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INTERACCIÓN DE LOS ASPECTOS GENÉTICOS Y FÍSICOS EN EL DESARROLLO DE AGREGADOS MULTICELULARES DE *MYXOCOCCUS XANTHUS*

TESIS
PARA OPTAR POR EL GRADO DE: DOCTORA EN CIENCIAS

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Índice

0. Resumen

Entender el origen de la variación biológica ha sido una motivación constante en la historia de la biología, y ha resultado en la construcción de un marco empírico y conceptual en el que diversas áreas de la ciencia se han encontrado. Dentro de este marco, figuran de manera importante el estudio del desarrollo de los organismos y su interacción con el ambiente, integrando aspectos de la ecología, el desarrollo y la evolución. Dependiendo del tamaño de los organismos y de la escala ambiental que es relevante para ellos, distintos factores pueden moldear las trayectorias de su desarrollo y los fenotipos resultantes. En esta tesis se aborda el papel del medio ambiente en el desarrollo de estructuras multicelulares microbianas. Específicamente, se hace una revisión acerca de la contribución de las fuerzas mecánicas en el desarrollo multicelular de algunos microorganismos. Además, se muestran resultados experimentales sobre el estudio particular del desarrollo multicelular de la bacteria *Myxococcus xanthus* sobre substratos con rigidez variable. Finalmente, se presenta una discusión sobre los posibles sesgos experimentales asociados a la elección de las variables ambientales y los organismos en el diseño de experimentos de laboratorio, y cómo estos sesgos dificultan la integración de la ecología en el estudio del desarrollo y la evolución de los organismos. Mediante la integración de esta evidencia, se concluye que 1) la plasticidad fenotípica en estructuras multicelulares microbianas resulta de la interacción de los factores genéticos, los ambientales y la interacción entre ellos, 2) que esta plasticidad se manifiesta de forma específica en distintos rasgos y escalas del desarrollo, sobre todo en ambientes extremos y 3) que el estudio de la plasticidad puede ayudar significativamente a comprender los eventos de transición a la multicelularidad.

1. Introducción

Desde el comienzo del desarrollo de los organismos, es decir, desde que están formados a partir de una sola célula, y hasta que culminan en estructuras multicelulares con formas y patrones característicos de su especie, distintos factores participan en los procesos de morfogénesis, contribuyendo, por un lado, a la formación de estructuras robustas, y por otro, a la variación en las características del organismo que se va moldeando. Incluso, después de la formación aparentemente completa de los organismos, el ambiente del que forman parte sigue contribuyendo al cambio en sus fenotipos, a la variación. ¿Cuáles son estos factores y mediante qué mecanismos participan en el desarrollo de los organismos?

Esta tesis es parte de una línea de investigación enmarcada dentro del área de la biología evolutiva del desarrollo, la cual estudia las variables y los procesos que participan durante el desarrollo de los organismos y su variación en la historia evolutiva. Este trabajo es un acercamiento al estudio de la interacción organismo-ambiente en donde se considera la contribución de los factores genéticos, los factores ambientales y su interacción en el desarrollo robusto y plástico de los organismos y, potencialmente, una aproximación a una de las grandes transiciones evolutivas: la transición a la multicelularidad. Específicamente, se aborda, para el caso de los microorganismos, el papel del substrato en la interacción con los microorganismos y las propiedades físicas de éste. La estructura de la tesis está conformada por tres artículos científicos: una revisión bibliográfica sobre la contribución de las fuerzas mecánicas en el desarrollo de los microorganismos (capítulo 2), un artículo con resultados experimentales respecto a la contribución del genotipo, de la rigidez del substrato, y de su interacción en el desarrollo multicelular de la bacteria *Myxococcus xanthus* (capítulo 3), y un artículo de opinión en el que se discute cómo los diseños experimentales y el uso de cepas de laboratorio pueden sesgar las conclusiones si se extrapolan los resultados de laboratorio hacia los contextos ambientales naturales; y cómo a su vez, esto dificulta la integración de la ecología en el estudio del desarrollo y la evolución de los organismos (capítulo 4). Finalmente, se presentan conclusiones generales y perspectivas que integran los capítulos anteriores (capítulo 5).

2. El eco-evo-devo y la microescala: el papel de las fuerzas mecánicas en el desarrollo de microorganismos multicelulares

¿Cómo se origina la variación biológica? Ésta es una de las preguntas centrales de la biología. Con el transcurso del tiempo, la evolución de nuevas técnicas y la integración de distintos campos de la ciencia, las aproximaciones al estudio de esta pregunta han ido modificándose. Durante la formulación de la Síntesis Moderna, por ejemplo, se aceptó que la fuente relevante de variación fenotípica y de variación en los procesos del desarrollo de los organismos era la variación genética, y que la información necesaria para el desarrollo de los organismos estaba contenida en ellos (Robert, 2004; Jablonka & Lamb, 2014). Más recientemente, la evidencia teórica y empírica ha expandido esta visión y se ha reconocido que otros procesos y factores como los sociales, ecológicos, fisicoquímicos (además de otros factores relacionados con el desarrollo de los organismos) contribuyen fundamentalmente a la variación fenotípica (Newman, 2012; Jablonka & Lamb, 2014; Arias del Angel *et al*., 2015; Gilbert & Epel, 2015; Sultan, 2015; Bonner, 2016).

El área de estudio eco-evo-devo (del inglés *ecological evolutionary developmental biology*) ha surgido reconsiderando la importancia de estudiar el desarrollo y la evolución de los organismos en sus contextos ecológicos naturales, o en condiciones semejantes a éstos, teniendo en cuenta la relevancia ecológica de las variables ambientales, así como la de sus intervalos o rangos de variación (Gilbert & Epel, 2015; Sultan, 2015). Bajo esta perspectiva, se ha observado que las propiedades específicas de cada contexto ambiental determinan, al menos parcialmente, las trayectorias del desarrollo de los organismos. Por ejemplo, la luz, la textura y la disponibilidad de nutrientes del suelo son variables que modifican el proceso de desarrollo en las plantas, reflejándose en cambios en sus rasgos fenotípicos; mientras que variables como la presencia de depredadores, o la densidad poblacional, son importantes para el desarrollo de las especies animales (Gilbert & Epel, 2015; Sultan, 2015).

Sin embargo, otras variables ambientales que podrían pasar aparentemente desapercibidas para organismos de escala métrica, adquieren relevancia en la microescala. Las variables

relacionadas con las propiedades reológicas o elasto-mecánicas del medio o del substratro son algunas de ellas (Persat *et al*., 2015; Even *et al.*, 2017). Por ejemplo, debido a su tamaño, los microorganismos habitan ambientes dominados por la viscosidad, y no por la inercia, como en el caso de los macroorganismos (Persat *et al.*, 2015). Además, dado que frecuentemente se encuentran en contacto con otras células y asociados a distintos tipos de superficies con propiedades físicas y mecánicas diferentes, no es sorprendente que los microorganismos sean sensibles a las propiedades físicas (e.g. dureza o deformabilidad) de su substrato (Guégan *et al*., 2014; Be'er *et al*., 2015; Persat *et al*., 2015). Por ejemplo, en el caso de la especie bacteriana *Myxococcus xanthus,* se sabe que cuando el sustrato en el que crecen es comprimido, sus células tienen la capacidad de alinearse de forma perpendicular a dicha fuerza de compresión (Fontes & Kaiser, 1999; Lemon *et al*., 2017). También, se ha reportado que la expansión de las biopelículas bacterianas puede estar regulada por la fuerza de tensión superficial en la interacción de la biopelícula y la superficie sobre la que reposa (Trinschek *et al*., 2017). En este sentido, es evidente que las fuerzas mecánicas que actúan sobre el medio y sobre los propios microorganismos, proveen de condiciones necesarias para el movimiento y la organización de las células microbianas.

El estudio de los microorganismos resulta aún más interesante considerando que algunos de ellos pueden desarrollarse en estructuras multicelulares estereotípicas que albergan distintos tipos celulares como parte de su ciclo de vida (Shapiro, 1988; Shapiro, 1998; Aguilar *et al*., 2007). Además, su ambiente y su escala asemejan los escenarios en los cuales pudo haber emergido la multicelularidad, lo cual es relevante si se piensa que la transición de la vida unicelular a la escala multicelular es uno de los eventos más sobresalientes en la historia evolutiva (Smith & Szathmary, 1995; Grosberg & Strathmann, 2007). El hecho de que la multicelularidad haya ocurrido múltiples veces de maneras independientes permite hacer comparaciones orientadas a identificar las generalidades y las particularidades de los eventos de transición a la multicelularidad, y con ello, proponer algunos principios que gobiernan los procesos del desarrollo (Bonner, 2009; Bonner, 2016). En ese sentido y debido a la relativa simpleza de su estructura y de su desarrollo en general, el estudio de los grupos microbianos que forman estructuras multicelulares puede contribuir de manera importante al entendimiento de las interacciones organismo-ambiente en la evolución de la multicelularidad.

En este capítulo, se presenta un artículo de revisión en el cual se integra la información de trabajos recientes en torno al papel de las fuerzas mecánicas en el desarrollo de estructuras multicelulares microbianas. A través de ejemplos de grupos microbianos bien estudiados o considerados como organismos modelo, se discute cómo estas fuerzas, en interacción con

otros factores ecológicos y bioquímicos, pueden actuar guiando y restringiendo el desarrollo de las estructuras multicelulares. Además, se discute cómo el estudio del desarrollo en la microescala permite y se apoya en la integración de distintas áreas de la ciencia como la física y la biología, pero también de enfoques empíricos, conceptuales y teóricos para la prueba de hipótesis, la identificación de huecos experimentales y la generación de predicciones que retroalimenten las observaciones que se tienen actualmente. Por ejemplo, se ha reportado que la rigidez del substrato afecta la adhesión de las bacterias con éste, y que, por lo tanto, los conglomerados de células que crecen en superficies con diferentes grados de rigidez varían entre sí en su tamaño y su disposición espacial (Guégan *et al*., 2013). Sin embargo, identificamos que aún es necesario entender y caracterizar la variación de los rasgos fenotípicos de estructuras multicelulares que resulta de esta interacción con el substrato, así como los mecanismos precisos detrás de estos procesos. Finalmente, se concluye que el contexto ambiental y sus propiedades pueden tener un papel como agentes causales, no sólo como variables contingentes, en el desarrollo de los organismos y en la transición de éste a través de las diversas escalas en la historia la vida.

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Current Opinion in Genetics & Development

Microbial multicellular development: mechanical forces in action

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Multicellular development occurs in diverse microbial lineages and involves the complex interaction among biochemical, physical and ecological factors. We focus on the mechanical forces that appear to be relevant for the scale and material qualities of individual cells and small cellular conglomerates. We review the effects of such forces on the development of some paradigmatic microorganisms, as well as their overall consequences in multicellular structures. Microbes exhibiting multicellular development have been considered models for the evolutionary transition to multicellularity. Therefore, we discuss how comparative, integrative and dynamic approaches to the mechanical effects involved in microbial development can provide valuable insights into some of the principles behind the evolutionary transition to multicellularity.

