$

"A_R" being the relative area (as percentage) occupied by spores and "n" the number of images in which each section was divided. With the values from the 10 colony-biofilm measured for each incubation time, mean, standard deviation (SD) and variation coefficient (VC) were calculated using the statistical package SPSS 11.

Results

Colony-biofilm external architecture

Macroscopic aspect and colony size showed variations according to the incubation period. After 24 h, colony-biofilm appeared white and opaque, with wavy borders, nearly circular in shape, with a denser central core (Fig. 1A).

After 48 and 72 h, colonies maintained the wavy borders and the central core had a convoluted aspect produced by the presence of vein-like structures, surrounded by one homogeneous band (Fig. 1B,C). After 20 days, colonies showed an aged aspect, with regular reinforced borders, eccentric core with less relief, lined by two bands, the inner being clear and homogeneous and the outer more dense (Fig. 1D).

Table 1 shows maximum diameter and area measurement values for both complete colony-biofilm structure and central core, at the different incubation times.

Colony-biofilm internal architecture

Light and electron microscopy images of transverse sections from the central colony-biofilm area showed definite architectural patterns, which evolved over time and were stratified. Specifically, 24 h colony-biofilm consisted of three strata (Figs. 2A, 3A): 1) the basal stratum, directly in contact with the agar, with cocci and coccobacilar Grampositive structures, without specific spatial organisation; 2) the clear stratum, intermediate, Gramnegative, containing dead bacilli and bacterial debris scarcely degraded, as confirmed by electron microscopy; and 3) the superficial stratum, Grampositive, with a slightly wavy surface, containing cords of bacilli of normal morphology, disposed at different spatial directions. Relative thickness values for each stratum are shown on Table 2.

Colony-biofilms of 48 and 72 h appear to have similar internal structure, with the presence of four strata (Figs. 2B,C, 3B,C): 1) the basal stratum with similar characteristics to those observed at 24 h, but with less bacterial density (Fig. 4A); 2) the waxy stratum, Gramnegative, formed by extracellular matrix and bacterial debris completely degraded, showing an homogeneous aspect (Fig. 4A); this layer is stained with Eosin (Fig. 5A) and is PAS-alcian blue positive (Fig. 5B), indicative of absence of nucleic acids and the presence of glycoproteins and neutral mucopolysaccharides; 3) the clear stratum, similar to that described for 24 h colony-biofilms, in which could be observed a predominance of empty bacterial walls which remember "cell ghosts" (Fig. 4B), but nucleic acids have not been degraded yet, and for that reason this layer was stained blue with Hematoxylin (Fig. 5A); 4) the superficial stratum, formed by bacilli with normal

Table 1. *B. subtilis* colony-biofilm external morphometry.

Incubation time	Maximum colony -biofilm Ø (mm)	Colony-biofilm area (mm^2)	Maximum core Ø (mm)	Core area (mm^2)	Core relative area (%)
24h	5.1±0.5	18.9±2.5	1.7±0.2	2.2±0.2	11.8±2.2
48h	6.2±0.3	28.1±2.9	3.6±0.3	10.4±1.8	37.1±5.1
72h	7.7±0.7	41.5±4.1	3.7±0.3	10.9±1.9	26.4±2.9
20d	8.4±0.7	55.5±3.1	3.0±0.5	6.9±1.9	12.3±2.7

Values expressed as mean ± standard deviation for the ten colony-biofilm analysed at each incubation time.

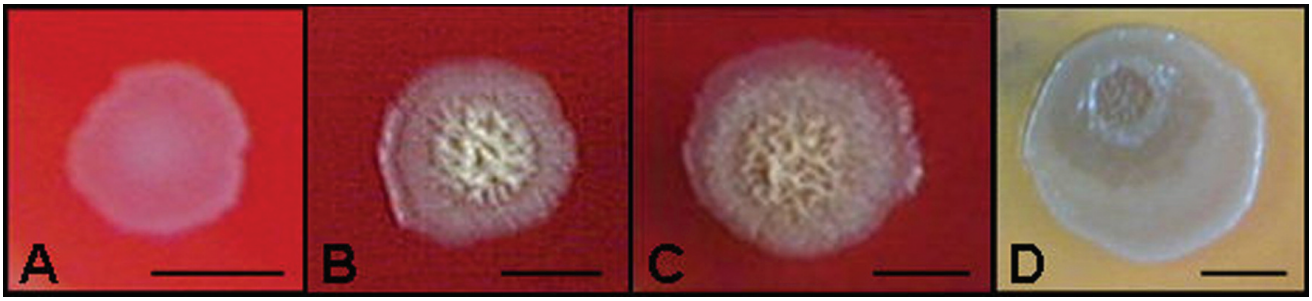

Fig. 1. Macroscopic images of *B. subtilis* colony-biofilm at different incubation times. **A.** 24 hours at 35°C. **B.** 48 hours at 35°C. **C.** 72 hours at 35°C. **D.** 3 days at 35°C and 17 days at room humidity and temperature. Scale bar: 3 mm.

