Colonial architecture and growth dynamics of *Staphylococcus aureus* resistant to methicillin

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ABSTRACT

The aim of the study was to explore the structure and growth dynamics of *Staphylococcus aureus* resistant to methicillin (MRSA) colonies using semithin sections visualized by light microscope. One *S. aureus* susceptible to methicillin (MSSA) and one MRSA clinical strains were studied. Colonies in agar plates were embedded in epoxy resin after each incubation period (24 h and 48 h) at 37ºC. Semithin sections of 0.5 μm were stained with toluidine blue and visualized by light microscope. Microscopically, no structural differences were observed between SASM and SARM colonies but differences were observed in both strains between 24 and 48 h incubation periods. Colonies showed two layers clearly differentiated at 24 h independently of the resistance to methicillin: (A) one basal layer with high density of population in contact with culture media, and (B) one superficial layer with a lower density of population. Colonies showed four layers at 48 h: (A) one basal layer with high density of population; (B) one clear layer constituted by very degraded bacterial remains in which can be observed cocci dispersed with their dyeing properties; (C) one mixed layer constituted by viable bacteria and little degraded bacterial remains (D) one superficial layer with a lower density of population than basal layer. Colonial architecture is a complex and time-dependent process.

Key words: ***Staphylococcus aureus*, light microscope, structure, colony**

Arquitectura colonial y dinámica del crecimiento de *Staphylococcus aureus* resistente a meticilina

RESUMEN

El objetivo del estudio fue observar la estructura colonial y la dinámica del crecimiento de *Staphylococcus aureus* resistente a meticilina (SARM) empleando cortes semifinos visualizados al microscopio óptico. Se estudiaron una cepa de *S. aureus* sensible a meticilina (SASM) y un SARM. Después de cada periodo de incubación (24 y 48 h) a 37ºC las colonias fueron incluidas en una resina epoxi. Cortes semifinos de 0.5 μm fueron teñidos con azul de toluidina y visualizados al microscopio. Desde el punto de vista microscópico, no se observaron diferencias estructurales entre las colonias de SASM y SARM. Si se observaron diferencias en ambas cepas entre las colonias de 24 y 48 h de incubación. En las colonias de 24 h se observaron 2 capas claramente diferenciadas: (A) una capa basal con alta densidad de población en contacto con el medio de cultivo y (B) una capa superficial con menor densidad de población. En las colonias de 48 h se observaron cuatro capas: (A) una capa basal con alta densidad de población; (B) una capa clara constituida por restos bacterianos muy degradados en cuyo seno se observan muy escasos y dispersos cocos que conservan sus propiedades tintoriales; (C) una capa mixta, constituida por una mezcla de bacterias vivas y restos bacterianos muy groseros y poco degradados y (D) una capa superficial con menor densidad de población que la capa basal. Las colonias forman estructuras altamente organizadas originadas por la disponibilidad de nutrientes y mecanismos de comunicación intercelular. La arquitectura colonial es un proceso complejo y tiempo dependiente.

Palabras clave: ***Staphylococcus aureus*, microscopio, estructura, colonia**

INTRODUCTION

Each bacterium is a biotic autonomous system with its

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own internal cellular capabilities (storage, processing and assessments of information). These features afford the cell certain plasticity to select its response to biochemical messages it receives, including self-alteration and broadcasting messages to initiate alterations in other bacteria. Hence, new features can collectively emerge during self-organization from the intra-cellular level to the whole colony. Collectively bacteria store information, make decisions (e.g. to sporulate) and even learn from past experience (e.g. exposure to antibiotics), features of a bacterial social behaviour. In addition, organisms have to extract latent information included in the environment. By latent information, Ben-Jacob refers to the non-arbitrary spatio-temporal patterns of regularities and variations that characterize the environmental dynamics[1]. In addition, it has been found bacteriocins are able to kill sibling cells that belong to the same colony[2].