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Introduction

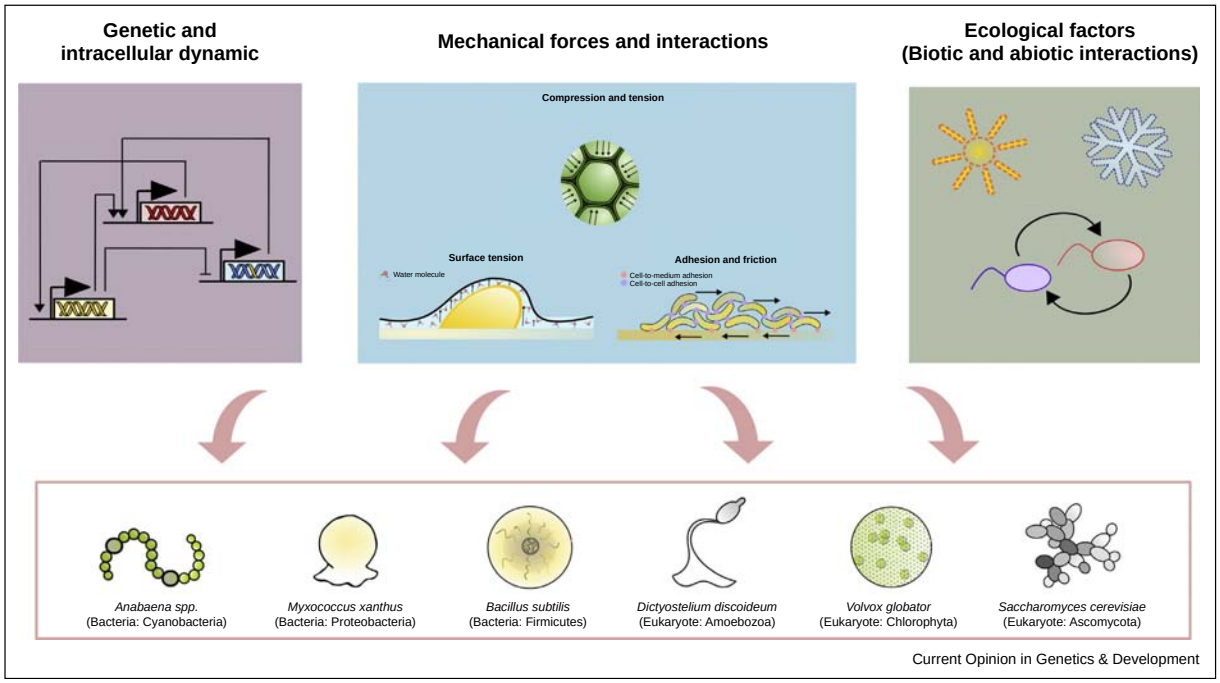
Multicellularity is considered to be one of the major transitions in evolution and it is estimated to have evolved independently at least 25 times [1,2]. Moreover, if one considers multicellular organisms as conglomerates conformed by multiple cells that are able to adhere to each other, coordinate via intercellular communication, and exhibit some degree of division of labor or cell differentiation, it can be argued that multicellular behaviors and structures continue to emerge in different environments with many ecological, evolutionary, medical and practical implications [3–5].

When thinking about multicellularity, plants and animals immediately come to mind. Nevertheless, some microorganisms can develop stereotypical multicellular structures harboring different cell types as part of their life cycles [6,7]. Indeed, multicellular phenotypes have been reported in several prokaryotic and eukaryotic lineages of microorganisms, such as cyanobacteria, actinobacteria, myxobacteria, dyctiostelids, yeasts, and algae, among others [2,8–10] (Figure 1). Within these diverse groups, multicellularity can be attained by incomplete cell division (clonal development, as observed in plants and animals) or by cell aggregation (sorocarpic development, as observed in some groups of fungi and bacteria). The fact that multicellularity has evolved in such distant lineages enables broad comparative studies, and the search for general principles in development [11]. Furthermore, due to the relative simplicity of their structure and development, multicellular microbial groups can provide key insights regarding the basic principles of the transition to multicellularity.

Genomic comparisons between extant multicellular taxa and their closest unicellular relatives suggest that the transitions from a unicellular to a multicellular lifestyle did not necessarily require large genomic changes, but rather that these transitions have involved the co-option of components and pathways likely to be already present in the unicellular ancestors [12–14]. Interestingly, despite the vast diversity of microorganisms, their known multicellular phenotypes can be reduced to a few convergent structures, such as spheric, stalked or branching fruiting bodies, many of which are reached by evolutionary convergence. Moreover, while traits such as intercellular communication and adhesion have been recognized to be shared by many or all the identified multicellular lineages, the genetic factors associated with these traits may be remarkably different among them [12,15,16]. This suggests that, besides genetic changes, developmental, social, ecological, environmental, physicochemical and mechanical factors are also determinant in the development and evolution of microbial multicellularity [17•,18,19,20••,21] (Figure 1). Nutrient availability, the presence of symbionts, or reaction-diffusion processes are examples of these factors [22–27].

From the diversity of factors involved in microbial development, physical processes and effects play a key role in

Figure 1



The interaction among different factors impacts the development of phenotypes in multicellular microorganisms. Diverse factors that interact during the generation of the phenotypic diversity observed across lineages are represented within boxes. Since mechanical forces involved in microbial multicellularity are the focus of this review, the corresponding box details specific examples of forces acting on microorganisms during development. Red arrows emphasize how the joint effect of genetic, mechanical and environmental factors give rise to the diverse multicellular phenotypes shown below. Examples for the organisms shown in this figure are discussed in the main text, Figures 2 and 3. For each model organism, the corresponding kingdom and phylum are noted.

the establishment of robust multicellular patterns and forms, and have been often considered to explain recurrent aspects of development and collective cellular behavior [14,17•,28•,29]. Mechanical forces in particular have been shown to largely influence the development and evolution of living beings [25,30,31•]. We review recent work — focusing on the last two years — that helps understand how these forces, in complex interaction with ecological and biochemical factors, can act both as drivers and restrictors in the development of microbial multicellular structures.

Mechanical forces involved in microbial multicellular development

Physical forces involved in multicellular development may vary across scales. Actually, even though these forces have been often been thought of as extremely generic, that is, affecting both living and nonliving matter, they can act in a very specific manner depending on the spatiotemporal scale and the nature of the materials on which they are exerted [28•] (Figure 2). For example, due to their remarkable differences in size, large animals experience distinct physical environments as compared to microorganisms: while the latter experience much larger inertial than viscous forces, microorganisms live in a 'world of low Reynolds numbers' in which viscous forces are larger than inertial ones, influencing their modes of mobility. Similarly, some mechanical effects associated with tension and compression, for example, could become very influential forces immediately after the transition to a multicellular lifestyle [32].

Diverse mechanical forces come into play at the change in scale that characterizes the transition from a cell measuring a few micrometers to three-dimensional microbial structures attached to a surface or suspended in a liquid medium [32]. Such forces are manifested in the single-cell morphology, the cell-to-cell and cell-to-surface interactions, as well as the size, shape and spatial arrangement of cellular conglomerates [33••,34•]. Below, we review some experimental and theoretical efforts to study the physical forces involved in prokaryotic and eukaryotic microbial systems, considering cases that illustrate the role of physical forces at different levels, from the single-cell to the conglomerate level, as well as cell-to-cell and cell-to-surface interactions.

