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Comparative spatio-temporal evolving histology of staphylococcus aureus and staphylococcus epidermidis colony/biofilm

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ABSTRACT

Microbial colonies are a special type of biofilm and as such should have a defined internal structure. The aim of this study was to assess the evolving architecture of *Staphylococcus aureus* and *Staphylococcus epidermidis* colonies. Isolated colonies from 16 hours up to 15 days were processed using histological techniques to visualize their internal structure by light and electron microscopy. A morphometric analysis of the biofilm components was performed by image analysis. Both species followed the same structural evolution at short and medium incubation times (up to 96 h), consisting of a sequential stratification in up to 4 layers with different compositions and staining properties. At 15 days, clear cut different architectural patterns were evident. *S. aureus* showed a multilayered pattern with additional lenticular-shaped invasions of the culture medium. *S. epidermidis* showed only three strata, with subpopulations regrowing from specific points on the surface and in the base of the colonies, but never invading the agar. Our results demonstrate that evolving architectural pattern of *Staphylococcus* colony/biofilm presents as a complex and time-dependant process that might be considered as a genus phenotypic characteristic in the short and medium term, while it would be species or even strain specific in the long run.

KEYWORDS

Staphylococcus;
Biofilm;
Histology;
Image analysis;
Light microscopy;
Electron microscopy.

INTRODUCTION

Biofilms are communities of cells held together by a self-produced extracellular matrix typically consisting of protein, exopolysaccharide, and often DNA[1]. These biofilms enable almost all bacteria and fungi to survive on surfaces in nature. Bacterial biofilms are resistant to disinfectants, antibiotics, and to several components of the inflammatory defence system, facilitating the development, persistence and recurrence of infection. There is a gradient of nutrients and oxygen in biofilms from areas of rich to poor concentration where bacterial cells have a decreased metabolic activity and increased doubling times. Thanks to the quorum sens-

ing phenomenon, bacteria in biofilms communicate by means of molecules, which activate certain genes responsible for production of virulence factors and, to some extent, of biofilm structure[2].

Gram positive cocci, particularly *Staphylococcus aureus* and coagulase-negative staphylococci, are the most frequent etiology of indwelling medical devices inserted through the skin. Both *S. aureus* and *S. epidermidis* have a high affinity for artificial surfaces and they frequently produce biofilm-mediated infections on medical devices such as vascular and urethral catheters, as well as on previously damaged mucosal surfaces as the bronchial mucosa in cystic fibrosis patients or in the middle ear in patients with chronic otitis[3,4]. The formation of thick biofilms is a very efficient means to evade recognizing and inactivation by antimicrobial molecules, phagocytes and antibiotics.

Classical systems to study biofilms involve two media: one of them usually solid, inert, and motionless on which the microbial growth takes place, and the other, liquid or gaseous that flows and provides the nutrients and oxygen. Some authors consider that bacterial colonies grown on agar plates are in fact a special type of biofilm in which the microbial growth develops between a solid culture medium that provides nutrients and a gaseous medium which provides the oxygen[5,6]. Although external morphology of the colonies has been widely studied in the past and it has been described cell subpopulations with distinct gene expression patterns[6-8], at present there are few data about the internal architecture of the colony/biofilm and spatio-temporal organization of those subpopulations.

Recently, our group has developed an histological methodology to analyse colony/biofilm internal structure and we have demonstrate that microbial colony/biofilms are not mere cells accumulations[9]. On the contrary, colonies show a complex internal structure that evolves with time. However, whether these evolving architectural patterns are genus-, species-, or strain-specific phenotypic characteristics is not known. In order to elucidate this question, short-, medium- and long-term colony/biofilm histological evolution of several strains of the two most representative species of the genus *Staphylococcus*, namely, *S. aureus* and *S. epidermidis*, was compared.

MATERIALS AND METHODS

Strains and cultivation techniques

Five *S. aureus* strains were studied: one ATCC 29213 strain, one Mu3 strain and three clinical isolates (MS92, MR378, MR347). Along with the *S. aureus* strains, five additional *S. epidermidis* strains were also studied: ATCC 12228, ATCC 35983, ATTC 35984, and two clinical isolates (UR147 and CT24). All clinical isolates proceeded from Department of Microbiology, School of Medicine, Complutense University, culture collection.