Colonies of bacterial cells can display complex collective dynamics, frequently culminating in the formation of biofilms and other ordered super-structures. Recent studies suggest that to cope with local environmental challenges, bacterial cells can actively seek out small chambers or cavities and assemble there, engaging in quorum sensing behaviour[3]. By using a microfluidic device, Cho et al showed that within chambers of distinct shapes and sizes allowing continuous cell escape, bacterial colonies can gradually self-organize[4]. The self-organization described by Cho et al may be crucial for the early stage of the organization of high-density bacterial colonies populating confined growth niches. It suggests that this phenomenon can play a critical role in bacterial biofilm initiation and development of other complex multicellular bacterial super-structures, including those implicated in infectious diseases[4].

Scientifics have highlighted the importance of the development of imaging techniques and their importance for research in biology and microbiology[6]. All novel techniques allow investigators to better knowledge of the organization of bacterial colonies. The aim of the study was to explore the structure and growth dynamics of ***Staphylococcus aureus*** resistant to methicillin (MRSA) colonies using semithin sections visualized by light microscope.

MATERIAL AND METHODS

One *S. aureus* susceptible to methicillin (MSSA) and one MRSA clinical strains were studied. Bacterial suspensions in saline solution from an overnight culture in Mueller–Hinton agar with 5% lysed sheep blood (Biomedics, Madrid, Spain) 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 24 and 48h at 37°C.

After each incubation period (24 h and 48 h), agar plates were flooded with 20 ml of 2% agar solution and were allowed to solidify at room temperature. Cylindrical agar samples containing isolated bacterial colonies were extracted with a punch. Bacterial colonies were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for two hours at room temperature. The colonies were then washed with cacodylate buffer plus 4 % saccharose, dehydrated in 50%, 70%, 90% and 100% alcohol and embedded in epoxy resin (Eponate 12). Semithin sections of 0.5 µm were stained with toluidine blue and visualized by light microscope (Leica, DM5000).

RESULTS

Macroscopically, colonies are circular and convex at 24 h (figure 1) and no differences were observed between MSSA and MRSA colonies. Microscopically, no differences were observed between MSSA and MRSA colonies at 24 and 48 h but differences were observed in both strains between 24 and 48 h incubation periods. After 24 h of incubation the most of the colonies showed two zones or layers clearly differentiated in transversal sections independently of the resistance to methicillin (figure 2): (A) one basal layer with high density of population in contact with culture media, and (B) one superficial layer, over the previous one, with a lower density of population. In some cases, a thin third layer situated between the basal and superficial layer was observed (figure 2). Two different external edges of the superficial layer were observed: smooth in MSSA (figure 3) and frayed in MRSA (figure 4).

Macroscopically, colonies are circular and convex or slightly umbonate at 48 h. In transversal section, most of the colonies showed four layers at 48 h (figure 5): (A) one basal layer with high density of population in contact with culture media; (B) one clear layer constituted by very degraded bacterial remains in which can be observed cocci dispersed with their dyeing properties; (C) one mixed layer constituted by viable bacteria and little degraded bacterial remains. Both components showed each of them reverse gradients through the thickness of the layer: bacterial remains predominate in the lower part in contact with the light layer and viable bacteria predominate in the higher part in contact with the next layer; (D) one superficial layer with a lower density of population than the basal layer and with few bacterial remains. As the colonies at 24 h, two different external edges of the superficial layer were observed: smooth in MSSA and frayed in MRSA.

DISCUSSION

The present work is a study of the colonial architecture and growth dynamics of *S. aureus*. Implicitly, it is accepted that a bacterial colony is the result of the "in situ" growth of a bacteria or cluster giving rise to the concept of colony-forming unit. In any case, it is the basis of the pure culture meaning by the equivalence of cellular clone. Traditionally, it is assumed that a colony consist in the homogeneous accumulation of bacterial cells. In this study, we focused on the structure and dynamics of growing three-dimensional colony collective behaviour of MRSA and MSSA colony-forming units through semithin sections visualized by light microscope. Colonial ar-

Figure 1 Gross appearance of *S. aureus* colonies onto Mueller-Hinton agar supplemented with 5% sheep blood after 24 h of incubation.