morphology (Fig. 4C), disposed according to a population density gradient, the latter being greater on the inner side and lesser on the outside. Excepting the basal stratum, which was adherent to the agar surface, all the strata formed folds and loops, producing the vein-like structures which give a convoluted aspect to the colony-biofilm central core (Fig. 2B,C). This phenomenon was more intense on 72 h colony-biofilm, with higher structures (up to 465 μm), folds and loops with greater complexity. Inside some vein-like formations an homogeneous PAS-alcian blue positive matrix was observed (Fig. 5B). Figure 6 compares height of vein-like structures at different incubation periods. The ANOVA test showed statistically significant difference in the biofilm height at different incubation times ($p<0.01$). Relative thickness values for each stratum are shown in Table 2.

Colonies maintained for 20 days at room humidity and temperature had flattened and the characteristic folds and loops on 48 and 72 h colony-biofilm had disappeared, although the wavy surface and the stratified structure persisted (Fig. 2D). Specifically, five strata could be identified, a new stratum appearing beneath the superficial, consisting exclusively of spores, as can be observed on the sections stained by the modified Wirtz-Conklin method (Fig. 7D). The other four strata correspond to those described on 48 and 72 h colony-biofilm, but with different composition, and all of them are Gramnegative (Fig. 3D). The basal, clear and superficial strata had the same microscopic aspect, being formed by "cell ghosts" and bacterial debris, more or less degraded (Fig. 4D). The waxy stratum showed the same characteristics as those described for 48 and 72 h colony-biofilm.