Figure 2

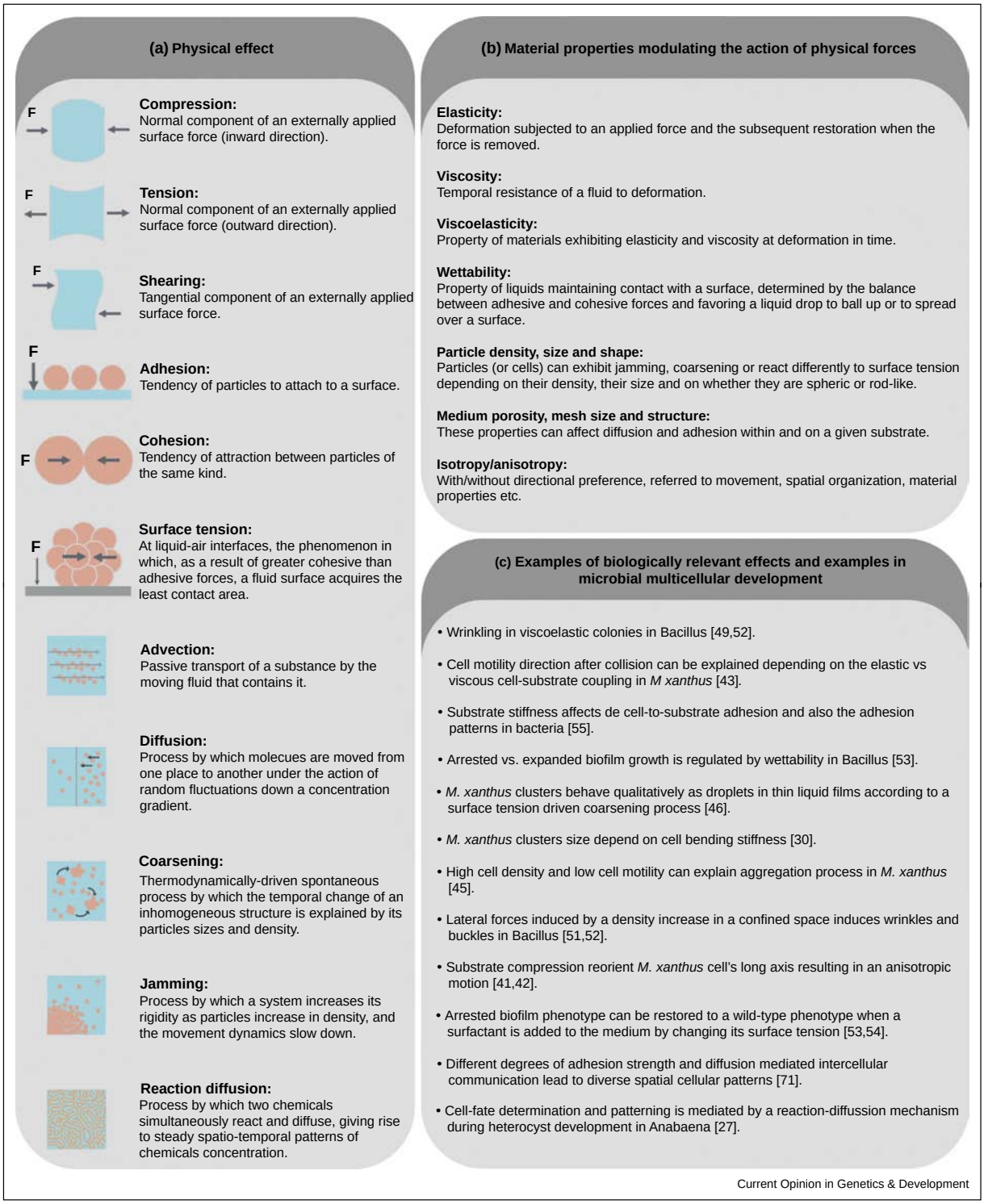

(a) Physical effect
F
Compression:
Normal component of an externally applied surface force (inward direction).
F
Tension:
Normal component of an externally applied surface force (outward direction).
F
Shearing:
Tangential component of an externally applied surface force.
F
Adhesion:
Tendency of particles to attach to a surface.
F
Cohesion:
Tendency of attraction between particles of the same kind.
F
Surface tension:
At liquid-air interfaces, the phenomenon in which, as a result of greater cohesive than adhesive forces, a fluid surface acquires the least contact area.
Advection:
Passive transport of a substance by the moving fluid that contains it.
Diffusion:
Process by which molecues are moved from one place to another under the action of random fluctuations down a concentration gradient.
Coarsening:
Thermodynamically-driven spontaneous process by which the temporal change of an inhomogeneous structure is explained by its particles sizes and density.
Jamming:
Process by which a system increases its rigidity as particles increase in density, and the movement dynamics slow down.
Reaction diffusion:
Process by which two chemicals simultaneously react and diffuse, giving rise to steady spatio-temporal patterns of chemicals concentration.
(b) Material properties modulating the action of physical forces
Elasticity:
Deformation subjected to an applied force and the subsequent restoration when the force is removed.
Viscosity:
Temporal resistance of a fluid to deformation.
Viscoelasticity:
Property of materials exhibiting elasticity and viscosity at deformation in time.
Wettability:
Property of liquids maintaining contact with a surface, determined by the balance between adhesive and cohesive forces and favoring a liquid drop to ball up or to spread over a surface.
Particle density, size and shape:
Particles (or cells) can exhibit jamming, coarsening or react differently to surface tension depending on their density, their size and on whether they are spheric or rod-like.
Medium porosity, mesh size and structure:
These properties can affect diffusion and adhesion within and on a given substrate.
Isotropy/anisotropy:
With/without directional preference, referred to movement, spatial organization, material properties etc.
(c) Examples of biologically relevant effects and examples in microbial multicellular development
• Wrinkling in viscoelastic colonies in Bacillus [49,52].
• Cell motility direction after collision can be explained depending on the elastic vs viscous cell-substrate coupling in M xanthus [43].
• Substrate stiffness affects de cell-to-substrate adhesion and also the adhesion patterns in bacteria [55].
• Arrested vs. expanded biofilm growth is regulated by wettability in Bacillus [53].
• M. xanthus clusters behave qualitatively as droplets in thin liquid films according to a surface tension driven coarsening process [46].
• M. xanthus clusters size depend on cell bending stiffness [30].
• High cell density and low cell motility can explain aggregation process in M. xanthus [45].
• Lateral forces induced by a density increase in a confined space induces wrinkles and buckles in Bacillus [51,52].
• Substrate compression reorient M. xanthus cell's long axis resulting in an anisotropic motion [41,42].
• Arrested biofilm phenotype can be restored to a wild-type phenotype when a surfactant is added to the medium by changing its surface tension [53,54].
• Different degrees of adhesion strength and diffusion mediated intercellular communication lead to diverse spatial cellular patterns [71].
• Cell-fate determination and patterning is mediated by a reaction-diffussion mechanism during heterocyst development in Anabaena [27].
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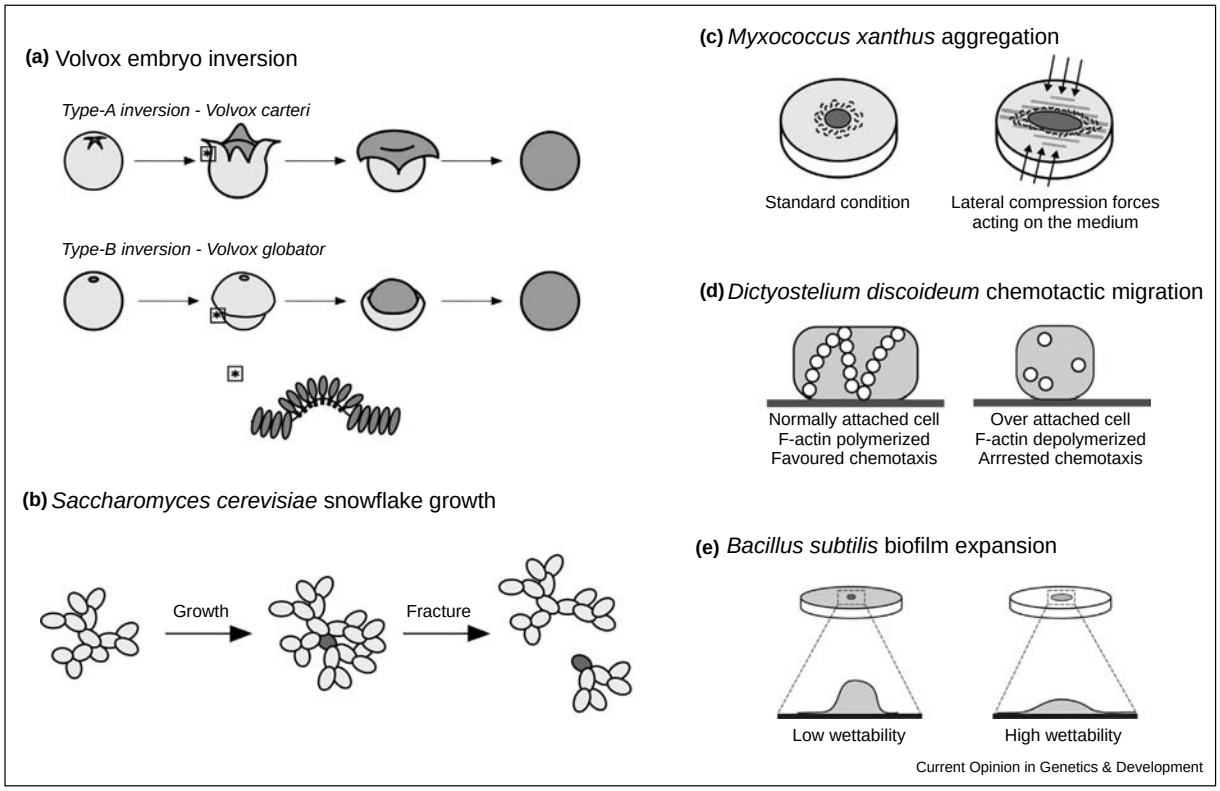
Figure 3



Mechanical forces effect in the multicellular development of model microorganisms. **(a)** During its development, Volvox embryo exhibits an inversion following either the A-type or B-type mechanism. Such inversion is initiated by local changes in the cell shape. **(b)** Multicellular yeast snowflakes grow by incomplete cell division and spontaneously fracture due to the accumulation of mechanical stress (cell with highest stress is indicated in dark gray). **(c)** Colonies of vegetative *Myxococcus xanthus* cells grow outwards and radially on agar plates. However, after the action of lateral mechanical compression over the agar substratum, cells reorient perpendicular to the compression axis resulting in an elongated colony. **(d)** During the chemotaxis-dependent aggregation phase of Dyctiostelum development, the medium properties affect the F-actin cytoskeleton structure and, concomitantly, the attachment strength and cell mobility. **(e)** The spreading or arrested spreading of a biofilm can be understood as a wetting phenomenon in which the extracellular matrix modifies surface tension. At high surface tensions (low wettability) the biofilm will have an arrested spreading, while at a lower surface tensions (high wettability) it will expand.

Volvox globator and elastic properties relevant for embryo inversion

The *V. globator* embryo is formed by a monolayer spheroid consisting of motile somatic cells that enclose non-motile germ cells [35]. At this stage, somatic cells are physically attached via cytoplasmic bridges and form an elastic sheet that defines the organismal boundaries and is able to experience stretching and bending [36,37•]. However, an anterior–posterior axis is defined by the presence of an opening called a phialopore, where cells are not connected to each other, at the anterior pole in the cell sheet [35,37•]. At early stages of the embryo's development, the flagella of the motile cells point toward the inside of the sphere, and the embryo must be inverted to bring flagella to the outside to be employed for organism motility [35].

In *V. globator*, inversion occurs through the so-called type-B mechanism. In this process, the cells near the embryo equator change from columnar-shaped to wedge-shaped, causing tensile stress to accumulate in the elastic sheet and favoring local bending (Figure 3). In turn, bending helps to alleviate the tension and finally triggers an initial

(Figure 2 Legend) Summary of different physical effects involved in the multicellular development of microorganisms. **(a)** Physical effects which influence the medium, its surface, the cell-to-medium and cell-to-cell interaction. **(b)** Material properties by which physical forces act over the medium and cells. **(c)** Some specific biological examples of how physical effects and material properties play key roles in microbial multicellular development.

invagination, leading to the inversion of the whole embryo [37•]. By using modeling approaches, it has been suggested that neither the elastic properties of the cell sheet nor the changes in cell shape alone explain the experimental observations, and that inversion requires both of them to occur. By contrast to the type-B inversion observed in *V. globator*, *V. carterii* and other Volvox species undergo type-A inversion. In this case, inversion is initiated by cells undergoing shape changes near the phialopore instead of the embryo equator [36]. In both type-A and type-B inversion, the joint effect of the elastic properties of the cell sheet and the local changes in cell shape is crucial for inversion [36,37•]. The mechanisms determining the position where cells initially change their shapes remain to be elucidated.

Saccharomyces cerevisiae jamming and size-related mechanical stress

S. cerevisiae is capable to develop a multicellular phenotype termed 'snowflake' consisting of a cluster of clonal cells [38]. As the 'snowflake' increases in number of cells and, concomitantly, aggregate size, mechanical stress accumulates within the cluster and when it reaches a certain threshold, spontaneous fractures occur and give rise to two or more independent conglomerates. Recently, Jacobeen and co-workers [31•] showed that multicellular yeast conglomerates may overcome this size limit, and increase their size up to 70%, by generating more elongated cells and ultimately delaying the accumulation of stress and fracturing. It is worth noting that the increase in cluster size it is not only due to increase in individual cell size, but also to a larger number of cells in the cluster. The authors argue that when cells become more elongated they are packed differently due to a jamming effect (Figure 2). Jamming diminishes the accumulation of stress and delays the spontaneous fracture because of cellular crowding inside the cluster. Additionally, previous work in this system shows that cells at the fracture points undergo apoptosis [38]. It could thus be speculated that mechanical stress influences cell fate determination during 'snowflake' cluster development (Figure 3).

Another study on budding yeast populations in confinement showed that single-cell forces add up into large mechanical pressures as the population grows [39], also resulting in cellular jamming and leading to a gradual formation of force chains. As in the study reviewed above, mechanical forces seem to feed back to the cells' properties because the confinement stress causes yeast cells to undergo shape deformation and to decrease their growth rate.

Myxococcus xanthus coarsening dynamics and response to medium changes

M. xanthus is a rod-shaped bacterium that moves across semi-solid surfaces in the direction of the cell's long axis [40]. Over nutrient rich substrates, vegetative cells swarm, expanding the colony outward. If nutrients are depleted, they glide inward, developing multicellular structures called fruiting bodies, where eventually, some cells differentiate into resistant spores [40]. In both cases, the structural and mechanical properties of the substrate affect their movement at the cellular and multicellular scale. When the agar mesh is mechanically compressed, agarose polymers rearrange and the cells reorient their long axes perpendicular to the direction of compression in a few minutes, which spontaneously gives rise to a collective anisotropic movement [41,42••] (Figure 3). Interestingly, this behavior is common in other bacteria (e.g. *Bacillus subtilis* and *Paenibacillus dendritiformis* [42••]). *In silico* and *in vitro* experiments also show that the clustering of individual cells can be explained largely by mechanical interactions among cells, and between the cells and the substrate. Specifically, as cells move and collide, they align in a direction determined largely by the interaction with a viscoelastic substrate [43,44]. *M. xanthus* development can also be partially reproduced by simulating the dynamics of simple interactions between cells as a phase separation process using a modified active-Brownian-particle model [45]. Moreover, the size, establishment and disappearance of cellular aggregates that eventually give rise to fruiting bodies has been accurately predicted — until some point in fruiting body development — by assuming that cell clusters behave according to a surface-tension-driven coarsening process similar to that configuring droplets in thin liquid films [46] (Figure 2).

Dictyostelium discoideum cell-to-medium interactions in chemotactic migration

Similar to *M. xanthus*, *Dictyostelium discoideum* cells aggregate to form fruiting bodies when nutrients in the medium are scarce, but in this case aggregation is triggered by chemotaxis towards the highly diffusible cyclic adenosine monophosphate (cAMP) [12]. For aggregation, *D. discoideum* requires a solid surface and thus the mechanics of cell-to-medium interactions impact the progress of development [25]. Interestingly, the cells perceive the strength of the interaction with the medium through the actin cytoskeleton and the PI3K signaling pathway, which in turn affects the synthesis of cAMP and the propagation of waves triggering chemotaxis [25] (Figure 3).

Biofilms, compression and interaction with the substrate

Biofilms can also form surface-attached structures involving properties such as cellular adhesiveness. However, it remains controversial whether they exhibit strict multicellularity or not, mainly because these cell conglomerates may not entail the stereotypic recreation of individuals through well-defined developmental stages [47,48]. Despite this controversy, studying the influence of mechanical forces acting over cell-to-cell and cell-substrate interactions on the organization of biofilms allows

further understanding of some principles present during the formation of complex three-dimensional patterns and metabolic integration within cell conglomerates, both of which are essential for multicellular development [3,20••]. Moreover, due to their importance in biomedical and technological applications, there is vast evidence regarding physical forces in biofilm organization and growth. We will briefly review some key examples, but there are in-depth reviews that focus on physical forces acting on biofilms [33••,34•,49].