Bacterial suspensions in saline solution from an overnight culture in Mueller-Hinton agar with 5% lysed sheep blood were adjusted to McFarland 0.5. At least five ten-fold dilutions of each sample were spread (20 µL) onto Mueller-Hinton agar supplemented with 5% sheep blood and incubated for 16 h, 24 h, 48 h, 96 h and 15 days at 37ºC. Humidity conditions were kept constant in order to avoid the long term media desiccation. Cultures were repeated three times.

Colonies processing

After each incubation period, agar plates were flooded with 20 ml of 2% agar solution at 45ºC and were allowed to solidify at room temperature. Agar blocks containing isolated colonies were extracted from culture plates and were processed according to the methodology previously described[9]. In brief, agar blocks were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for two hours at room temperature. Blocks were then washed with cacodylate buffer plus 4.5% saccharose, dehydrated in increasing gradation alcohols and embedded in epoxy resin (Eponate 12), directing some colonies parallel to the major axis of the mold and other colonies perpendicular to this axis. Semithin sections (0.5 µm thick) were stained with toluidine blue and visualized by light microscope (Leica, DM5000B). Ultrathin sections (50 nm thick) were stained with lead citrate and visualized with an electron microscope (Hitachi H-7000). At least 10 isolated colonies were processed for every culture plate.

Ten additional isolated colonies from 48 h, 96 h and 15 d cultures were fixed in buffered formaldehyde for 24 hours and embedded in paraffin by routine histological techniques. Thin sections (4 µm thick) were stained with Hematoxylin-Eosin and Gram dyes.

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Image analysis

Morphometric analysis of colony/biofilms was made by use of Leica QWin image analysis software. Macrophotographs of the culture plates were taken with a conventional digital camera to assess the colonies diameter.

Biofilm height and thickness of the different components of their internal structure were quantified by means of digitized microphotographs of transversal semithin sections, stained with toluidine blue, and taken with a digital camera Leica DC 300, coupled to a light microscope Leica DM 5000B. Specifically, biofilm height was measured on each section as distance between the agar line and the outermost point of the biofilm surface. Similarly, five different measures of the thickness of every stratum on the central area of stage IV colonies (as defined in the "Results" section) were performed. In this case, suitable filters implemented in image analysis software were used for improved visualization of the strata. All the measures were recorded as files for statistical analyses. For each strain and incubation period ten sections where analysed, corresponding to the same number of colony-biofilms, and the mean value for those 10 measures was considered the thickness for each architectural component and the biofilm height.

Statistical analysis

GraphPad Prism (version 5.04, GraphPad Software, San Diego California USA) for Windows was used for all analyses. The Kolmogorov-Smirnov test was used to test for normality. We used the Kruskal-Wallis test with the Dunn's post-test for intra-strain and intra-species multiple comparisons, and the Mann-Whitney test for comparison of the two different species of non-normal data. Significance was taken as $p<0.05$.

RESULTS

Macroscopically, *S. aureus* and *S. epidermidis* colonies were circular and convex at 24 h and 48 h, and slightly flattened at 15 days of incubation. The diameters of the colonies grown on blood agar at 24 h, 48 h and 15 days are shown in TABLE 1.

The observation by optical microscopy of transversal semithin sections, perpendicular to the agar surface, showed the existence of specific patterns in the internal structure of the colonies, defined by the presence of strata based on different population densities of microorganisms, their arrangement and the proportion of dead cells. At short and medium incubation times (up to 96 h), all strains, regardless of bacterial species, showed the same evolving pattern. This allowed us to define four different stages (Figure 1):

Stage I, characterized by a homogeneous distribution of cocci in all the thickness of the colony (Figure 1A).

Stage II, formed by two strata: 1) a basal stratum with a high population density in contact with the culture media, and 2) a superficial stratum with a lower density (Figure 1B).

Stage III, formed by three strata: 1) the basal stratum with a high population density, 2) a clear stratum, intermediate, composed of a waxy or slightly granulated material within which a few cocci were dispersed, and 3) the superficial stratum with a high density similar to the basal stratum (Figure 1C). Electron microscopy showed that the clear stratum was predominantly constituted by degraded bacterial remains (Figure 2A).