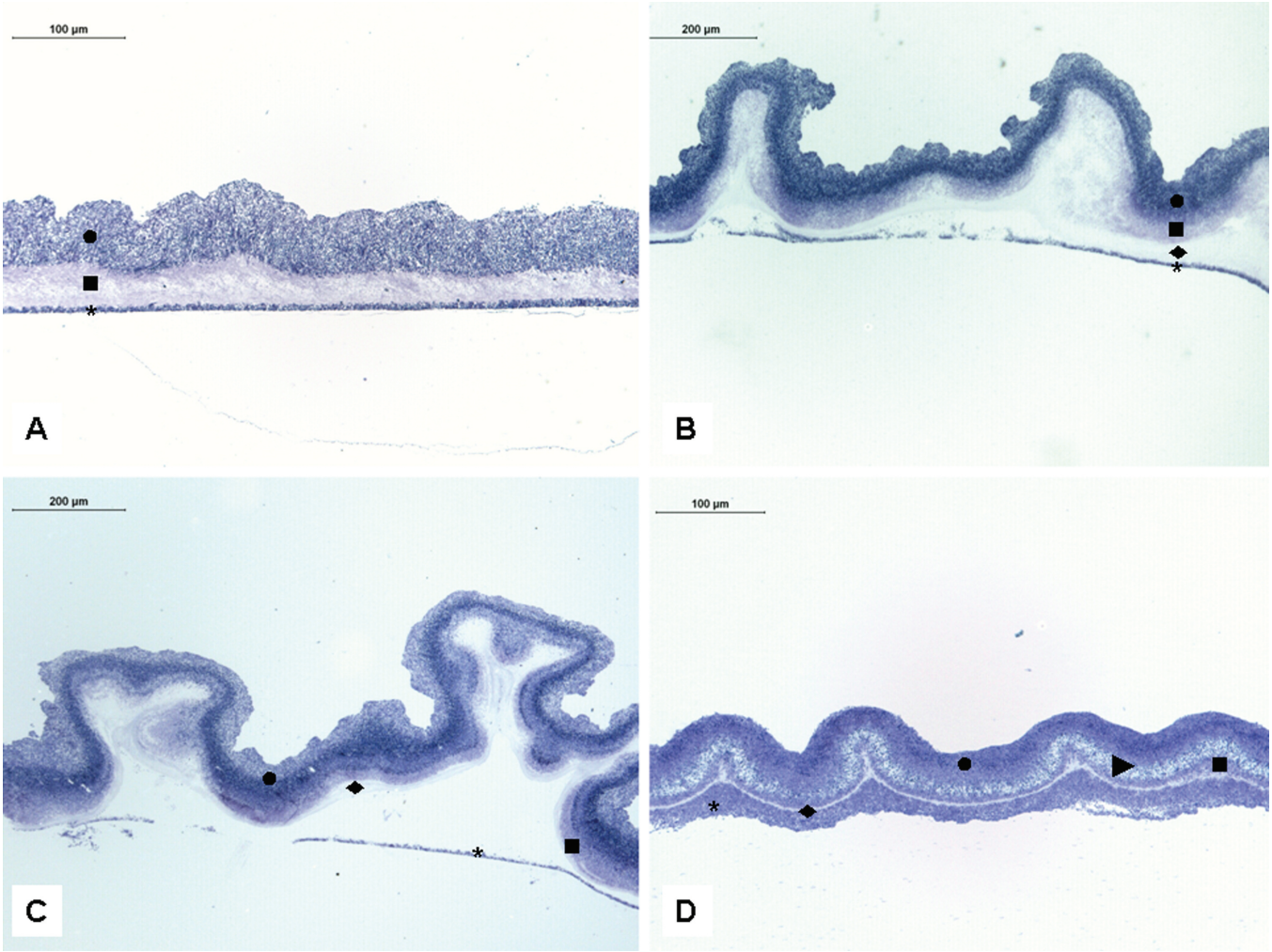

Fig. 2. Microscopic images of *B. subtilis* biofilm semithin sections stained with toluidine blue at different incubation times. The architectural patterns observed are stratified, evolving in time from three strata at 24 h (**A**), four strata at 48 and 72 h (**B and C**), and five strata at 20 days (**D**). ★: basal stratum; ♦: waxy stratum; ■: clear stratum; ►: spore stratum; ●: superficial stratum.

Sporulation dynamics

Analysis of sections stained by the Wirtz-Conklin modified technique demonstrated sporulation mainly at the interface between the superficial and clear strata (Fig. 7). No spores were observed in any of the three

Table 2. *B. subtilis* colony-biofilm internal morphometry.

Stratum	24h		48h		72h		20 d	
	m±sd	%	m±sd	%	m±sd	%	m±sd	%
Superficial	54.6±7.3	58.8	60.2±5.7	54.7	84.1±17.4	67.7	29.6±1.8	46.6
Spores							10.3±1,1	16.2
Clear	32.9±6.3	35.5	27.6±5.3	25.1	19.9±3.4	16.1	7.0±0,8	11.1
Waxy			15.0±1.8	13.6	13.7±1.5	11.0	2.7±0.5	4.3
Basal	5.3±0.7	5.7	7.2±1.0	6.5	6.4±1.0	5.2	13.8±1.3	21.8

For each incubation time, the column on the left shows the mean values (m) in μm ± standard deviation (sd) of measures registered from 10 colony-biofilm analysed; the column on the right shows the strata relative percentage with respect to the total thickness of the biofilm. At 24 h only three strata were identified; at 48-72 h four strata were identified and at 20 days five strata were observed.