Recent work shows that cell-to-cell compression through the increase of cellular density in a confined space generates lateral forces that can in turn lead to cellular alignment within the biofilm extracellular matrix (ECM). As the ECM fills in the extracellular space, cellular rotational movement and Brownian diffusion are suppressed, further stabilizing cellular alignment (nematic-like order) [50]. Also, the patterns of wrinkles and buckles of *Bacillus subtilis* biofilms in an air–liquid interface have been described as a consequence of mechanical instability due to the lateral compression caused by increased cellular density [51]. Similar patterns have been described as the result of spatially-focused lateral forces promoted by cell death [52].

Forces associated with the cell–substrate interaction may also modify biofilm growth and patterning. The interplay between cell-to-cell cohesive forces and cell-to-surface adhesion forces (wettability (W)) can determine the expansion or arrest of a biofilm (Figure 3). Using a theoretical model, Trinscheck and coworkers [53] showed that at high wettability, that is, when the contact area between the surface and the biofilm is larger, the biofilm will have a low surface tension and consequently, expand horizontally and grow. By contrast, for lower wettability the biofilm will remain in an arrested state because of a higher surface tension. Furthermore, when the surface tension is changed by adding a surfactant to the medium, arrested biofilms expand [53,54]. Additionally, substrate stiffness can modify electrostatic attractive and repulsive forces, Van der Waals forces and Brownian motion, which in turn, may influence microbe-surface interaction and adhesion. For instance, *Pseudoalteromonas* sp. colonies adhere better to a 3% than to a 0.75% agarose hydrogel, while *Bacillus* sp. forms larger clusters in 0.75% than in 3% agarose hydrogels [55]. Also, variations in cell shape may lead to different clustering patterns. Indeed, the interaction of wild-type elongated *Escherichia coli* cells and spherical cells from a mutant strain, lead to the spontaneous emergence of a layered structure consisting of the spherical cells laying at the top and the elongated ones at the bottom of the structure [56]. Overall, biofilm expansion, growth and spatial patterning can be explained by considering mechanical forces and effects, and not only bioactive processes or intrinsic single-cell motion.

Towards an integral study of the development and evolution of microbial multicellularity

From these and other examples, it is possible to postulate some common themes in microbial multicellular development. For instance, mechanical forces can act both at short-range and long-range scales to provide robust cues or conditions for cellular motion, organization and differentiation. Also, ECM, slime and other substances at the unicellular or multicellular interface appear to be key integrators of biochemical and mechanical processes, largely mediating local cell-to-cell and cell–substrate interactions and contributing to the generation of specific microenvironments. Terms that refer to the joint mechanical and biochemical effects in microbial development are currently emerging or becoming commonly used, such as elasticotaxis (cellular reorientation response to elastic forces within the substrate) and polymertropism (cellular response to the arrangement of the polymers in the substrate) [41,42••]. Also, we have focused on mechanical forces involved in the development of microbial structures, but these forces interact in a dialectic way with other factors [49,57]. On the one hand, mechanical forces modify cellular attributes, as reviewed above for different examples. In this context, mechanosensing emerges as a promising field to further understand these processes [25,58]. On the other hand, unicellular and multicellular organisms may modify their physical environment or enable certain scale-dependent physicochemical processes, which in turn interact with biochemical and ecological factors to define multicellular form and function. For instance, fungi and bacteria can produce surfactants or amyloid fibrils that allow them to locally lower the water surface tension and grow their multicellular structures apically into the air [59–61]. Mechanical forces can also shape ecological interactions, for example, by preventing the contact among bacterial colonies that can generate a tension border as they move [62, but see 21].

These complex and multiscale interactions and the nature of cellular conglomerates (soft, excitable materials that pose a challenge to the characterization of physico-chemical effects [28•]) call for an integrative and interdisciplinary approach to understanding the multicellular development of microbial structures. Thus, we require conceptual, mathematical and computational models that enable us to articulate biological and physical evidence and study the spatiotemporal dynamics of multicellular development. Ideally, such models would contribute not only to rendering a dynamic account of this process, but also to testing hypotheses, identifying empirical gaps, and generating novel predictions that feed back to experimental work.

Among the conceptual frameworks that we find extremely useful in this context is that of 'dynamical patterning modules' postulated by S. Newman and collaborators [14,28•] to put forward a set of physico-chemical processes that, in association with specific molecules,

give rise to the basic patterns and forms observed in plants, animals and microorganisms [63–65]. In these cases, researchers have postulated a set of modules conformed by relatively well-conserved molecules in association with the physicochemical processes they mobilize. In animals, for example, some of these modules may account for multilayering, segmentation or lumen formation. The combined action of these modules is proposed to render the basic organismic shapes in different lineages and, since they involve conserved molecules, they are suggested to have been co-opted from processes that were already present in the unicellular ancestors of multicellular lineages. In addition to this and other conceptual frameworks, several mathematical and computational formalisms have also been developed to consider the role of both biochemical regulatory pathways and mechanical forces [66–69]. The model proposed by Marée and Hogeweg [70] illustrates this approach, as it contributes to understanding the development of *D. discoideum* fruiting bodies by integrating the effect of mechanical properties with regulatory and signaling pathways.

As mentioned above, several microbial multicellular phenotypes are convergent. An example of this convergence is the presence of similar fruiting bodies in prokaryotes and eukaryotes. Given the key role of physical aspects involved in the motility, growth and patterning, fully understanding phenotypic convergence, as well as the restrictions, biases and potentialities of development in this microscale, will be possible only when the forces and material properties involved in multicellular organization are considered. Moreover, these systems have been considered models to study the evolutionary transition to multicellularity. Indeed, studies considering genetic, environmental and physical aspects of development in microbes are providing valuable insights into ongoing evolutionary debates regarding this transition [17•,23,28•]. For instance, taking physical effects into account could help explain the stabilization and transgenerational recreation of relatively complex phenotypes even before robust genetic systems evolved [71]. Similarly, this approach could shed light into long-standing, yet unexplained, observations, such as the morphological and structural differences between multicellular organisms that develop through aggregation (usually smaller and with fewer cell types) versus incomplete division (usually larger and with more cell types) [65]. Overall, considering physical forces as an integral and causal agent in development, and as drivers (not only constraints) of phenotype development, will help understand multicellular development in the microbial world, proving invaluable insights into many developmental, ecological and practical issues, as well as into the evolution of multicellularity itself.

Conflict of interest statement

Nothing declared.

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3. Plasticidad fenotípica en el desarrollo de *Myxococcus xanthus* en medios con rigidez variable

Independientemente de que un organismo sea un animal, una planta o una bacteria, debido a que su desarrollo ocurre en contextos ambientales cambiantes y heterogéneos, su adaptación o incluso su supervivencia, pueden resultar de la flexibilidad de las respuestas de su fenotipo, su fisiología, o incluso su conducta, en relación con dicha variación ambiental. A este cambio en uno o más rasgos de su fenotipo, consecuentes de la interacción organismo-ambiente, se le llama plasticidad fenotípica (Sultan, 2015). Al repertorio fenotípico de un genotipo dado a través del rango de alguna variable ambiental se le denomina norma de reacción, y su caracterización constituye la aproximación más común al estudio de la interacción organismo-ambiente (Schlichting & Pigliucci, 1998; Sultan, 2015).

El estudio de la plasticidad fenotípica mediante normas de reacción se ha abordado principalmente a través de modelos experimentales de plantas y animales (Gilbert, 2015; Sultan, 2015). Sin embargo, como se mencionó en el capítulo anterior, debido a los múltiples ambientes que habitan, a su propia variación fenotípica, a su escala y a la naturaleza de las variables de sus contextos ambientales, el estudio de los microorganismos puede contribuir al entendimiento de los principios detrás de las interacciones de los organismos con su ambiente, así como al planteamiento de posibles escenarios e interacciones durante la transición a la multicelularidad.

Myxococcus xanthus es una delta proteobacteria que habita ampliamente los suelos del mundo. Como otras especies microbianas, puede formar estructuras multicelulares tridimensionales en las que coexisten varios tipos celulares (Yang & Higgs, 2014). En el caso de *M. xanthus*, la formación de estas estructuras, llamadas cuerpos fructíferos, ocurre en respuesta a la falta de nutrientes en el substrato, y su desarrollo incluye la agregación de las células y su posterior diferenciación en alguno de tres destinos celulares: esporas, células periféricas y células que alcanzan la lisis mediante muerte celular programada (Yang & Higgs, 2014; Arias Del Angel *et al*., 2018). Es interesante notar que el desarrollo de los cuerpos fructíferos es estereotípico, es decir, que mantienen tiempos de desarrollo determinados y formas y tamaños similares (Escalante *et al*., 2012). Contrariamente, las

células se mantienen como vegetativas y sin diferenciar cuando se encuentran en medios ricos en nutrientes (Yang & Higgs, 2014). Para su crecimiento vegetativo o su desarrollo en el laboratorio, se deposita una gota de medio de cultivo líquido sobre un substrato de agar con o sin nutrientes, respectivamente, sobre el cual crecerá o se desarrollará una población (Yang & Higgs, 2014). En cualquiera de los dos casos, las células se mueven arrastrándose sobre el substratato de agar y, al menos para el caso de su movimiento vegetativo, pueden percibir y responder a la compresión mecánica y la estructura del substrato (Fontes & Kaiser, 1999; Lemon *et al*., 2017). Considerando estas evidencias, resulta razonable pensar que las propiedades mecánicas y estructurales del substrato representan variables que modifican la interacción entre las células mismas y entre éstas con su medio; y que por lo tanto, pueden modificar también los procesos de desarrollo de esta bacteria (Be'er *et al*., 2009; Guégan *et al*., 2014; Lemon *et al*., 2017).

En este capítulo se presenta un experimento de norma de reacción en el que células de diferentes genotipos de *M. xanthus* son puestas a desarrollarse en substratos con condiciones de rigidez variable. A través de la cuantificación de los rasgos fenotípicos de los cuerpos fructíferos, así como la de sus poblaciones, se muestra que los factores genéticos, las propiedades mecánicas del substrato (rigidez) y su interacción, contribuyen a la variación en el desarrollo de *M. xanthus*. Mediante análisis multivariados y normas de reacción de dichos rasgos, se muestra que además de la organización multicelular que ocurre en la escala de un sólo cuerpo fructífero, el desarrollo de *M. xanthus* involucra también el arreglo espacial de la población de cuerpos fructíferos en patrones estereotípicos. Además de esta organización en dos distintas escalas, puede verse que la plasticidad fenotípica es específica de cada rasgo y de la escala característica del rasgo. En conjunto, lo anterior sugiere que, tanto a nivel de los cuerpos fructíferos individuales como a nivel de la población, la contribución del componente ambiental no es contingente, sino es robusta, es decir, que dada una condición ambiental y un genotipo específico, el proceso de desarrollo es estereotípico, así como el fenotipo resultante. Finalmente, se discute sobre las limitaciones analíticas de las normas de reacción, en el sentido de que no permiten el estudio aditivo o no aditivo de dos o más variables ambientales, por lo que resulta necesario desarrollar nuevas técnicas para el análisis de la interacción organismo-ambiente en contextos que asemejen las condiciones naturales.

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Plastic multicellular development of *Myxococcus xanthus*: genotype–environment interactions in a physical gradient

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In order to investigate the contribution of the physical environment to variation in multicellular development of *Myxococcus xanthus*, phenotypes developed by different genotypes in a gradient of substrate stiffness conditions were quantitatively characterized. Statistical analysis showed that plastic phenotypes result from the genotype, the substrate conditions and the interaction between them. Also, phenotypes were expressed in two distinguishable scales, the individual and the population levels, and the interaction with the environment showed scale and trait specificity. Overall, our results highlight the constructive role of the physical context in the development of microbial multicellularity, with both ecological and evolutionary implications.

1. Introduction

Developmental patterns, and phenotype in general, have often been considered as univocal outcomes of the genome. Thus, research has focused—conceptually and experimentally—on studying genetic mechanisms in invariable environmental conditions. However, phenotype has been shown to involve complex, bidirectional interactions between organisms and their environment. For example, light, moisture and nutrient availability in plants, as well as temperature, population density and predator presence in animals, partially determine developmental trajectories [1–6]. The phenotypic repertoire of a given genotype across a range of environmental conditions is called reaction norm [7] and its characterization constitutes the most common approximation to the study of organism–environment interactions. Since development occurs at changing and often organism-modified environmental conditions, increased survival, adaptation and phenotypic innovation can result from flexible phenotypic responses (phenotypic plasticity). Plasticity itself contributes as a cause and not just as a consequence of development and phenotypic transitions in evolution [1,5,8–10].