Stage IV, formed by four strata: 1) the basal stratum with a high population density, 2) the clear stratum, similar to that observed in stage III, 3) a mixed stratum, composed of cocci embedded within a slightly granular material, and 4) the superficial layer with a density similar to the basal layer (Figure 1D).

TABLE 1 : Diameters in mm of the *Staphylococcus* colony/biofilms grown on blood agar plates. The table shows the mean values ± standard deviation for the ten colony-biofilm analysed at each incubation time.

	Strain	24 h	48 h	15 d
S. aureus	ATCC 29213	2.8 ± 0.1	6.0 ± 0.2	11.00 ± 1.1
	MS92	2.5 ± 0.1	5.6 ± 0.3	12.3 ± 1.4
	MR378	1.6 ± 0.1	3.8 ± 0.1	10.5 ± 1.2
	MR347	1.7 ± 0.0	4.0 ± 0.1	10.0 ± 1.1
	Mu3	0.8 ±0.1	2.3 ± 0.1	6.0 ± 0.5
S. epidermidis	ATCC 12228	1.3 ± 0.1	3.9 ± 0.2	15.8 ± 0.5
	ATCC 35983	0.9 ± 0.1	2.5 ± 0.3	6.2 ± 0.6
	ATCC 35984	0.9 ± 0.1	2.3 ± 0.2	8.6 ± 0.7
	UR147	1.0 ± 0.1	2.8 ± 0.2	8.4 ± 0.7
	CT24	1.1 ± 0.1	3.0 ± 0.3	9.9 ± 0.8

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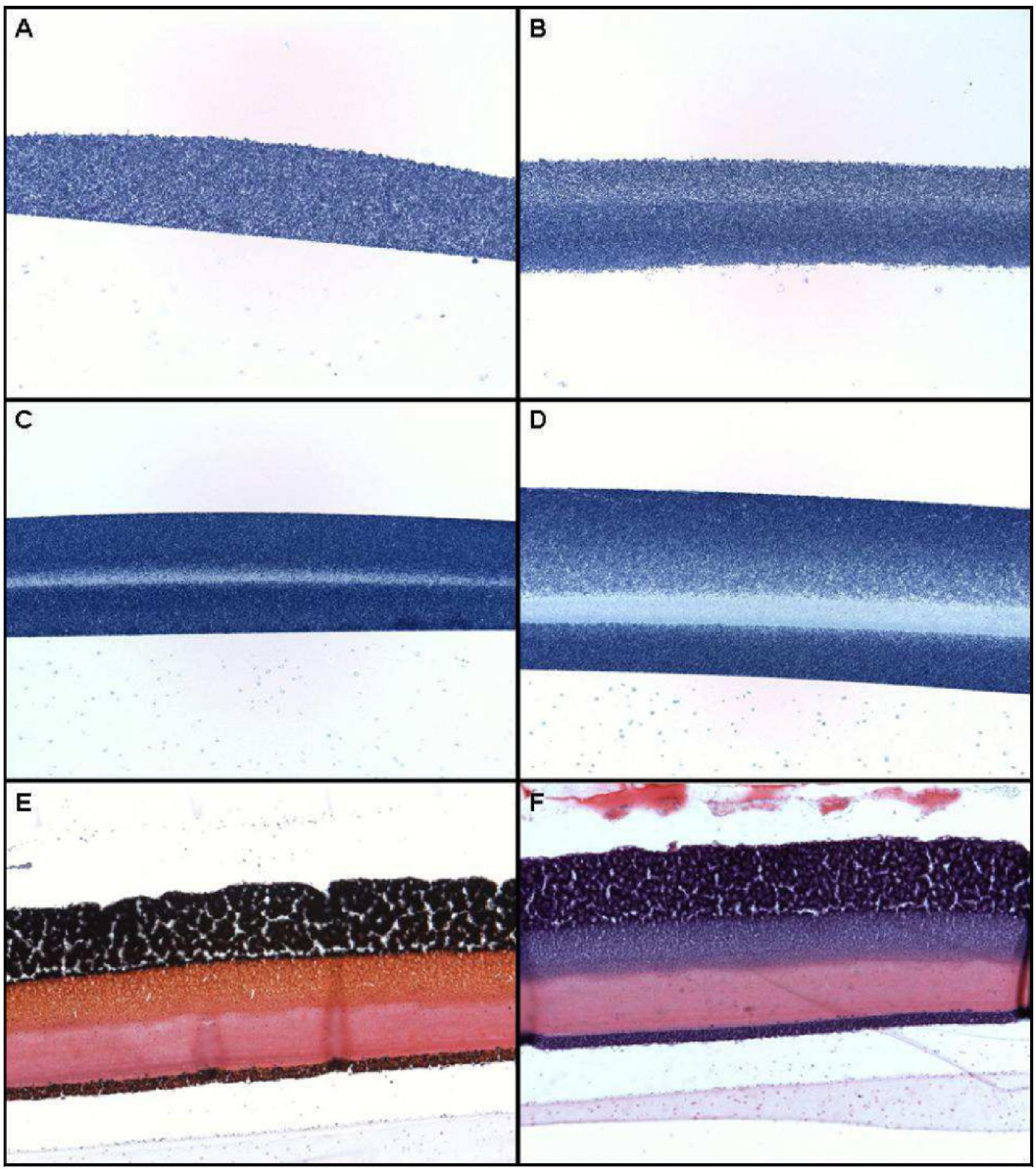