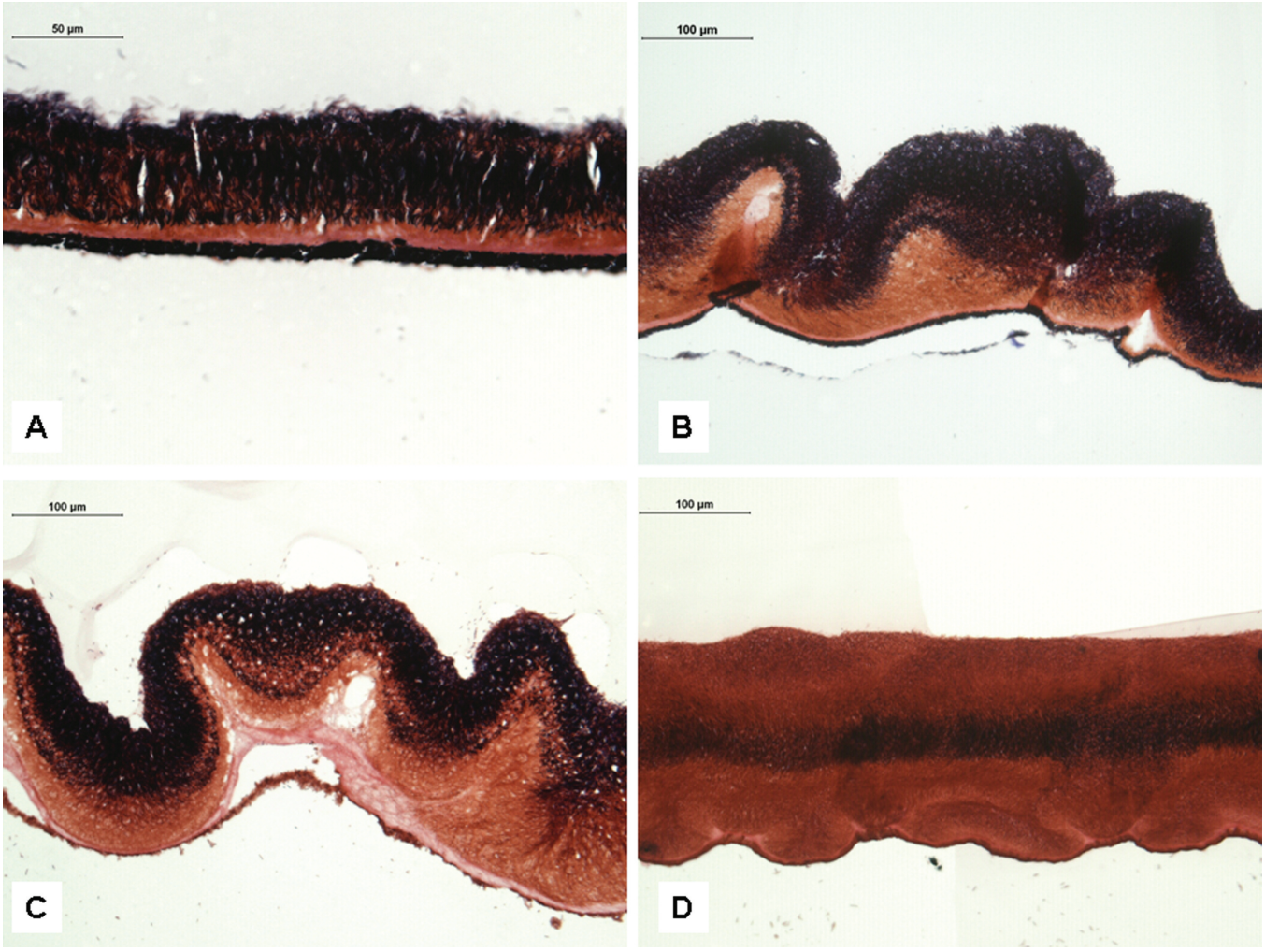

Fig. 3. Microscopic images of *B. subtilis* biofilm thin sections stained with Gram at different incubation times. **A.** 24 hours. **B.** 48 hours. **C.** 72 hours. **D.** 20 days.

strata described for colony-biofilm at 24 h incubation (Fig. 7A). Spores were identified at the first instance on 48 h colony-biofilm, precisely at the crests of the vein-like structures, that is to say, at the farthest site from the culture medium (Fig. 7B). On the 72 h colony-biofilm, spores were identified also at the valleys of the folds, forming a continuous layer (Fig. 7C). On the 20 days colony-biofilm, spores remained collected in a central stratum within the colony depth, protected by layers of extracellular matrix, dead cells and bacterial debris (Fig. 7D). Table 3 details the relative values of the area occupied by spores on the complete colony-biofilm, together with the corresponding standard deviation and variation coefficient.

Table 3. Spores relative area in percentage at different incubation times.

Incubation time	AR (%)	SD	VC
24h	0	---	---
48h	1.7	0.2	12.9
72h	2.7	0.3	11.6
20d	7.5	0.4	5.8

AR: spores relative area; SD: standard deviation; VC: variation coefficient

Discussion

In this study we have analysed colony-biofilm internal structure and sporulation dynamics of a *Bacillus*

