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Invasion of solid culture media: a widespread phenotypic feature of clinical bacterial isolates

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ABSTRACT

Objectives. The in-depth growth in solid culture media is a common feature in filamentous fungi and yeasts. However, there are very few bacterial species in which this phenomenon has been documented. The aim of this work was to assess the agar invasiveness of a wide range of Gram-positive and Gram-negative bacterial species of clinical interest.

Material and methods. Three different clinical isolates for each of eleven bacterial species were plated onto Columbia blood agar and let grow up to 15 days. Isolated colonies were processed by histological methods, embedded in epoxy resin, and then, semithin sections were stained with toluidine blue and visualized by light microscopy.

Results. Growth within the agar was observed in at least one strain in 9 of the 11 studied species. Invasions of Gram-negative rods were small, not plentiful, and round or triangle-shaped. In Gram-positive cocci, invasions were of big size, abundant and of variable shape (lentiform, globular, irregular, arrowhead) depending on the species.

Conclusions: We propose that the growth within the agar can indicate a survival strategy common to many bacterial species, and so far, not previously reported. This strategy could be either a nutrient gradient tropism or the spread and colonization of new ecological niches, with potential implications in pathogeny.

Keywords: Agar invasion, gram-positive, gram-negative, morphological pattern, epoxy resin.

Invasión del medio de cultivo sólido: un carácter fenotípico frecuente en especies bacterianas de interés clínico

RESUMEN

Objetivos. El crecimiento en profundidad en medios de cultivo sólidos es un fenómeno habitual en hongos filamentosos y levaduras. Sin embargo, son muy escasas las especies bacterianas en las que se ha documentado este fenómeno. El objetivo de este trabajo fue comprobar la capacidad de invasión del agar de un amplio abanico de especies bacterianas grampositivas y gramnegativas de interés clínico.

Material y métodos. Se cultivaron tres cepas de cada una de once especies bacterianas sobre agar Columbia hasta un máximo de 15 días. Colonias aisladas fueron procesadas e incluidas en resina epoxi por métodos histológicos y secciones transversales semifinas teñidas con azul de toluidina fueron visualizadas por microscopía óptica.

Resultados. Se observó crecimiento en el interior del agar en al menos una de las cepas de nueve de las especies estudiadas. En bacilos gramnegativos, las invasiones eran escasas en número, pequeñas y con morfología redondeada o triangular. En cocos grampositivos las invasiones eran de gran tamaño, numerosas y de morfología variable (lentiforme, globular, irregular, en forma de punta de flecha, etc.) según la especie.

Conclusiones. En nuestra opinión, el crecimiento en el interior del agar puede significar una estrategia de supervivencia común a muchas especies bacterianas y hasta ahora prácticamente no descrita. Esta estrategia podría ser el reflejo de un tropismo a favor de gradiente de nutrientes, o bien un fenómeno de diseminación y colonización de nuevos nichos ecológicos, con posibles implicaciones en la patogenia.

Palabras Clave: Invasión del agar, grampositivo, gramnegativo, patrón morfológico, resina epoxi.

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INTRODUCTION

The in-depth growth within agar solid culture media is a common feature in filamentous fungi and also relatively frequent in yeasts or leavduriform fungi such as *Candida* and *Saccharomyces*^{1,2}. In these latter cases, a creamy colony on the surface of the agar is observed, mainly made up by yeasts and pseudomycelia invading the agar medium located just below the colony.

Up to now, however, this feature had not been reported in the vast majority of the bacterial species with clinical importance. Hence, there are very few bacterial genera that are known to invade culture media, sometimes because they grow eroding the media, such as *Eikenella* and *Mycoplasma*, and others because they are able to grown in the thickness of the agar such as *Actinobacillus*.

Recently, a new methodology that enables to study the internal structure of bacterial colonies has been developed^{3,4} and the isolated feature of agar microinvasion of aging colonies of *Escherichia coli* has already been mentioned⁴.

The purpose of the present article was to evaluate the potential for agar invasion of culture media in a wide array of both gram-positive and gram-negative bacteria of clinical interest.

MATERIAL AND METHODS

Three different clinical strains of each of the following eleven species were studied (33 isolates): *Staphylococcus aureus*, *Enterococcus faecalis*, *Streptococcus mitis*, *Streptococcus agalactiae*, *Streptococcus oralis*, *Streptococcus pneumoniae*, *Bacillus subtilis*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Stenotrophomonas maltophilia*. Cultures were done on Columbia Blood Agar (Biomerieux, France) for 1, 3, 7, and 15 days, at a temperature of 35 °C, in constant moisture conditions in order to avoid desiccation of the solid media. Once incubation times were due, a minimum of ten isolated colonies from each plate were processed for light microscopy as described elsewhere⁴. In brief, agar blocks containing isolated colonies 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). Transversal semithin sections (0.5 µm) of colonies were finally stained with toluidine blue dye and visualized by light microscopy.