Figure 1 : Transversal (perpendicular to agar) sections of staphylococcal colony/biofilms showing the evolution of their internal structure up 96 hours of incubation. The distribution of cells in strata let us define four different and sequential evolving stages, according to the observed number of strata. A) Stage I (a single stratum), B) Stage II (two strata), C) Stage III (three strata), and D-F) Stage IV (four strata). Strata description is given in the text. (A-D) Toluidine blue stain, 20x; E) Gram stain, 20x; F) Hematoxylin-eosin stain, 20x).

Basal and superficial strata were Gram positive, whereas the central colony zones (clear and mixed strata) were Gram negative (Figure 1E). These two latter layers were easily distinguishable with the hematoxylin-eosin stain, since the clear stratum was acidophilic (pink or strained with eosin), and the mixed stratum was basophilic (blue or stained with hematoxylin) (Figure 1F). Electron microscopy showed that the mixed stratum was constituted by morphologically normal cocci with degraded bacterial remains (Figure 2B). Both components showed inverse gradients through the thickness of the stratum, while cocci predominated in the upper part in contact with the superficial stratum, the bacterial remains were predominant in the area in contact with clear stratum.

The thickness of every stage IV strata for the different strains is shown in TABLE 2. The first thing to note was that the thickness of the strata that make up the colonies was not uniform. In *S. aureus*, the clear strata. were thinner whereas the mixed strata were wider than the others ($p<0.001$). However, in *S. epidermidis*, the superficial strata were the thickest ($p<0.05$). The

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comparison between the two species showed that the mixed strata were thicker in *S. aureus* ($p<0.01$), while the superficial strata were wider in *S. epidermidis* ($p<0.05$). When comparing each strain individually, there were no significant differences in the intra-species relative strata thickness of *S. aureus* and *S. epidermidis*, except for MR347 *S. aureus* and ATCC 35983 *S. epidermidis* that showed thinner clear and thicker superficial strata ($p<0.01$), and for ATCC 12228 *S. epidermidis* with a basal stratum significantly thinner than the rest ($p<0.05$).