The origin of multicellularity is a major evolutionary transition in organismal development. Plant and animal models have guided research in organism–environment interactions as well as multicellular development and evolution. However, microorganisms are largely missing in these integrative efforts, despite their ubiquity across environments and the fact that some of them can develop stereotypical multicellular structures. Microbial groups can provide new insights regarding the contribution of the organism–environment interactions to the development and evolution of multicellularity since they develop in a scale and environment similar to those in which multicellularity might have emerged [11]. Importantly, physical processes associated with environments at the microscale happen to be relevant as both driving and restrictive forces in the development of microbial multicellular phenotypes [12–14]. These forces and their effect on living matter vary across scales, leading to reconsideration of the meaning of environment and phenotype at different organization levels.

Myxococcus xanthus is a cosmopolitan soil bacterium that has a multicellular life history and moves by gliding across surfaces. In nutrient-rich substrates, cells divide and swarm outwards expanding the colony radially. When nutrients become scarce, *M. xanthus* cells come together and develop into three-dimensional multicellular structures called fruiting bodies (FBs) that contain differentiated cells (spores) [15]. The genetics underlying this process have been studied intensively [15,16]. In addition to the multicellular organization at the level of a single FB, the development of *M. xanthus* involves a stereotypical, but largely unexplored, spatial arrangement of FBs [17]. Given their characteristic gliding motility, cell-to-substrate interaction is an important aspect of organism–environment interaction in myxobacteria and is susceptible to variation by mechanical properties of the substrate [18–20]. We therefore hypothesize that changes in the substrate stiffness may affect velocity and aggregate formation, uncovering phenotypic plasticity in different scales and traits of the multicellular structures of *M. xanthus*.

In the present study, we determine reaction norms of different *M. xanthus* genotypes in multicellular development under varying substrate stiffness environments. Our results show that phenotypic plasticity for multicellularity in *M. xanthus* involves multiple levels of biological organization: single FBs and FB groups.

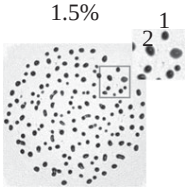
2. Methods

2.1. Strains, growth and developmental conditions

To evaluate the contribution of the genotypic and environmental context to the phenotypes, five genotypes (strains) were assayed for development over five different substrate stiffness conditions, which were modified by varying agar concentrations (parental strain: DZF1; in-frame deletion mutants: Δ*mkapC*, Δ*mkapA*/Δ*mkapC*, Δ*mkapA*, Δ*pktC2*/Δ*pktD1*, kindly provided by S. Inouye; agar concentrations: 0.5%, 1.0%, 1.5%, 2.0%, 2.5%) [21]. DZF1 is a standard laboratory *M. xanthus* strain. *ΔmkapC*, *ΔmkapA*/*ΔmkapC*, *ΔmkapA* and *ΔpktC2*/*ΔpktD1* are DZF1-derived in-frame deletion mutants of the network of PSTKs and the associated scaffold proteins Mkaps, which participate during the development of FBs [16,22]. An important feature of these mutants is that they do not arrest development and thus allow tracking of multicellular phenotypic changes at this scale. Previous studies suggest that deletion of *pktC2*, *pktD1* and other components of this network (*pktA2*, *pktD9*) impacts development and FB formation [17,23–26]. MkapA and MkapC are scaffold proteins in this network, but there are not previous reports regarding the phenotypic consequences of their deletion.

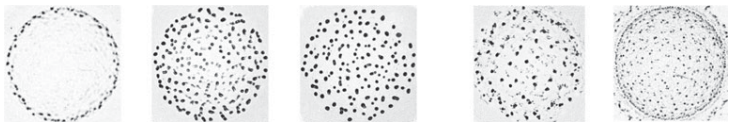
Following the protocol described in Yang & Higgs [15], strains were taken from frozen stocks by spotting 50 µl of each onto a CYE agar plate and incubated at 32°C for 2 days. Cells from the resulting

(*a*)



FB phenotypic trait	FB 1	FB 2
Area	0.04132	0.05983
Circularity	0.865	0.871
Aspect Ratio	1.198	1.274
Roundness	0.835	0.785

(*b*)



drop phenotypic trait	Δ*pktC2/D1* 0.5%	Δ*pktC2/D1* 1.0%	Δ*pktC2/D1* 1.5%	Δ*mkapA/C* 1.0%	DZF1 0.5%
Ring	4.2	2.2	1.6	1.7	2.2
Distance	2.94	2.36	2.46	2.70	2.70
Num. FB	83	191	147	181	406
FB out	0	0	0	0	1
Maturation	0	0	1	1	1
Scars	0	0	0	1	0
Dev. time	5	5	4	4	5

Figure 1. Micrographs to exemplify the quantification of *M. xanthus* phenotypic traits at (*a*) FB scale (Area, Circularity, Aspect Ratio, Roundness), and (*b*) population scale (Ring, Distance, Num. FB, FB out, Maturation, Scars, Dev. time). Black dots correspond to developed FBs at 96 h after starvation. Phenotypic trait units are provided in electronic supplementary material, table S1.

colonies were transferred to 25 ml of CYE liquid medium and incubated at 32°C, shaking at 250 r.p.m. overnight. Culture dilutions of each strain were grown from 0.2 OD550 until they reached 0.7 OD550 (nutrient-rich liquid culture). Prior to the bacteria development assays, cells were harvested by spinning them at 8000 r.p.m. for 5 min. The resulting pellet was washed twice with TPM solution and resuspended in 1/10th of the original volume. Fifteen microlitres were spotted onto the different agar concentration TPM plates. TPM plates were prepared by filling each with 30 ml TPM/agar media, at the appropriate agar concentration, and storing them overnight at 32°C before use. After the spots dried, the plates were incubated at 32°C for 96 h, until aggregates fully developed into matured FBs, recognized by their invariant shape, size and complete darkening [17]. In order to measure the motility of each strain, 15 µl of nutrient-rich liquid culture (CYE, described above) was spotted onto the different agar concentration CYE plates. After the spots dried, plates were incubated at 32° for 96 h. Each strain/substrate condition of TPM and CYE plates were conducted in triplicate for statistical support.

2.2. Measurement of phenotypic traits

Micrographs of the resulting FB development on nutrient-poor substrates, or growth on nutrient-rich substrate, were taken at 370.8 pixels mm^{-1} using a LEICA m50 stereomicroscope with an ACHRO 0.63× objective lens and a Canon-EOS Rebel T3i camera. For image processing, micrographs were binarized into black/white images and phenotypic traits were measured using FIJI (ImageJ) software v. 2.0.0 [27]. For each FB image, phenotypic traits were classified and quantified at two scales: individual and population level. Single FB scale traits included Area, and Circularity, Aspect Ratio and Roundness as shape descriptors. Population scale traits, from all the FBs resulting from a single drop, included ring formation at the edge of the drop (Ring), standard distance between FBs (Distance; note that this variable might change in unexpected directions when the FBs exhibit an uneven distribution), number of FBs (Num. FB), maturation of FBs considered as complete darkening of clearly defined aggregates (Maturation), presence of vestiges of not clearly defined aggregates (Scars), position in the sequence of maturation speed, being 1 the fastest agar % and 5 the slowest agar % for each genotype (Dev. time), and mature FBs beyond the edge of the drop (FB out). Outer FBs are formed during development from cells that migrated beyond the initial drop edge. In order to analyse the images systematically, we cropped the FBs placed outside the drop, but recorded their presence under the categorical variable 'FB out' (figure 1; electronic supplementary material, table S1)

Table 1. PERMANOVA analysis to test the contribution of genotype, agar concentration and their interaction on *M. xanthus* phenotypes.

	d.f.	sums of squares	mean of squares	pseudo-*F*	R^2	*p*-value
genotype	4	35.51	8.87	4321.8	0.571	0.000999
agar	4	10.35	2.58	1259.9	0.166	0.000999
genotype – agar	16	11.48	0.71	349.6	0.185	0.000999
residuals	2305	4.73	0.002		0.076	
total	2329	62.08			1.000	

[28]. The edge of the drop was defined at time 0 h, when it is clearly seen, but it did not expand or change its position during development on nutrient-poor media.

For assays of growth on nutrient-rich substrate, the diameter of colonies was measured in micrographs taken from 0 to 96 h, every 24 h. We calculated the growth rate as the percentage that the colony diameter increased over 96 h. For this study, this rate was used as a proxy for motility. These measurements were performed using FIJI (ImageJ) software v. 2.0.0 [27].

2.3. Data analysis

In order to test contributions of genotype, substrate and their interaction, a PERMANOVA analysis of a representative sample was performed using the *adonis* function in the R package *vegan* (10% of the total data, $N = 2400$) (table 1) [29]. This is a multivariate test suitable to distinguish sources of variation in non-parametric datasets. To investigate the phenotypic differences in micrographs of figure 2, phenotypic traits were grouped by genotype and by agar concentration, and a factorial analysis of mixed data (FAMD) was performed for each group through the *FAMD* function in the R package *FactoMineR* [30]. This multivariate analysis allows us to analyse numerical and categorical variables and generates a multidimensional space where the first and second axes explain the largest proportion of variances as a combination of phenotypic traits (figure 2*b*,*c*; electronic supplementary material, table S2).

To characterize the trait-specific phenotypic variation, reaction norms were constructed connecting median values for each phenotypic trait. A linear regression fit was superimposed to visualize tendencies, although *p*-values were not significant in all cases (figure 3*a*; electronic supplementary material, figure S2 and table S3). A second set of reaction norms based on the coordinates of the first and second axes of the FAMD analyses as a statistical synthesis of phenotypic trait variation was also performed (figure 3*b*).

Finally, to test if the motility was altered in response to agar concentration or in response to genotype, and whether such variability might contribute to the observed developmental plasticity, we assessed colony growth over nutrient-rich substrates measuring the increase in its diameter and plotted in function of agar concentration (figure 4*a*) and of time (figure 4*b*). These values were normalized according to the colony diameter at 0 h. Then, we plotted the motility rate considering the proportion in which the colony diameter increases after 96 h and performed a two-way ANOVA to test the statistical significance of genotype and substrate conditions in such rate (figure 4*c*). Note that FBs do not develop in the presence of nutrients.

All analyses were conducted in R program (v. 3.2.3) through RStudio [31,32]. ggplot2 package v. 3.0.0 was employed for visualization [33].