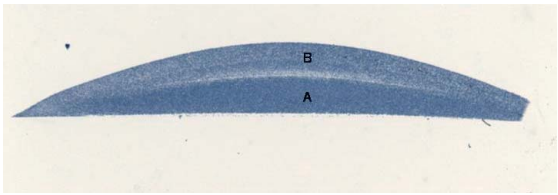

Figure 2 Microscopic appearance of a *S. aureus* colony at 24 h. Colonies show two layers: (A) basal layer, and (B) superficial layer. (Blue toluidine, 20x)

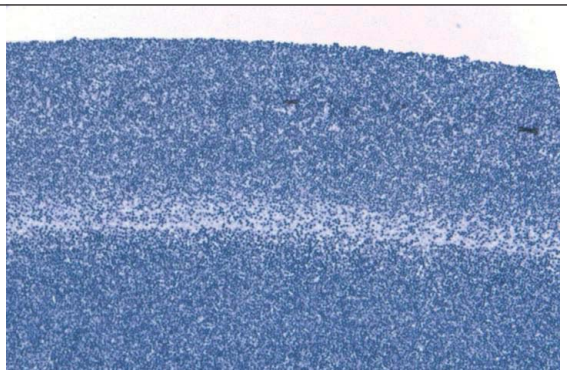

Figure 3 Microscopic appearance of the smooth external edge observed in a MSSA colony at 24 h. (Blue toluidine, 40x)

Figure 4 Microscopic appearance of the frayed external edge observed in a MRSA colony at 24 h. (Blue toluidine, 40x)

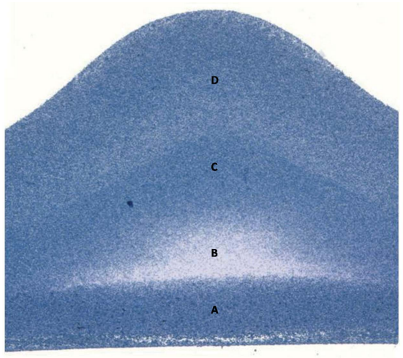

Figure 5 Microscopic appearance of a transversal section of two *S. aureus* colonies at 48 h. i) convex colony, and ii) slightly umbonated colony
Colonies show four layers: (A) basal layer, (B) clear layer, (C) mixed layer, and (D) superficial layer (Blue toluidine, 20x)

chitecture is complex and time-dependent process. Thus, colonies of MRSA and MSSA showed two layers clearly differentiated at 24 h (figure 2) and four layers at 48 h (figure 5). These growth patterns could be explained by nutrients availability. Thus, the A layer with high density of population and more homogeneous is in contact with culture media (high nutrient availability) while the superficial B layer at 24 h shows lower density of population and heterogeneous (low nutrient availability). A mixed layer (C) was observed at 48 h constituted by viable bacteria and little degraded bacterial remains. Bacteria belong to this transition layer could feed due to a nutrient release by lysed cells from B layer. Thus, different layers could be related to different steps in the nutrient availability. This fact could be explained by a cannibalism mechanism, and/or heterogeneity among cells. Finkel et al have demonstrated that *Escherichia coli* is capable of consuming double-stranded DNA as a sole source of carbon and energy and that this ability to "eat" DNA is necessary for the competitive survival of *E. coli* during long-term incubation[5]. Second, Strovas et al have showed the importance of heterogeneity among cells within a population that is responding to environmental changes and develop detailed descriptions of physiological states for individual cells[6]. In our study, those layers could be produced by the different level of cellular specialization. These cells, using complex signaling mechanisms, could be responsible for the distinct shapes and the formation of a highly organized system observed in this study and the self-organization described by Cho et al[4].

This methodology could be used to investigate the evolution of the biofilm formation or to study the interaction among antibiotics and bacteria or mixed populations (bacterial consortium). Thus, this new tool will allow us to study the adaptation of the colonial architecture to the environmental changes since it is a highly evolved and complex process.

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