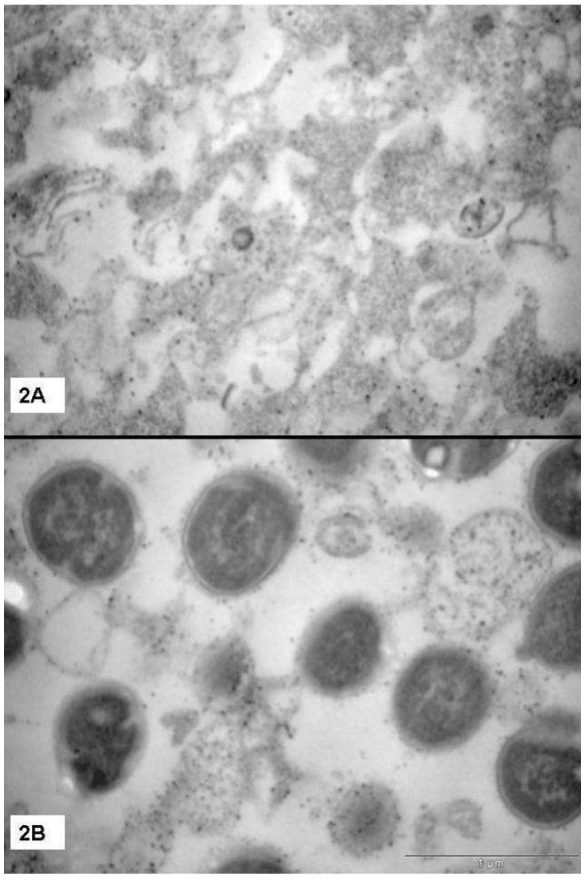

Figure 2 : Electronic microphotographs of the clear stratum in stages III and IV (A) and of the mixed stratum in stage IV (B) of staphylococcal colony/biofilm. The clear stratum is made of degraded bacterial remains, whereas there are alternating bacterial remains along with morphological normal cocci in the mixed stratum.

TABLE 2 : Relative thickness of the four strata observed in stage IV *Staphylococcus* colony/biofilms. Each value represents the mean value ± standard deviation of a given stratum regarding total height of the colony as a percentage.

	Strain	Basal	Clear	Mixed	Superficial
S. aureus	ATCC 29213	21.7 ± 0.1	15.8 ± 2.8	37.2 ± 1.2	25.4 ± 1.7
	MS92	29.6 ± 9.3	20.0 ± 0.9	35.0 ± 6.2	15.4 ± 4.0
	MR378	28.4 ± 2.0	13.8 ± 1.9	35.1 ± 5.9	22.7 ± 5.8
	MR347	22.9 ± 1.6	7.6 ± 0.7	37.9 ± 6.6	31.6 ± 4.3
	Mu3	27.9 ± 3.8	15.3 ± 1.6	32.5 ± 3.7	19.3 ± 2.0
S. epidermidis	ATCC 12228	10.4 ± 1.1	31.7 ± 2.6	31.3 ± 3.3	29.7 ± 2.9
	ATCC 35983	24.8 ± 0.8	8.5 ± 0.5	23.9 ± 3.5	42.9 ± 3.2
	ATCC 35984	27.0 ± 1.4	20.4 ± 0.7	22.7 ± 2.9	30.0 ± 2.6
	UR147	21.7 ± 1.0	17.1 ± 1.2	25.0 ± 3.0	36.2 ± 2.9
	CT24	19.8 ± 0.9	21.1 ± 1.6	26.9 ± 3.1	32.2 ± 2.8

The observation of semi-thin sections, parallel to the agar surface, localized in central zone of the thickness of colonies in stage IV, showed a pattern of concentric arrangement formed by three zones: a central clear zone that corresponded to the clear stratum, an intermediate zone corresponding to the mixed stratum and an outer zone equivalent to the superficial stratum (Figure 3).

All strains studied went through the same four different stages. This was an evolutionary process regardless of the bacterial species and strains. The time required to reach stage IV was a characteristic of each strain and depended on the growth rate of each of them. Thus, it took *S. aureus* ATCC 29213 and *S. epidermidis* ATCC 12228 strains 16 h to reach stage I, 24 h to reach stage II and 48 h to reach stage IV. However, it took *S. epidermidis* ATCC 35983 strain 24 h to reach stage I, 48 h to reach stage II and 96 h to reach stage IV.

In *S. epidermidis* ATCC 35983 and ATCC 35984 strains a wrapping membrane was observed on the surface of the colonies (Figure 4).

After 15 days of incubation there was a clear cut divergence in the evolving pattern of both species. *S. aureus* colonies evolved in order to form a multi-stratified structure, with six or seven clearly defined strata, whose image reminded of two superimposed colonies separated by a clear central layer (Figure 5A and 5B). There were also small and focal lenticular microbial regrowths within and invading the agar, underneath the colonies (Figure 5B).