3. Results

We observed that most of the phenotypic variation revealed in micrographs of figure 2*a* is explained by genotype (adj $R^2 = 0.571$), substrate stiffness (adj $R^2 = 0.166$) and their interaction (adj $R^2 = 0.185$). Statistical significance for each factor is strongly supported by the PERMANOVA analysis: pGenotype $= 9.9 \times 10^{-4}$, pEnvironment $= 9.9 \times 10^{-4}$, pInteraction $= 9.9 \times 10^{-4}$ (table 1). The FAMD analyses provided clarity in discriminating phenotypes. Specifically, by fixing each substrate condition (agar %) and assessing the differences among the genotypes, phenotypic differences can be distinguished (figure 2*b*). For example, all the genotypes (ellipses) are well distinguished at the lowest

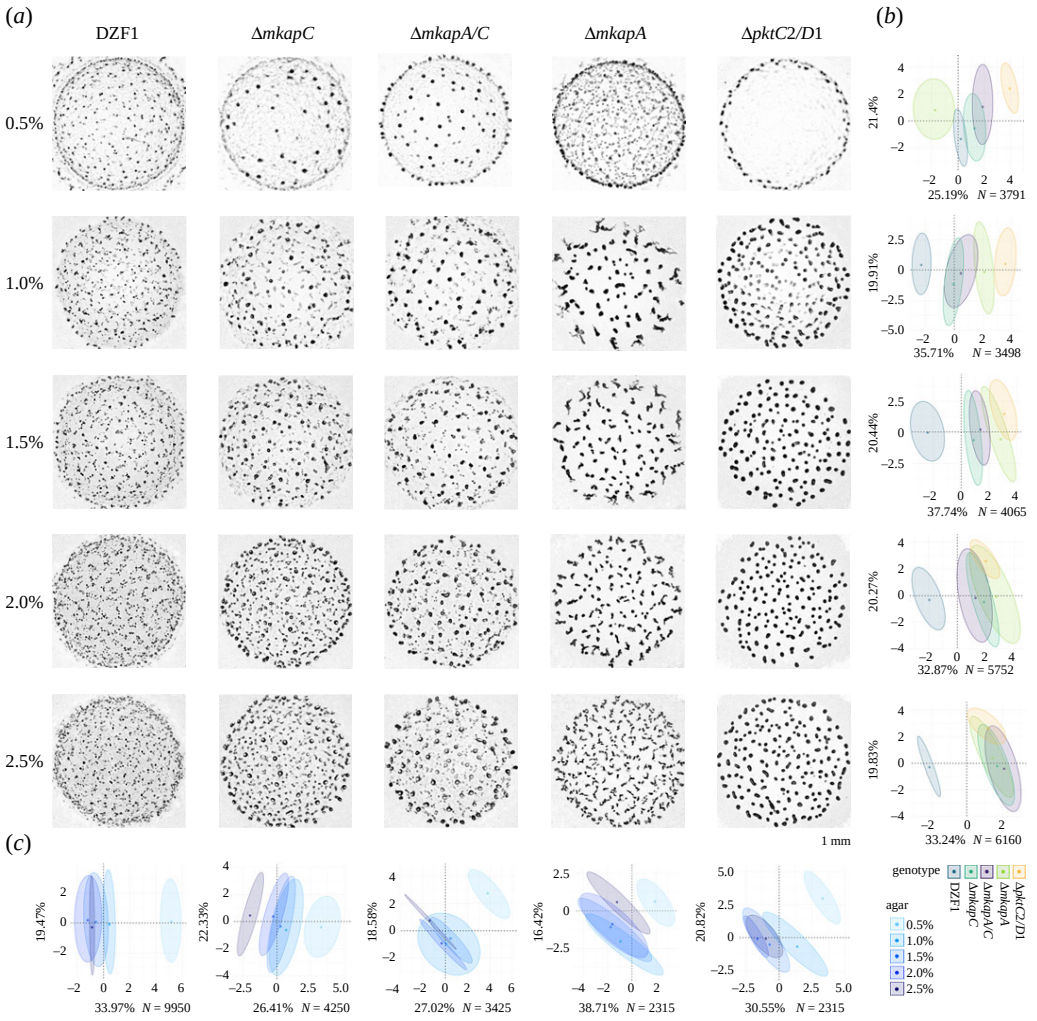


Figure 2. Phenotypic variation of *M. xanthus*. (*a*) Phenotypes (population scale) micrographs with mature FBs (dark spots). Columns correspond with the genotypes (parental strain and knock-out mutants), rows correspond with different agar concentrations. FAMD multivariate analyses of phenotypic variation for (*b*) all genotypes per agar concentration, and (*c*) all agar concentrations per genotype. DZF1: parental strain; *ΔmkapC, ΔmkapA/ΔmkapC, ΔmkapC, ΔpktC2/ΔpktD1*: mutants; 0.5%, 1.0%, 1.5%, 2.0%, 2.5%: agar concentrations. 95% confidence interval ellipses enclose data centroids.

agar concentration (0.5%), but tend to overlap as agar concentration increases. However, the parental strain ellipse (DZF1) is clearly apart from that of the other phenotypic variants, except for 0.5%. Looking across agar concentrations, the arrangement of the genotypes follows equal directions along the *X*-axis (axis of major variation) in all agar concentrations, except for 0.5%, with larger contribution from the number of FBs and the standard distance between them (electronic supplementary material, table S2 and figure S3). Finally, while the Δ*mkapA/C* and Δ*mkapC* phenotypes overlap in all the cases, correlating with their similar phenotypic profiles shown in the micrographs, there is a non-additive effect of the double mutant, as it does not resemble the Δ*mkapA* phenotype.

By contrast, we observed more overlap when assessing agar concentrations (ellipses) for each genotype (figure 2*c*). There is not a clear sequence in the ellipse disposition and overlapping is present between almost all of the ellipses, except for 0.5% ellipse, which is well distinguished in all genotypes. We also did not observe a pattern of traits contributing to *X*- and *Y*-axes within each genotype, although ring formation is predominant in all cases (electronic supplementary material, table S2 and figure S3). Altogether, our results show that at 0.5% agar concentration, there are clear phenotypic differences within and among genotypes, and that phenotypic identity is blurred as agar concentration increases. Furthermore, the consistency of variables explaining the phenotypic variation at *X*-axis (Dim. 1) and the constancy in the sequence of genotype ellipses when considering each agar concentration suggest that the expressions of the genotype are enabled in a given environmental range. These patterns are notably robust across replicates, which is evident from the marginal

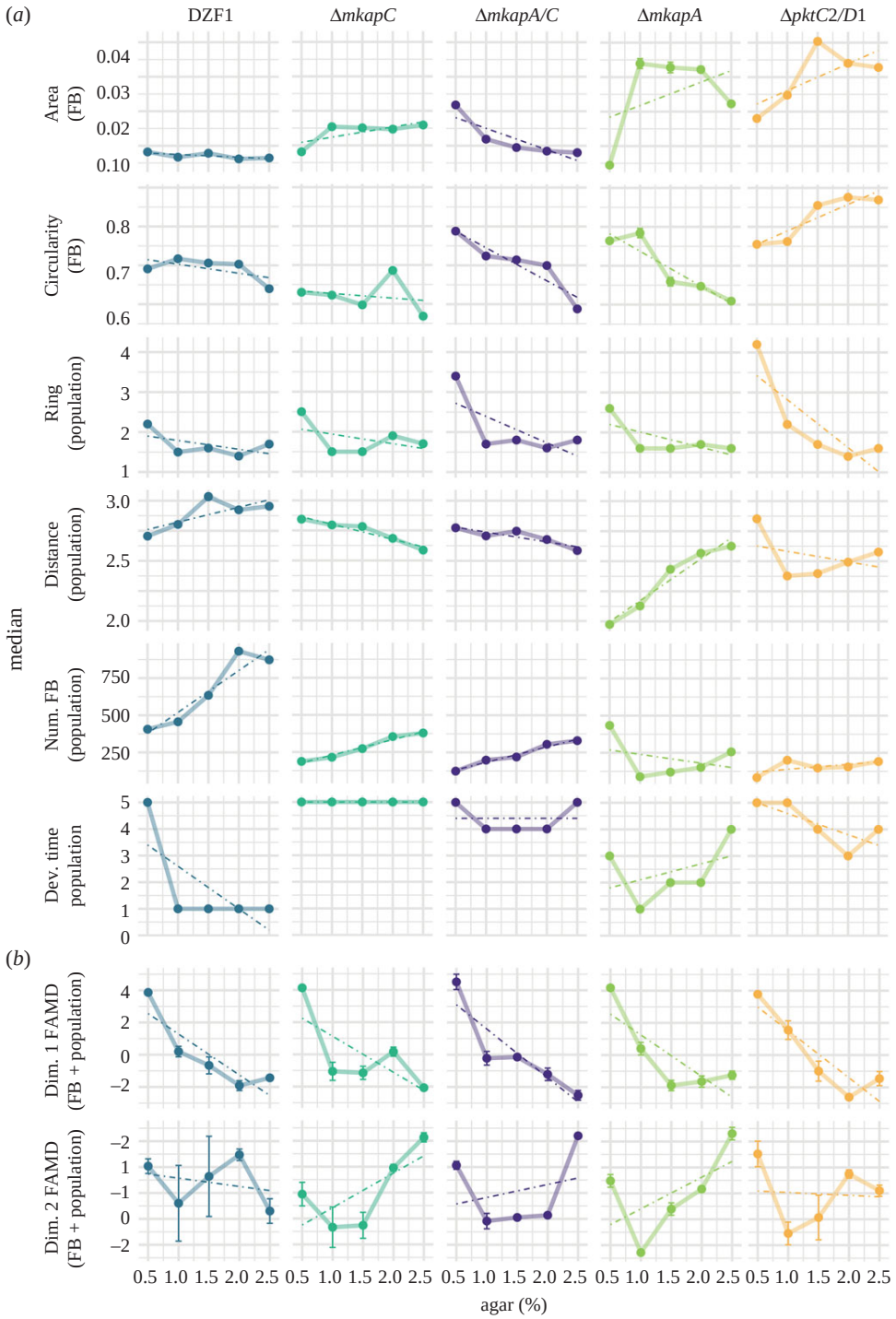
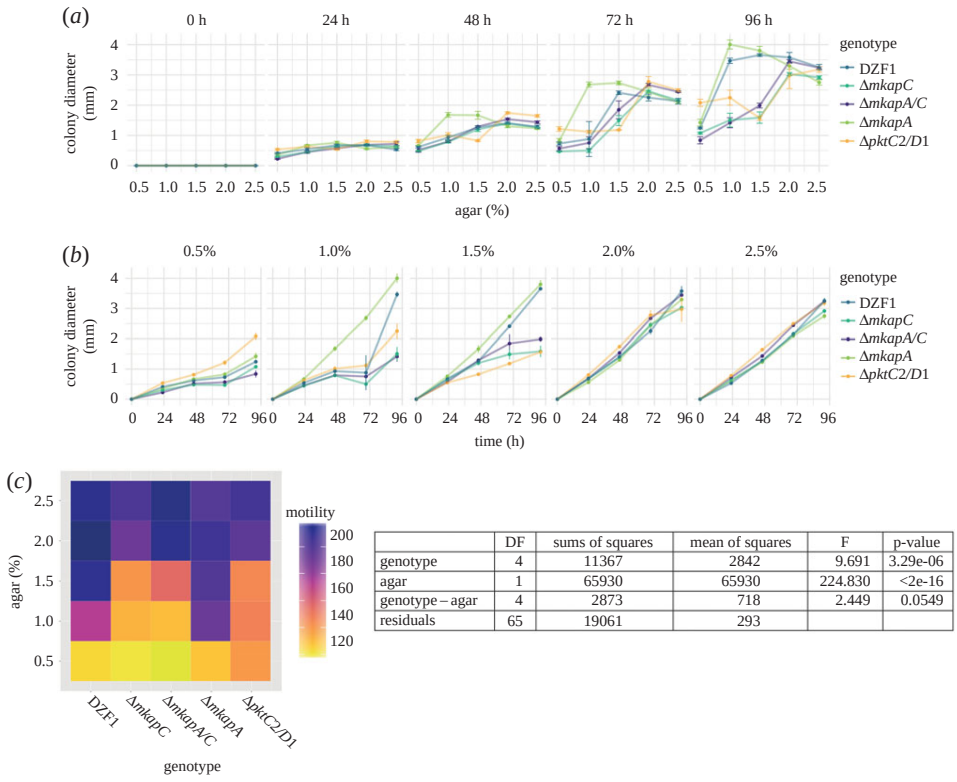


Figure 3. Phenotypic plasticity among *M. xanthus* parental and mutant genotypes in response to substrate agar concentration. Each line represents the reaction norm of a single genotype (columns) based on the median ± s.e. of (*a*) single phenotypic traits (rows) and (*b*) coordinates of dimension 1 and dimension 2 of the FAMD multivariate analysis. Linear regression fit with a dotted line, although *p*-values are not significant in all cases.

contribution of replicates to the overall phenotypic variation (electronic supplementary material, table S2 and figure S3).

Reaction norms show that there is substantial phenotypic variation among genotypes for individual traits across agar concentrations (figure 3*a*). Each trait × genotype combination has its own pattern, for both the single FB and population scales. For example, in Δ*mkapA*, 'Circularity' and 'Distance' are almost linear but with opposed slope signs, while 'Area' does not show a linear behaviour.