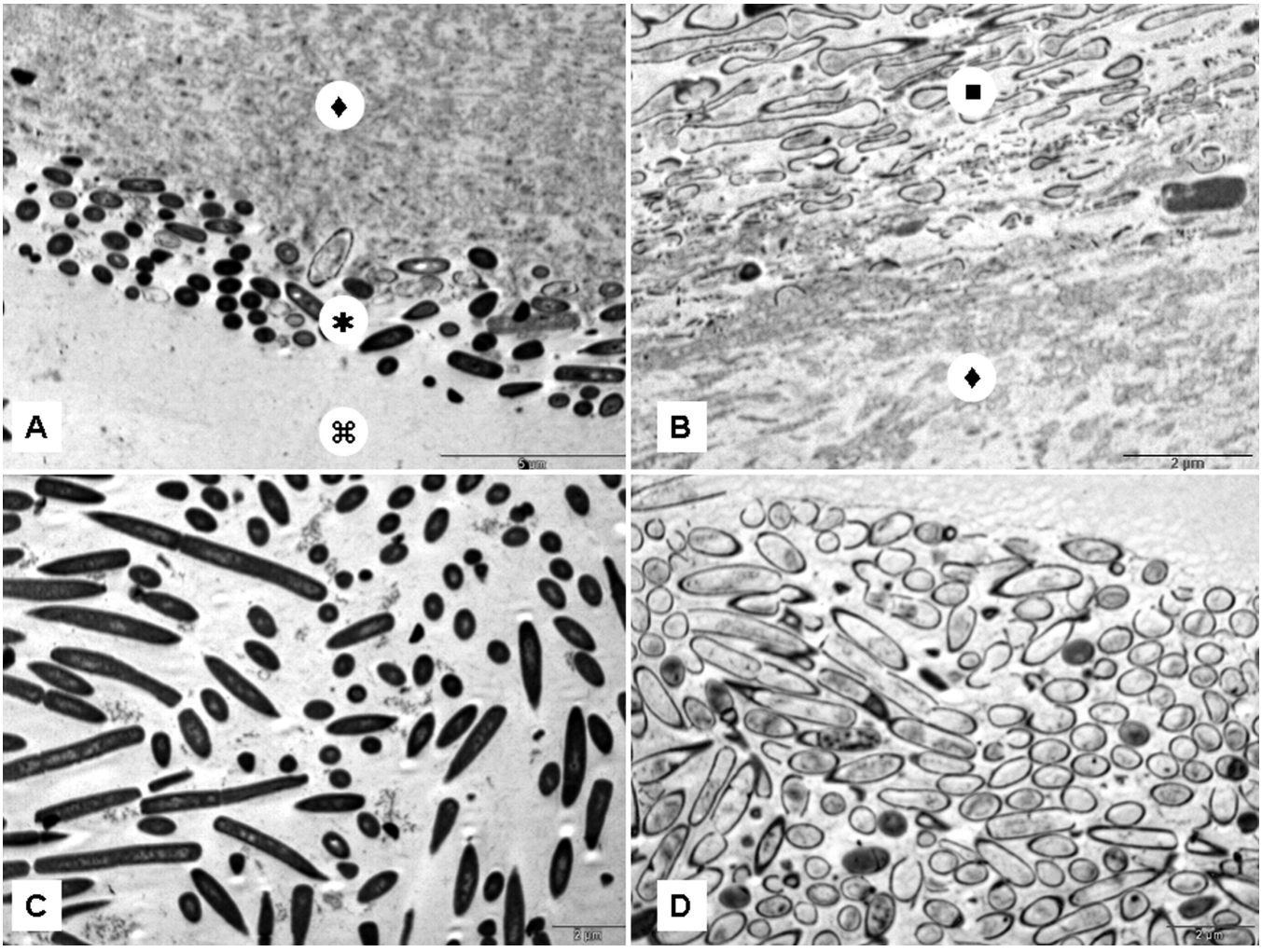

Fig. 4. Electron microscopy images of *B. subtilis* biofilm showing different strata ultrastructure. **A.** 48 h biofilm: ⌘: culture medium; ✱: basal stratum, in which the predominance of cocci and cocobacillar forms are observed; ♦: waxy stratum, consisting of highly degraded cellular debris and extracellular matrix. **B.** 48 h biofilm: ♦: waxy stratum; ■: clear stratum, formed by cellular debris slightly degraded and "ghost cells". **C.** superficial stratum on 48 h biofilm, in which predominance of mature bacillary forms without apparent spatial arrangement can be observed. **D.** superficial stratum on 20 days biofilm, consisting mainly of "ghost cells".

subtilis collection strain, by means of histological techniques. The idea came about by seeing parallelism between eukaryotic tissues and bacterial biofilms. Classically, a tissue is defined as a set of differentiated cells with a common embryonic origin, organised within an extracellular matrix to perform a function. All these features are fulfilled by the *B. subtilis* colony-biofilm grown on a solid medium, since all the cells that comprise it have a common origin, and are bound together by an extracellular matrix produced by the cells themselves (Branda et al., 2006; Vlamakis et al., 2008; López et al., 2010), and differentiate into subpopulations with distinct patterns of gene expression and function (Vlamakis et al., 2008). In this context, the colony-biofilm can be likened to a prokaryotic tissue, which can be processed like eukaryotic tissue to analyse the internal structure. Thus, fixation with aldehydes and embedding in solid media such as paraffin or epoxy resin, guarantee structural preservation and permit microscopic observation of sections of different thickness, from 4 μm up to 50 nm. The use of histochemical stains can also provide information on the chemical composition of the different structures observed. As far as we know, our group is pioneer in the application of these stains on biofilm histological sections.