On the contrary, *S. epidermidis* colonies evolved towards an evident stage III, with a basal stratum with a higher population density, a wide central stratum (around 40% of the colony thickness) and a superficial stratum similar to the mixed one described in stage IV but upside-down, that is, with a larger proportion of cocci in the closest zone to the clear central stratum (Figure 5C and 5D). Additionally, two different patterns of focal regrowths with larger population density

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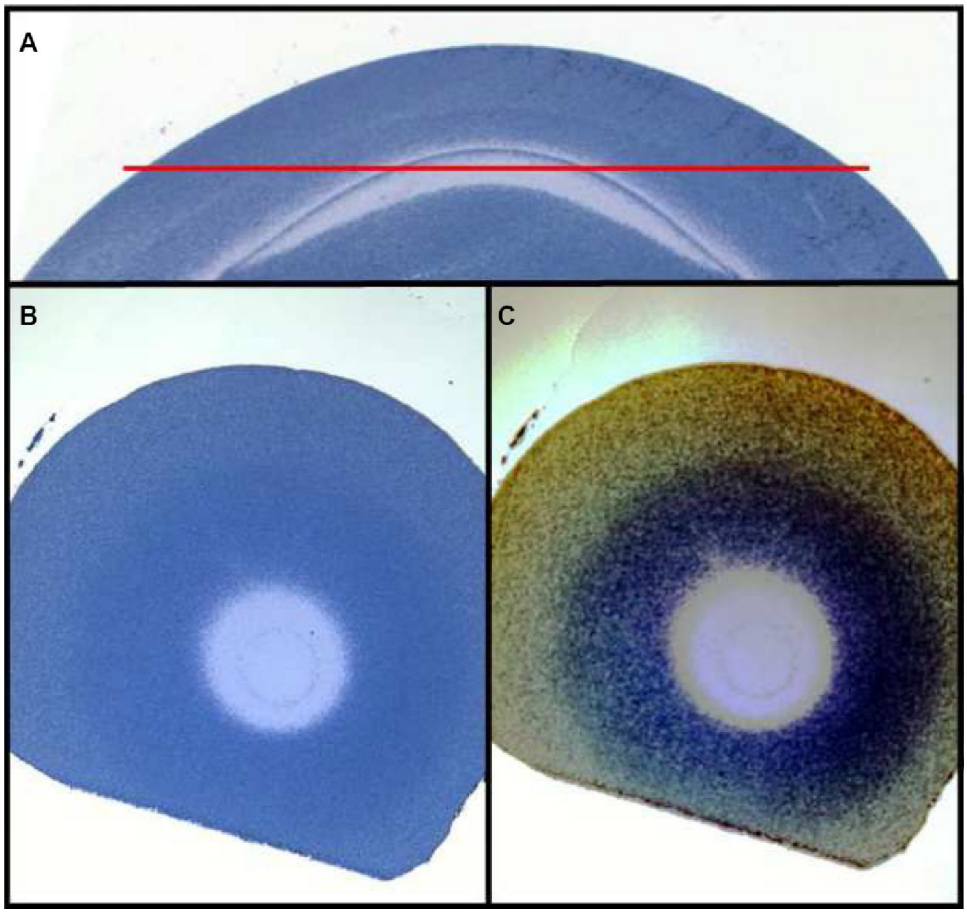

Figure 3 : Internal structure of an *S. aureus* ATCC 29213 colony/biofilm after 48-hour incubation. A) Transverse semithin section showing a typical Stage IV arrangement (toluidine blue, 10x). B) Longitudinal (parallel to agar) semithin section, following the red line in A) with a typical disposition in concentrical rings, corresponding to the strata seen in transversal sections (toluidine blue, 20x). C) Image of B) applying filters implemented in image analysis software so as to better visualize the arrangement of cells within the colony.

Figure 4 : Detail of a transversal semithin section of a *S. epidermidis* colony ATCC 35983 that shows a membrane-like structure on the surface of the colony (arrows). (Toluidine blue, 40x).

were observed: 1) superficial regrowths, that budded from the inner zone of the superficial layer and projected to the outside with a round or globular shape (mushroom-like structures) (Figure 5C); 2) basal regrowths, that appeared at the base of the colony and on the surface of the agar, with no agar invasion and with an umbonate shape, originated from the basal layer and deforming the whole colony (Figure 5D).

DISCUSSION

The present work is a study of the colonial patterning and stage evolution of *S. aureus* and *S. epidermidis*. Our results support the hypothesis that staphylococcal colonies are a static kind of biofilm, similar to colony/biofilms obtained from a bacterial suspension spot[10]. However, bacterial colonies present clear differences regarding dynamic biofilms. Colonies have a slower growing speed, a concentric expansion from the initial seeding point, but also a larger thickness/height than classical biofilms.