RESULTS

The invasion of the culture media was observed in 13 out of the 33 studied strains (39.4%). Except for *B. subtilis* and *S. pneumoniae*, the agar invasive growth behavior was seen in at least one strain of the other 9 species (81.8%, table 1). The size and morphology of the invasions varied according to the species. In Gram-negative rods, the invasions were already seen after 3 days of incubation, as small and few round- or

Table 1 Number of invasive and non invasive strains in every studied species.

Species	Number of invasive strains	Number of non invasive strains
<i>S. aureus</i>	2	1
<i>E. faecalis</i>	2	1
<i>S. mitis</i>	1	2
<i>S. agalactiae</i>	1	2
<i>S. oralis</i>	2	1
<i>S. pneumoniae</i>	0	3
<i>B. subtilis</i>	0	3
<i>E. coli</i>	2	1
<i>K. pneumoniae</i>	1	2
<i>P. aeruginosa</i>	1	2
<i>S. maltophilia</i>	1	2

triangular-shaped bulges in the interior of the agar. After 7-15 days of incubation, these invasions detached from the base of the colony as globular or irregular microemboli, without an increase in either size or extending further downward in the agar below the colony (figure 1). Invasions of *E. coli* isolates were more frequent than those of the other three species.

In Gram-positive cocci, the agar invasions were seen later, from days 7 to 15. The morphology and evolution of these invasions also varied among species. In *S. aureus* and *S. agalactiae*, the invasions had a vertical lentil-like shape, and were very numerous in *S. aureus* but scarce in *S. agalactiae*. They grew as time went by and ended up detaching from the colony and extending downward in the agar (figure 2A). Colonies of *E. faecalis* presented a lot of invasions, they also grew with time and ended up detaching from the colony as microemboli but with very variable morphologies (irregular, elongated, globular, croissant-like) and not digging the agar any further (figure 2B). Colonies of *S. mitis* deformed the agar surface when they grew and invaded the agar at two to three points, developing large irregular protruding masses of cells, extending in the thickness of the agar, and reaching a size even larger than the original colony (figure 2C). Finally, as for *S. oralis*, a lot of vertical elongated arrowhead structures extending downward the agar surface after 15 days of incubation and freeing irregular microemboli were observed. The distance that those structures within the agar reached was several times larger than the height of the aerial part of the colony (figure 2D).

DISCUSSION

So far, the invasion of solid culture media has been reported in a very limited number of bacterial species, and it has