Our results demonstrate that *B. subtilis* colony-biofilm has a complex and organised internal architecture, based on stratification, evolving over time. Stratification consists of alternating layers of live bacilli, with normal morphology, and layers of dead cells at different stages of degradation. Thus, the basal and superficial strata consist mainly of live bacilli, but with marked differences. In the basal stratum are predominant cocci and coccobacilar forms, characteristic of populations with high replication rate and intense metabolic activity (Vlamakis et al., 2008). Probably, the individuals in this stratum obtain nutrients directly from the culture medium, which would explain that as time passes the population density decreases, and after 20 days incubation at ambient conditions is a dead layer with virtually no spore production. In the superficial stratum, however, bacillary forms are predominant, characteristic of mature cells with lower metabolic activity and replication rate (Vlamakis et al., 2008). It is likely that individuals from this stratum use as nutrients cellular components from dead bacilli situated at the clear stratum, immediately beneath the superficial layer, by the phenomena of cannibalism previously described and documented (González-Pastor et al., 2003; López et al., 2009a). Cellular debris of this clear stratum would degrade gradually to produce the homogenous aspect of the waxy layer.

A stratified architectural pattern has been observed also in more simple colonies of *Staphylococcus aureus* and *Escherichia coli* (Gómez-Aguado et al., 2011). Thus, the three strata disposition observed in *B. subtilis* at 24 h is similar in layer number and composition to 48 h *E. coli*; however in *E. coli*, the outer layer bacilli were all oriented perpendicularly to the agar surface, while in *B. subtilis* biofilm there was not any predominant orientation. Also, the four layer disposition observed in *B. subtilis* at 48-72 h is similar in strata number and composition to the *S. aureus* 48 h biofilm. The internal stratified biofilm structure conserved by different bacterial species suggests that this architectural pattern provides evolutionary advantages for the establishment and development of complex bacterial communities.

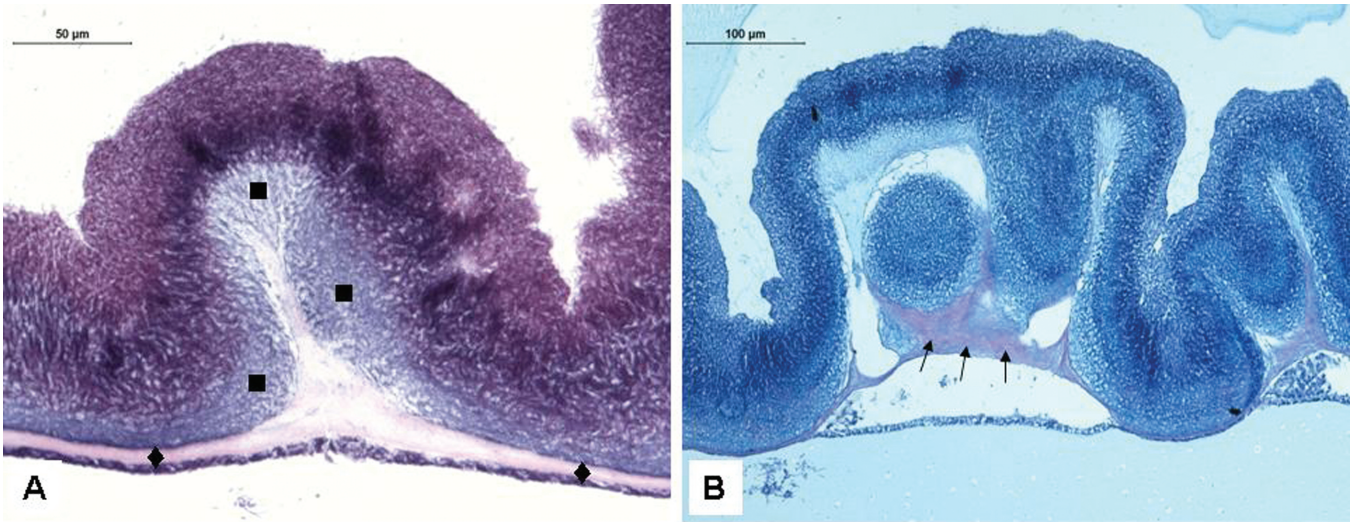

Fig. 5. Microscopic images of *B. subtilis* biofilm thin sections histochemically stained. **A.** 48 h biofilm stained with Hematoxylin and Eosin, showing the four strata described in text; waxy stratum (♦) stained pink by Eosin, indicating the absence of nucleic acids; clear stratum (■) instead, stained blue by Hematoxylin indicating that nucleic acids have not yet been degraded despite being a stratum of dead cells. **B.** 72 h biofilm stained with PAS-alcian blue; inside the vein-like structures accumulation of PAS-alcian blue positive extracellular matrix was observed (arrows).