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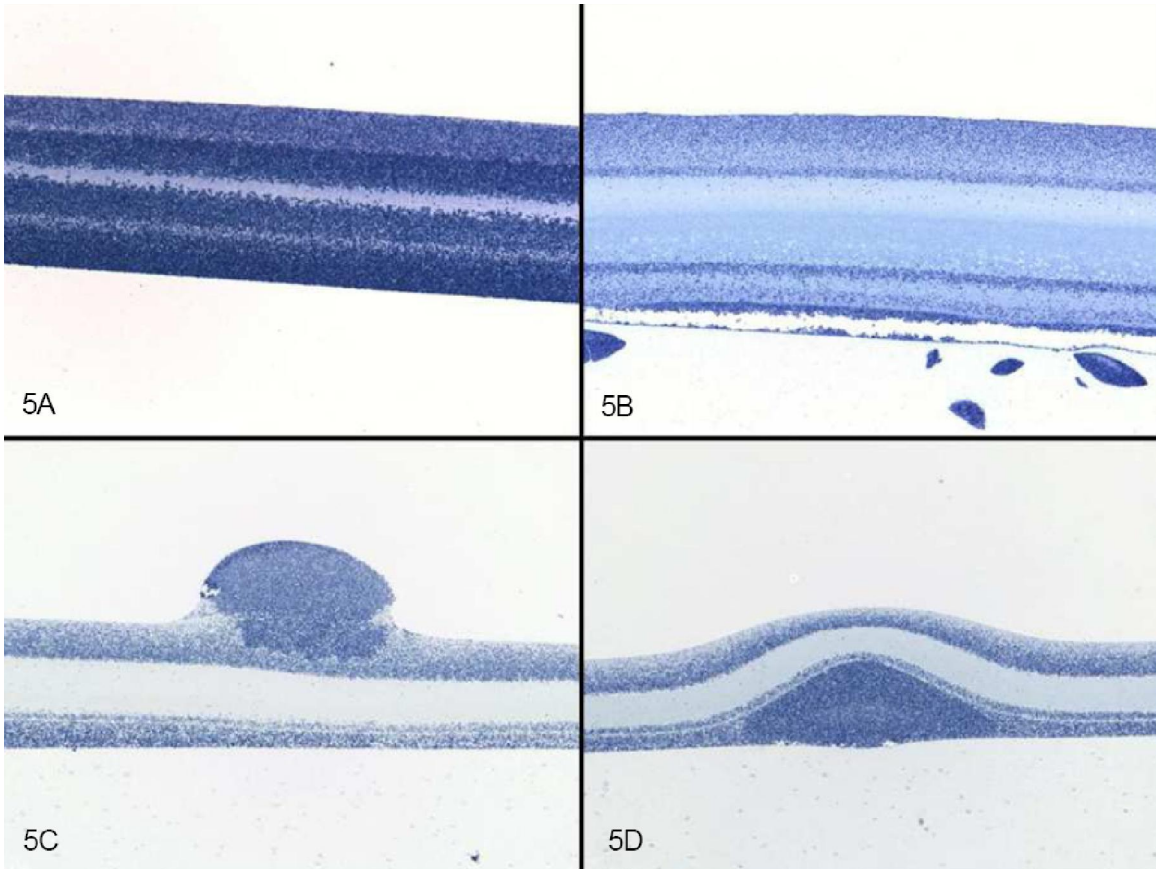

Figure 5 : Transversal semithin sections of long evolving colony/biofilms. A) and B) *S. aureus* colony/biofilm showing a characteristic multi-stratifie pattern. In B) small lenticular structures invading the agar are observed. C) and D) *S. epidermidis* colonies showing a typical stage III structure with focal regrowths on surface (C) or at the base (D). (Toluidine blue, 20x).