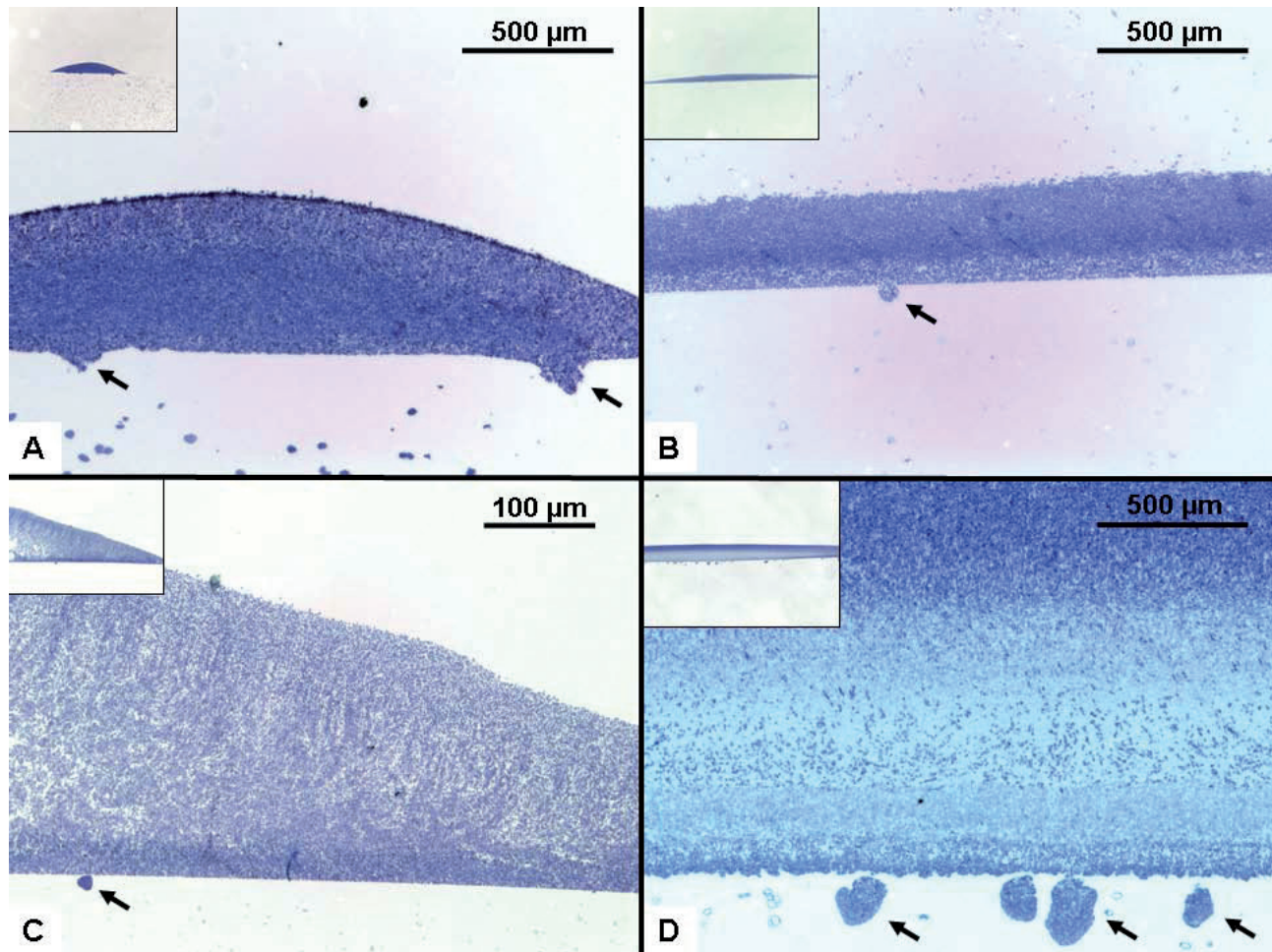


Figure 1

Transverse semithin sections of Gram-negative rods colonies with toluidine blue staining. A) *S. maltophilia* colony after 3 days of incubation. B) *P. aeruginosa* colony after 3 days of incubation. C) *K. pneumoniae* colony after 7 days of incubation. D) *E. coli* colony after 15 days of incubation. Panoramic view (low magnification, 50x) of the colonies in the upper left corner of each picture. Arrows indicate invasive growths within the agar.

been used as an additional macroscopic distinct property in the description of the colony growth to distinguish phenotypically those species. In this work, the use of histological methods for the microscopic internal structure of colonies has enabled us to demonstrate for the first time the existence of this "rare" phenomenon in 9 out of the 11 studied species. Therefore, we seem to face a new and unsuspected phenotypical feature, common to many bacterial species, which might be important in ecological and population dynamics terms. From an evolutionary biology standpoint, the early invasions of Gram-negative bacilli (diderms) opposing to the delayed but much more efficacious invasions of Gram-positive cocci (monoderms) is remarkable, and it might be considered an evolved phylogenetic trait as their cell walls changed from those less complex of Gram-positives and *Archaea*⁵.

This feature is not expressed in all the strains of a same

species, so rather than distinguishing between invasive and non-invasive species, it enables to differentiate between invasive and non-invasive strains. And what is more, our results suggest that this attribute is not simultaneously and uniformly expressed in all the members of the bacterial community that make up a colony, but in specific subpopulations. This might give account for the fact that the growth in the thickness of the agar is not massive from the bottom of the colony, but from concrete spots or locations, more or less frequent in number depending on species and strain.

The fact that the invasion is only observed in colonies at least grown after 3 days or more (7-15 days for gram-positive cocci), might also indicate that the expression of this trait is usually repressed, only unblocking under certain circumstances. It is possible that the impoverishment of the culture media on the surface gives place to the activation of enzymatic