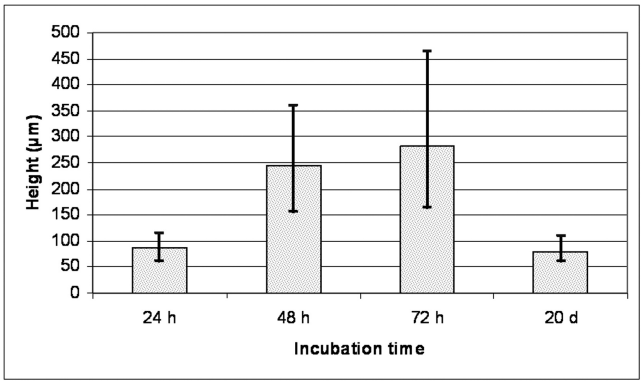

Fig. 6. *B. subtilis* biofilm height at different incubation times. Bars show the mean values as explained in the text. Each bar shows a line indicating maximum and minimum values. For 48 and 72 hours biofilm, values correspond to the height reached by the vein-like structures.

Therefore, it would seem that the clear and waxy layers, consisting of extracellular matrix and debris of dead bacteria, serve as support for maintaining the three-dimensional structure of the colony-biofilm, acting as an endoskeleton.

The main difference observed between *B. subtilis* biofilm and those from *S. aureus* and *E. coli*, is the greater central core three-dimensional complexity. While in *S. aureus* and *E. coli* the external morphology is convex or flat, *B. subtilis* develops "vein-like" structures. These formations are considered by Veening et al. (2006) as elevated bundles of cells, but they really are the superficial, clear and waxy strata, separated from the basal layer, folded over and over like a ribbon, producing more or less complex elevations, held in place by extracellular matrix.