	DF	sums of squares	mean of squares	F	p-value
genotype	4	11367	2842	9.691	3.29e-06
agar	1	65930	65930	224.830	<2e-16
genotype – agar	4	2873	718	2.449	0.0549
residuals	65	19061	293		

Figure 4. Colony growth rate of *M. xanthus* parental and mutant genotypes in response to agar concentration over nutrient-rich substrates. In the first two panels, dots connected by lines represent the median ± s.e. of the colony diameter (*a*) among time points from 0 to 96 h for each agar concentration, and (*b*) among agar concentration for each time point. Values were normalized according to colony diameter at 0 h. (*c*) Motility is approximated as the colony growth rate, considered as the proportion in which the colony diameter increases after 96 h, and depicted in the heatmap; darker colours represent high motility, while lighter ones represent low motility.

Remarkably, threshold variation is observed for some traits with particular condition and strain combinations, such as 'Ring' at the 0.5% condition. These effects are not general, especially for the parental strain (DZF1), in which the agar concentration is less strong in affecting 'Ring' and has no effect in FB traits and 'Dev. time', except at 0.5% agar. Finally, the large effect of substrate conditions, especially at the extreme ones (0.5% and 2.5%), is evident when plotting the FAMD coordinates as a statistical synthesis of phenotypic trait variation (figure 3*b*).

Regarding the colony growth rate in nutrient-rich substrates, there are strong interaction effects of agar concentration. At the lowest and highest agar concentrations, there was almost no difference among genotypes for colony growth rate (figure 4*b*,*c*). We observed a much lower motility on 0.5% compared to the rest of the agar concentrations (figure 4*b*,*c*), in line with previous reports [34]. Overall, when we track the colony growth, we observe that the strains slightly diverge according to their growth and that, as development proceeds, strains form two groups (DZF1 and *ΔmkapA* form one group and the rest of the strains another one (figure 4*a*,*c*)). However, we found that in such nutrient-rich substrates, the agar concentration is the most significant factor variable for the colony growth rate (see ANOVA results in figure 4*c*).

4. Discussion

We experimentally measured reaction norms of bacterial multicellular development across substrates with increasing stiffness. Phenotypic plasticity in *M. xanthus* development was revealed, involving changes in cell-to-substrate interaction due to increasing agar concentration in culture media plates. In this study, we focus on the potential for changes in morphology during development. These include changes in development time, spore maturation and FB size and number, which are important fitness-associated traits upon which selection may subsequently act. Previous studies with the same regulatory network reported statistically significant phenotypic differences when comparing mutants

with parental strain (DZF1), including differences in paradigmatic functional traits, such as spore count and viability [17]. Our current results, and those from previous studies, suggest that the joint effect of the physical environment and genetic background on morphologic and developmental traits is likely to be of evolutionary relevance.

The experimental design allowed for statistical discrimination of phenotypic changes at two scales: 'FB scale' and 'population scale'. In general, for each combination of genotype and substrate condition, FBs conforming the population exhibit little variation in their shape and size (figure 2*a*). Nevertheless, we found genotypic and substrate effects on phenotype at the 'population scale', which have been overlooked or dismissed when considering variation only at 'FB scale'. Remarkably, phenotypic identity over the genotypic and substrate conditions is not observed when considering only the FB scale (electronic supplementary material, figure S1; versus figure 2*b*,*c*).

To our knowledge, mechanisms behind the organization at the FB population scale have not been explored, although it is known that mechanical forces and living matter interact in a bidirectional way giving rise to robust patterns [13,14]. Based on our results and taking into consideration the influence of mechanisms such as substrate fibres alignment in colony formation [18] and the surface-tension-driven coarsening process of aggregation [35], we can suggest that the mechanical properties of the medium constitute a key element when thinking about environment at microscales. Also, substrate changes could determine the individual and collective motility, which could in turn drive aggregation. In this case, the developmental process occurring in nutrient-poor substrates would be mainly determined by the agar concentration. We found that the growth rate is largely determined by the agar concentration, with slight differences among strains (figure 4*a*,*b*). This is especially clear for the strain-independent low and high growth rate at the 0.5% and 2.5% conditions, respectively. Nevertheless, overall, the multicellular phenotypes at the FB and population scales are largely explained by agar concentration, genotype and the genotype–substrate interaction (table 1), which reflects the complexity of the developmental process under study.

Our results also demonstrate the value of investigating phenotypes from multi-level perspectives [11]. Phenotypic variation existed at both the level of single multicellular structures and the collective level of groups of these structures. It is important to note that specific environmental conditions have trait- and scale-specific effects. For instance, the substrate seems to play a much stronger role in certain ranges of agar concentration and for particular traits. We report a strain-independent ring formation, at population phenotype, surrounding the initial drop area at 0.5%. This could be explained by a higher cellular density at the edge of the drop forming when it dries at time 0 h, as in the coffee-ring formation physical phenomenon [36]. It is possible that at this agar concentration, cells cannot glide inwards easily, developing a ring of FBs at the edge. This hypothesis, and the precise mechanisms behind different phenotypes, remain to be tested.

In this work, we considered several points within the widest possible range of substrate modification which enabled a good approximation to the shape of reaction norms. We initially included a broader range of agar concentrations (electronic supplementary material, figure S4), but excluded those values where there was no development of FBs. This allowed us to notice that phenotypic change is usually greater at the extremes of the agar concentration range (figure 3*b*; in good agreement with previous theoretical proposals [37]). However, some limitations to this work and to the current study of plasticity in general should be considered. For instance, we modified stiffness by varying agar concentration, but other variables affecting the substrate properties such as water availability could be correlated. Indeed, reaction norms have provided useful perspectives regarding the paired relationship between a trait and an environmental factor for a specific moment in the developmental trajectory. However, new approaches considering the dynamical interaction among numerous phenotypic and environmental variables, across time and in ecologically meaningful ranges, are required. Moreover, plasticity is often studied in laboratory-adapted strains, and at least in this work, the parental laboratory-strain is not representative of the plastic responses for the rest of the genotypes (figures 1 and 2). Completely unexpected phenotypes may arise when considering natural environments or non-domesticated strains.

Overall, our study highlights the importance of the physical environment in the development of robust, yet plastic, aggregative microbial structures in different genotypic contexts. This in turn calls for systematic approaches to integrate the different factors and mechanisms (genetic and environmental) behind phenotypic plasticity and its consequent role in the emergence and evolution of multicellularity.

Data accessibility. Data are available from the Dryad Digital Repository: http://dx.doi.org/10.5061/dryad.308hs50 [28].
Authors' contributions. N.R.-Y., A.E.E., M.T. and M.B. conceived the study, N.R.-Y. and A.V.A. performed the experiments, all authors analysed the data and wrote the article.

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4. Pistas desde la microescala en la integración del eco-evo-devo

Cuando el genocentrismo empezó a ser dominante en la biología evolutiva y del desarrollo, la relación entre el genotipo y el fenotipo de los organismos se aceptaba como unívoca, es decir, que a un genotipo le correspondía un fenotipo (Robert, 2004; Jablonka & Lamb, 2014; Sultan, 2015). A pesar de que esta idea se ha reconocido como una simplificación y de que se ha ido modificando, su planteamiento implicó también la aceptación de que la información necesaria para el desarrollo de un organismo se encuentra principalmente contenida en sus propios genes (Robert, 2004). Por otra parte, en la relación de los organismos con su ambiente, el papel del contexto ambiental se consideraba como filtro de la variación biológica, o como agente que afectaba el desarrollo y la formación de los organismos de manera unidireccional y contingente. Bajo este esquema, los enfoques teórico y experimental se centraron en entender con detalle el papel específico de ciertos genes involucrados en el desarrollo de los organismos, minimizando la importancia de la variación fenotípica que resulta de la interacción de los organismos con su ambiente. Como consecuencia, los diseños experimentales se han basado en la selección de determinados organismos modelo y cepas de laboratorio, los cuales son reproducidos o desarrollados en condiciones de laboratorio constantes y controladas (Ashburner *et al*., 2000; Robert, 2004; Kaletta & Hengartner, 2006). Si bien el uso de estos organismos y cepas conlleva numerosos beneficios prácticos, la experimentación con ellos y los diseños experimentales más comunes restringen, por su naturaleza y de manera intencional, la variación biológica y la ambiental. Así, es difícil pensar que los resultados que se obtienen mediante este enfoque puedan ser directamente extrapolados al desarrollo y a la evolución de los organismos en sus hábitats naturales, lo cual ha dejado algunas preguntas abiertas. Por ejemplo: ¿cómo se origina y cómo se mantiene la robustez de los procesos de desarrollo en contextos naturales heterogéneos y cambiantes? ¿Mediante qué mecanismos la plasticidad fenotípica moldea las interacciones ecológicas? ¿Con qué frecuencia la plasticidad fenotípica contribuye en los procesos evolutivos como fuente de variación fenotípica dentro de las poblaciones naturales?

El panorama restringido que resulta de estudios basados en organismos modelo y en condiciones de laboratorio orientadas a anular o minimizar la variación ambiental es particularmente notable para el caso de las bacterias, representantes de la mayor diversidad

biológica en el planeta, en cuanto a sus capacidades metabólicas y a los ambientes que pueden habitar (Johri *et al*, 2005). Su enorme diversidad, ligada a nuestro limitado entendimiento sobre la relación con su contexto ecológico, se ha visto reflejada en la dificultad que representa lograr su cultivo en condiciones de laboratorio, en donde sólo se ha podido aislar un poco más del 1% de la diversidad natural (Pham & Kim, 2012; Nai & Meyer, 2017; Pande & Kost, 2017).

En este capítulo, se presenta un artículo de opinión en el cual se discute acerca de los sesgos implicados en los diseños experimentales convencionales para el estudio de microorganismos, clasificando dichos sesgos en dos grupos: aquéllos que tienen que ver con el uso de cepas que han sido manipuladas por largo tiempo bajo condiciones de laboratorio, y que por lo tanto han pasado por un proceso de domesticación; y aquéllos relacionados con la elección de las condiciones ambientales. Estos sesgos y la importancia de considerarlos se ilustran mediante la variación asociada a distintos contextos experimentales de la bacteria *Myxococcus xanthus*, mostrando cómo la modificación conjunta de dos variables ambientales permite una diversidad fenotípica que no se esperaría de esta misma bacteria en las condiciones estándar de laboratorio en las que normalmente es desarrollada. Además, proponemos que, como consecuencia de estos sesgos, la integración de los factores ecológicos en el entendimiento de la evolución de los fenotipos o el desarrollo, en otras palabras, eco-evo-devo, se ha limitado a escenarios específicos de laboratorio.

Laboratory biases hindering eco-evo-devo integration: hints from the microworld

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Keywords: developmental plasticity, gene-centrism, laboratory biases, multicellularity, *Myxococcus xanthus*

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Eco-evo-devo at the microscale

How biological variation originates is a longstanding question, and the interest on the variation generated by developmental processes occuring in different environmental conditions, as well as its evolutionary significance, is not new (Schmalhausen, 1949; Gupta & Lewontin, 1982; Scheiner & Goodnight, 1984; Sarkar 2004). However, genetic variation has been considered the major cause behind phenotypic variation, and the organismal interaction with varying environments has been often reduced to noise or to deviations from a norm, assuming a univocal genotype-phenotype relation (Lewontin, 2001; Robert, 2004; Sultan, 2017). Concomitantly, the goal of much research in developmental biology has been to distentagle phenotypic differences by looking at genetic ones.