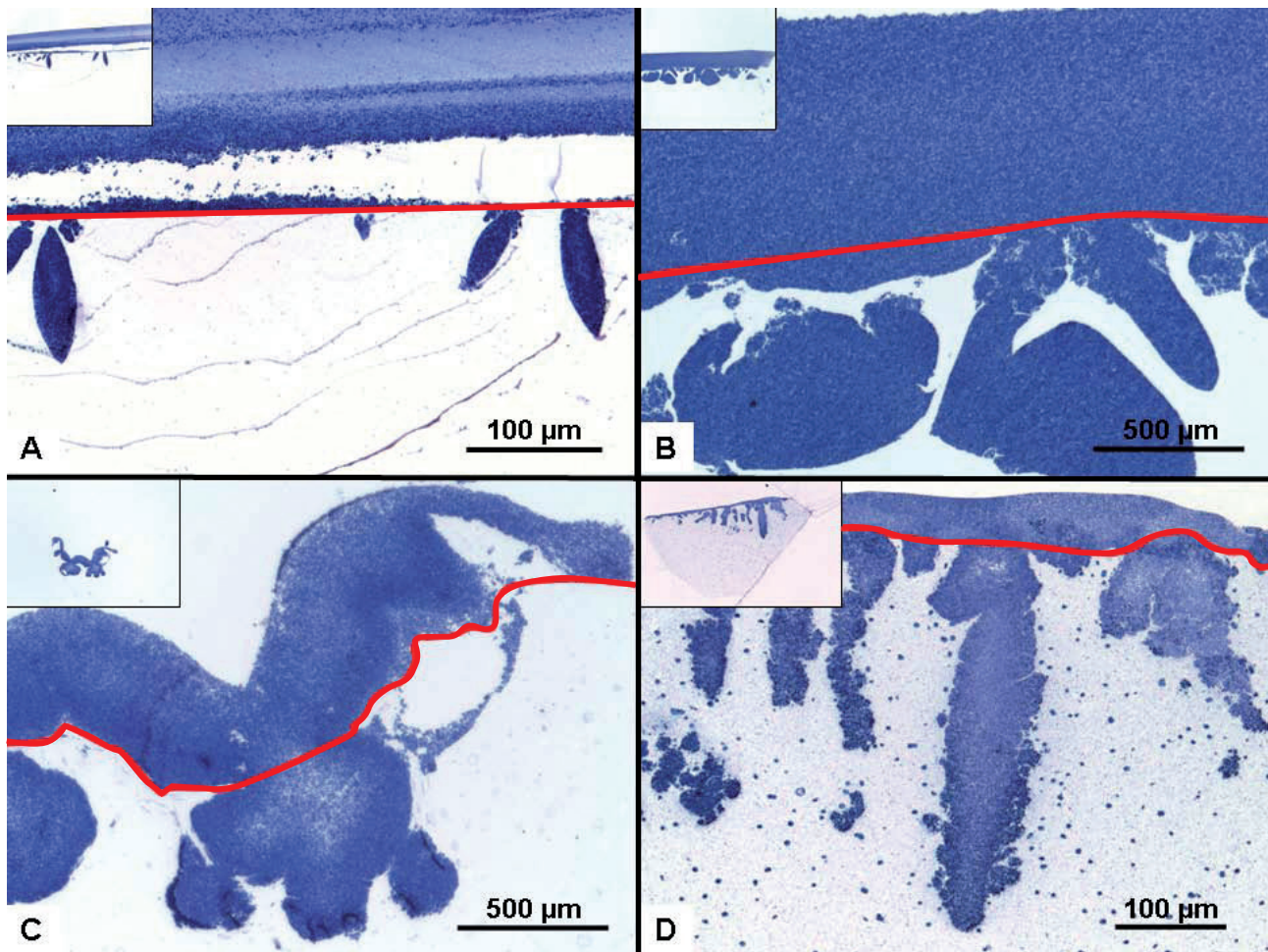


Figure 2

Transverse semithin sections of Gram-positive cocci colonies with toluidine blue staining after 15 days of incubation. A) *S. aureus*. B) *E. faecalis*. C) *S. mitis*. D) *S. oralis*. Panoramic view (low magnification, 50x) of the colonies in the upper left corner of each picture. The red line indicates the surface of the agar.

systems involved in the degradation of agar. In this case, the invasion would be related to a nutrient gradient tropism.

But it might also be possible that invasions represent a strategy of dissemination and colonization of new ecological niches, triggered by ageing indicators such as the population density or the accumulation of wasting products. This could have interest in bacterial species of clinical importance regarding the pathogeny of infection, especially in surface-colonizer and biofilm-maker species. In *Candida*, for instance, in which the invasion of culture media is well documented, there is some correlation between this property and the *in vivo* tissue colonization, since in both cases it is carried out by the pseudomycelium⁶. In fact, some authors consider this invasive feature as a potential virulence factor in molds^{7,8}. The expression of this invasiveness might be of great interest for *S. oralis* as a starter microorganism for the complex multimicrobial biofilm that constitutes the dental plate by anchoring it to the teeth and easing its spread.

We are aware that there are many more questions than answers coming up from our results regarding the ecological and pathogeny importance of agar invasiveness, its triggering factors^{2,9}, genetic systems and molecules involved^{10,11}, and the potential role of antibiotics on the inhibition or selection of invasive subpopulations¹², that will have to be dealt with in future works.

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