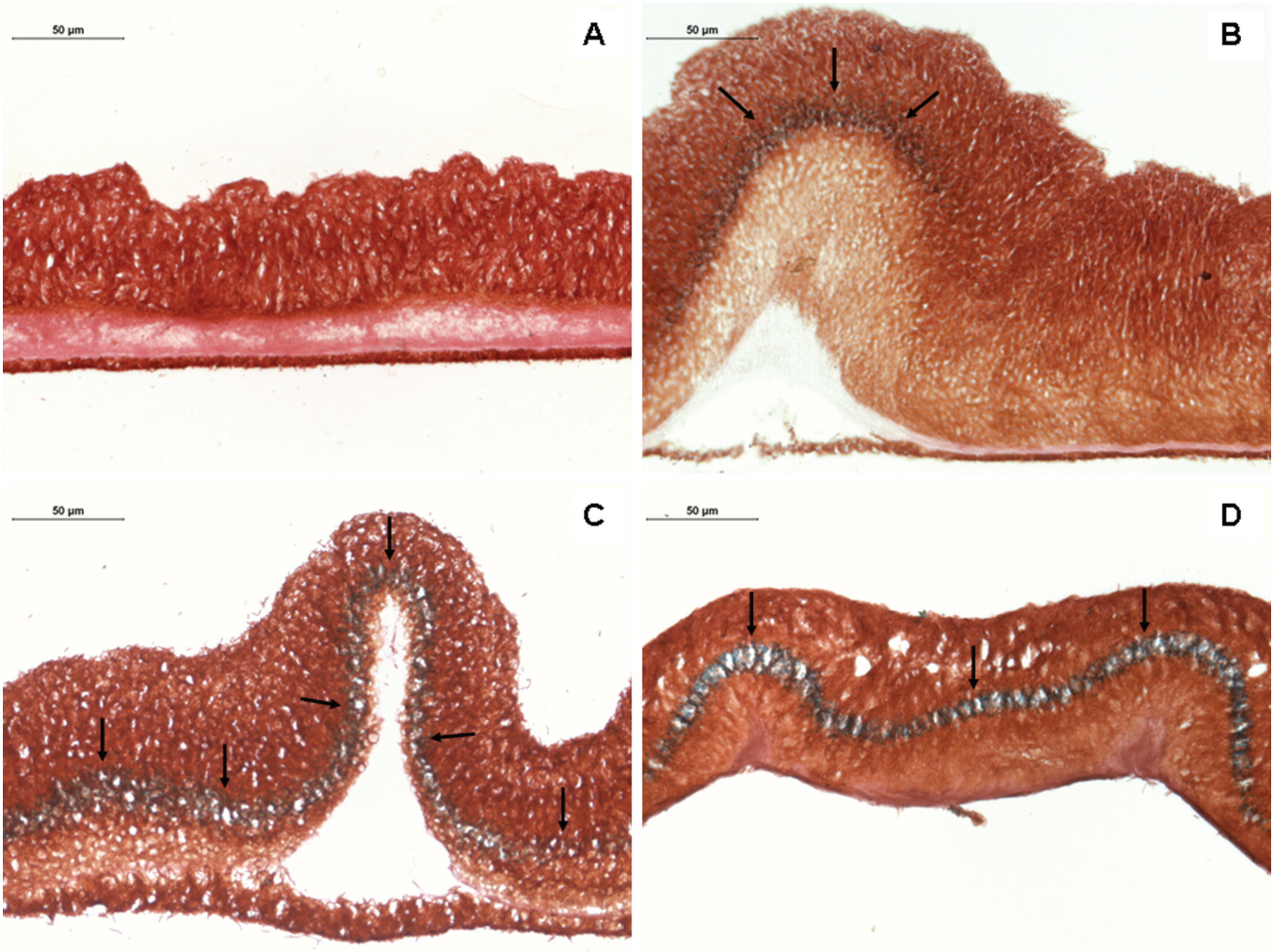

Figure 7. Microscopic images of *B. subtilis* biofilm thin sections stained with modified Wirtz-Conklin. **A.** 24 h biofilm with no spores observed. **B.** 48 h biofilm where spores were present at the crests of vein-like structures at the interface between superficial and clear strata (arrows). **C.** 72 h biofilm where spores were observed all along the interface between superficial and clear strata, both at crests and valleys of the vein-like structures (arrows). **D.** 20 days biofilm showing a continuous layer of spores inside the biofilm body (arrows).

Other authors using genetic molecular techniques (Branda et al., 2001; Veening et al., 2006; Bridier et al., 2011) have mentioned that sporulation starts at the elevated formations of *B. subtilis* biofilm. Our results confirm those observations and are even more precise as to spatial-temporal spore initiation. Sporulation starts after 48 h incubation at the top of the crests of the vein-like structures, on the interface between the superficial and clear layers. Over time (72 h), the sporulation phenomenon extends along the interface between both layers, including the valleys of the vein-like structures or areas nearer to the culture medium. Indirect spore quantification by means of image analysis of the relative colony area occupied by spores described in this study seems to be an appropriate method, as the variation coefficients are close or inferior to 10% and could permit the establishment of temporal sporulation curves, as a possible distinguishing feature between different strains of the same species. These curves may also be of interest in other sporulated species such as *Clostridium difficile*, in which the dynamics of sporulation is of great public health significance (Akerlund et al., 2008, Merrigan et al., 2010). To this respect, *in situ* spore quantification on histological sections could be a good alternative to count spores using phase-contrast microscopy and counting chambers such as the haemocytometer (Burns and Minton, 2011). With the counting chamber we can calculate the total spore number in a colony-biofilm after a determinate incubation period, but it does not provide information about to the topographic distribution of spores within the biofilm, neither about the spores proportion compared to the overall cell population. The absolute and relative measurements of the area occupied by spores in a biofilm obtained by means of image analysis provides this additional information.

Finally, it seems evident that the complex architectural development of the colony-biofilm observed in this study involves synchronous behaviours of bacterial groups, probably related to cell-cell communication processes called quorum sensing (Camilli and Bassler, 2006; Ng and Bassler, 2009). These processes would require, on the one hand, production and release of chemical autoinducers and, moreover, detection and response to these signaling molecules as control mechanisms of cell population density and to coordinate biological activities. In fact, for *B. subtilis* there have been described quorum sensing processes related to sporulation (Lazazzera, 2001) and extracellular matrix production (Lopez et al., 2009b), both very important events in colony-biofilm development, as evidenced by our results.

In summary, the application of fast, simple and inexpensive histological techniques to the study of *B. subtilis* biofilm has allowed us to clarify the internal architecture, its evolution over time, and sporulation dynamics, complementing and explaining previous knowledge obtained through molecular biology and genetics. Furthermore, as for eukaryotic tissues, the use of thin sections of colony-biofilm embedded on solid media would permit studies on gene expression by immunohistochemistry and *in situ* hybridization techniques, provided there are available specific antibodies and nucleic acid probes.

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Accepted May 2, 2013