In both studied staphylococcal species, the short- and medium-term growth evolution involved a stratified configuration with alternate alive cocci and cellular debris layers. Stage I, in which there is a more or less homogeneous distribution of cocci, would represent the exponential growth phase in a liquid medium. In stage II, the superficial stratum probably is the result of a deficient nutrient concentration due to mere distance. This relative lack of nutrients would probably cause death and lysis of the individuals located in the centre of the colony, with the outcome of degradation and liberation of cellular content, as demonstrated by electron microscopy, and therefore originating the characteristic middle clear stratum of stage III, that could be used to keep nourishing the superficial stratum. With time, bacterial death continues to expand from the central zone to the surface, causing the mixed layer in stage IV, as shown with electron microscopy again. The even staining of the mixed stratum with hematoxylin can be due to the presence of partially-degraded nucleic acids incorporated to the extracellular matrix after cellular lysis. The staining with eosin in the clear stratum can indicate that those nucleic acids have already been fully degraded. Both stages III and IV would represent the stationary growing phase in liquid media. The same structural evolutionary process up to stage IV repeats in every single assessed strain, with only differences regarding time needed for completion and the thickness of layers. This fact evidences minor strain-specific differences in the initial colony development, within a common global process.

In the long run, *S. aureus* and *S. epidermidis* colonies show a completely different population dynamics. Those of *S. aureus* evolve following a multi-stratified strategy that seems to warrant the survival of the population by means of likely cannibalism mechanisms. In this species, the population growth takes place not only on the surface but there are also lenticular-shaped agar invasions, from the basal layer of the colony, in depth in favor of the gradient of nutrients and possibly in microaerobic conditions. To our known, invasions of this kind had only been reported so far for yeasts, and

they have been considered as a putative virulence trait[11,12]. Whether they could also be a potential virulence trait in *S. aureus* remains to be elucidated.

In *S. epidermidis* colonies all the individuals in the mixed layer on stage IV ended up dying, so widening the clear layer and stabilizing colony. It is possible that there appeared focally nutritional variant mutants in the basal and superficial layers giving rise to new subpopulations in exponential growing, what could explain the presence of the mushroom-like structures springing from the surface of the colony, and the umbonate noninvading structures seen at the base of the colony. These subpopulations are likely able to develop on poor media or even scavenging bacterial remains, as it has been reported for *Bacillus subtilis*[13]. Mushroom-like structures with a complex net of intertwined channels that warrants the distribution of nutrients to every bacterium have also been described in mature biofilms[14]. However, we have not documented any channels in the mushroom-like structures observed in *S. epidermidis* colonies after 15 days of incubation.

In our study, those strata could be produced by the different level of cellular specialization. These cells, using complex quorum sensing signalling mechanisms, could be responsible for the distinct shapes and the formation of the highly organized architecture observed in this study[15]. We propose a hypothesis for the phenomenon of the different strategies of colony arrangement: colony growth would be a developmental process in which bacteria undergo a regulated lifestyle switch from a nomadic unicellular to a sedentary multicellular state where subsequent growth results in structured communities and cellular differentiation[16]. Hence, a colony could be seen as a multicellular entity made up of different layers with a given arrangement in order to provide protection and to optimize the distribution of nutrients, and in a way, microbial colonies might be therefore considered as "supraorganisms". Collective behaviour requires that individuals act in the interest of the colony and the evolutionary process is relevant to each particular system of interest for each bacterial species. And this is what seems to be inferred from the observed population dynamics in our work; the regrowth on dead layers ensures survival and the population structure remains constant in colonies of the two species.

Regarding collective behaviour, the segregation of strata in *Staphylococcus* species within colonies is consistent (although to an individual level) with the classical ecological principle of "competitive exclusion" whereby competition pushes each species, or cluster of similar species, into a discrete niche[17]. The stratified pattern of colonies also is in accordance (but again to the individual level) with the principle of "insurance effect" based on the morphological diversity of different bacterial species and how this protects communities from unstable environmental conditions providing a form of biological insurance as it occurs in biofilms[18].

In conclusion, this study shows that the evolving colony pattern is a specific to species feature. Stratification of *Staphylococcus* colony/biofilm could constitutes an important survival strategy because at least some of the cells, which represent a wide variety of different metabolic states, are almost certain to survive any metabolically directed attack as nutrient limitation in the outer layer of the colony, what in turn could be related to bacterial persistence and recurrence of infections, commonly observed particularly in patients with indwelling medical devices[19].

Finallly, the histological approach used in this study could be useful as a surrogate of more complex biofilm models in order to gain insight into a better understanding of the mechanisms regulating potential persistent infections, or to study the interaction between antibiotics and bacteria or mixed populations (bacterial consortia).

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