To reveal the phenotypic effects of genetic change, the dominant experimental approach has included two key elements. First, experimental designs have relied on a limited set of model organisms, sometimes restricted to particular laboratory lines or strains of those species (Kaletta & Hengartner, 2006; Robert, 2004; Ashburner et al., 2000). Second, these studies have deliberately excluded realistic environmental variation, instead rearing organisms in controlled, constant conditions that may be very different as well as steadier than those in natural environments (Gilbert and Bolker, 2001). Although a wealth of valuable results has been obtained with this approach, it has also limited biological understanding to the extent that (a) model organisms do not capture key aspects of biological diversity, and (b) laboratory conditions intentionally restrict potential effects of natural environments (Gasch et al., 2015; Minelli & Baedke, 2014; Gilbert, 2001; Bolker, 1995). Thus, the

approach to the developmental and evolutionary processes occurring within specific ecological contexts has kept some important questions unsolved or largely unattended. For instance, how do both plastic and robust processes arise during development at varying natural contexts? What are the mechanisms by which phenotypic plasticity itself shapes ecological interactions? With what strength and how commonly does plasticity contribute to evolutionary processes such as phenotypic innovation in natural populations?

Minding these questions, increasing attention in phenotypic variation beyond single genetic sources has reconsidered with interest on the role of the environmental context in the expression and evolution of phenotypes (Bateson and Gluckman 2012; Moczek et al. 2011; Levis & Pfennig, 2016). This recognition has been recently renewed as Eco-Evo-Devo approach, bringing back to the discussion the relevance of the reciprocal interactions between ecology, evolution and development, and providing insights into multicausal development occurring in the "real-world" (Sultan, 2003; Gilbert, 2001). Despite the great efforts and progress on the conceptual approaches of Eco-Evo-Devo, its conforming fields may not be completely integrated due to the very nature of the traditional experimental designs.

For reasons that might be obvious, such as the practical evaluation of phenotypic outcomes, plants and animals have been the main targets of Eco-Evo-Devo research (Gilbert, 2015; Sultan, 2015). In contrast, microorganisms have been invaluable genetic and biotechnological laboratory models due to their short generation times, small size, amenability for genetic manipulation, experimental controllability and long-term storage resistance (O'malley et al., 2015; Jessup et al., 2004), but they are largely missing from eco-evo-devo efforts. However, their growth and development offer remarkable examples of the restrictions implied by studies based on model organisms and standard laboratory conditions, which tend to cancel, minimize or underestimate the causal role of environmental variation in development. In fact, microorganisms represent huge biological diversity regarding their metabolic capabilities and the wide range of ecological contexts they habit (Johri et al., 2005). Nevertheless, only approximately 1 % of the microbial diversity has been cultured using these experimental design strategies, visibilizing our limited understanding about the organism-environment interaction required to reproduce microbial species (Pham & Kim, 2012; Nai & Meyer, 2017; Pande & Kost, 2017).

Furthermore, in view of their ubiquity across environments and their diverse uni- and multi-cellular lifestyles, microorganisms can provide invaluable insights to further understand organism-environment interactions and the processes generating the variation that enables evolution. Moreover, multicellular microbial groups can inform about the organism-environment interactions in the evolution of multicellularity since they develop in a scale and environment similar to those in which multicellularity presumably emerged (Bonner, 2009; Rivera-Yoshida et al., 2018; Arias Del Angel et al., 2017). Focusing on microorganisms also leads to the study of environmental variables that are less evident or relevant at the macroscale, such as the mechanical properties of the cell-to-cell and cell-to-medium interactions (Persat et al., 2015).

Altogether, microbial models can help to unmask biases regarding experimental designs implemented mostly in plants and animals, contributing to a better integration and experimental planning within the Eco-Evo-Devo agenda. Here, we focus on microbial models to illustrate how gene-centered experimental designs harbor two layers of possible biases: one associated with the use of laboratory strains, and another associated with laboratory environmental conditions. In particular, we gather evidence from different microorganisms and use our own results for *Myxococcus xanthus* development in different environmental conditions to exemplify and comment on these biases. In our opinion, such biases should be explicitly considered when interpreting results and extrapolating them to natural contexts and should be overcome at least in some empirical approaches.

Laboratory standard strains vs. natural populations

Choosing a model organism is often limited to some well-established options. Among microorganisms, *Escherichia coli*, *Bacillus subtilis* and *Saccharomyces cerevisiae* are some of the widely used models (Blount, 2015; Love & Travisano, 2013). The use of laboratory standard strains implies following certain protocols associated to undeniable practical advantages that, in turn, reinforce the use of particular strains and species. Among such advantages are, for instance: 1) there is an important technological investment including complete genome sequencing, protein and metabolite quantification methods, and mutant construction for such organisms; 2) these strains consist of pure genetic lines and robust phenotypes which have been domesticated to grow on simple and standard laboratory conditions; 3) because of its minimum variation, results are tractable, systematic

and reproducible, leading to confident comparisons; 4) data and techniques can be shared among the researchers community; and also, 5) using model organisms offers popularity and facility at the granting systems (Gasch et al., 2016; Leonelly & Ankeny, 2013; Ankeny & Leonelli, 2011). All these features provide insight power into genetic mechanisms as they help control the influence of non-genetic factors.

Nonetheless, while using standard strains can be of great value in microbiology, molecular biology or some evolutionary studies, it becomes a limitation for other scientific purposes, such as those related with the Eco-Evo-Devo frame. Since biological questions should match the model organism and experimental choices, questions about phenotypic plasticity and its mechanisms could not be properly addressed plasticity has been intentionally or indirectly suppressed through invariant environmental conditions in already relatively unplastic organisms (Love, 2010; Travis, 2006). Indeed, model organisms often exhibit rapid development and developmental canalization (a specific developmental outcome is expressed regardless of minor variations in environmental conditions; Waddington, 1942), a well-known phenomenon in animal models (Gilbert, 2001; Bolker, 1995).

“Domestication” is commonly used to refer to the adaptation of wild strains to new, human-created habitats. When laboratories are the new habitats, domestication occurs in long-term, stable cultures or during repeated passaging (Eydallin et al., 2014; Palková, 2004; Kuthan et al., 2003; Branda et al., 2001). For microorganisms, laboratory-domesticated strains express robust phenotypic traits and apparently decreased phenotypic plasticity, as compared to short-time manipulated strains (Eydallin et al., 2014). However, whether these traits are actually canalized or not remains to be explored as reaction norm experiments are just starting to become available for microbial systems (Rivera-Yoshida et al., 2019). Despite domesticated strains enable important scientific and technical advances, relevant variation possibly occurring naturally at ecological complex scenarios, could be encrypted in these strains, as can be the factors and processes underlying such variation (Eydallin et al., 2014; Palková, 2004; Kuthan et al., 2003; Branda et al., 2001).

Laboratory domestication has been reported for several microbial species. Interestingly, common phenotypic traits regarding different laboratory strains are shared across species when exposed to similar experimental contexts (Table 1). For instance, in laboratory conditions, *E. coli*, *B. subtilis* and *S. cerevisiae* standard strains present a smooth biofilm

phenotype compared with the rough one observed in wild type strains (Eydallin et al., 2014; Palková, 2004; Kuthan et al., 2003; Branda et al., 2001). Moreover, in these two cases the smooth phenotype is related to the loss of complexity in the extracellular matrix structure (Table 1). Also, pathogen laboratory strains present lower virulence compared to the newly isolated strains (Sommerville et al., 2011; White & Surette, 2006; Barak et al., 2005; Heddleston, 1964). The phenotypic convergence shared between species that have suffered independent domestication processes reminds of the well-known domestication syndrome observed in crops (Burke et al., 2007; Gepts & Papa, 2002).

It should also be considered that domestication and genetic modification processes also involve the unintended selection of non-target traits (e.g. Hernández-Terán et al., 2017). Laboratory strains for the study of microbial multicellularity represent a clear example. *Myxococcus xanthus* and *Bacillus subtilis* wild strains can develop resistant complex structures in response to adverse environmental conditions. For these multicellular structures to occur, social behavior is needed. However, when culturing them in the laboratory, easily dispersed individual cells or colonies are chosen, and then grown within unstructured environments at liquid mediums, which is reported to be associated to a reduction in social behavior (Aguilar et al., 2007; Velicer et al., 1998). This domestication pathway actually makes them suboptimal for the study of multicellular development (Aguilar et al., 2007).

Remarkably, it is difficult to generalize about domestication processes and outcomes as very different dynamics underlie each specific case. For instance, populations or ecotypes of the same microbial species can be widespread in completely dissimilar environments, and can exhibit different domestication trajectories (Eydallin et al., 2013). Laboratory domestication processes, phenotypes and also metabolic changes depend on the ancestor strains, on physical and chemical properties of the culture medium, e.g. if it is a liquid medium or a hard agar plate, and also on how long they have been exposed to such certain medium (Eydallin et al., 2013). Finally, studying systems with standardized strains and environmental conditions has the objective of supporting confident comparisons across different research groups. However, due to the sensitivity of microbial strains to small variations on experimental treatments and also due to their long laboratory life history, sublines of the same laboratory standard strain could present phenotypic and genetic differences (Bradley et al., 2016).

To conclude, comparisons between laboratory strains and generalization to wild strains should be done with caution as domestication processes occurring in association to widespread experimental approaches could impose important biases. The rapid domestication in laboratory conditions highlights the importance of working with recently isolated wild strains, at least for some research questions. This is critical because in some cases, we do not even know if phenotypes that are commonly observed in laboratory strains actually exist in nature and are ecologically and evolutionarily relevant. For example, are we sure about how fruiting bodies of *Myxococcus xanthus* look when they develop in their natural soil environments? Further studies considering the repeated, well-documented and already ongoing lab-domestication processes could also contribute to a better understanding of organism-environment interactions, phenotypic variation and robustness in a wide phylogenetic context (Bradley et al., 2016).

Laboratory settings vs. natural environments

Laboratory strains are good proxies of their wild ancestors if comparisons of their phenotypes and genotypes are not biased due to their history of experimental manipulation. This situation could only be reached if phenotypic outcomes of these strains were invariant with respect to the environment or if laboratory conditions could mimic the natural ones. The latter is clearly an unrealistic assumption, because as soon as an organism is isolated from its media to be reared in the laboratory, several environmental variables are modified. Moreover, as explained above, experimental designs have not focussed on re-creating natural environments, but on generating “controlled environments” in which selected variables, often genetic ones, can be modified within a constant background (Robert, 2004). In this approach, controlled environments are assumed to function as neutral ones, but they are actually conformed by several biotic and abiotic components contributing to the organism-environment interaction, which in turn gives rise to particular phenotypes (Lewontin, 2001; Sultan, 2017).

Within this controlled-environment designs, development -and its plastic nature- can not be fully understood since it represents a single realization in specific environmental conditions, from a wide repertoire of possible ones. Furthermore, beyond the constant background, experimental settings where single variables are selected to be modified could also be misleading in at least three ways. First, selected variables may not be ecologically

meaningful for the studied organisms and developmental moment. Second, these variables could be ecologically meaningful but tested in non-significative ranges. Third, selected variables and unconsidered ones could be dynamically interacting and modifying the whole developmental system (see, for instance, Box 1). Indeed, meaningful environmental features could be the result of additive effects and complex interactions among variables, but it has been usually considered convenient to test a few but "key" variables, mostly in independent experimental sets (Rivera-Yoshida, et al., 2019). This approach, commonly associated to reaction norm studies, leads to interpreting the environment as a sum of major variables and, consequently, to limited conclusions.

Microorganisms have been considered important experimental models partially due to their ease of manipulation (Love & Travisano, 2013; Jessup et al., 2004). However, natural history for most species is unknown, even for cultivable ones, so that their successful growth in laboratory conditions tells us about their adaptation capacity to laboratory conditions but does not necessarily furthers our understanding of their ecologically meaningful conditions. For instance, the design of culture media is specially focussed on chemical components for nutrient supply, while physical or ecological factors are often overlooked. Remarkably, choosing the correct media chemical properties is not an easy task and may itself uncover interesting environmental dependencies (Uphoff et al., 2001).

The uncultivability phenomenon can provide clues about meaningful variables and ranges of natural settings neglected in current experimental designs, for example, by contrasting experimental properties with the natural ones. Here we identify some key experimental conditions that differ from natural contexts. Growth media is restricted to either solid agar plates or liquid cultures commonly kept at constant agitation, which in turn, is known to favor loss of social behavior after several generations (Velicer et al., 1998). For agar plates, stiffness is standardized fixing the agar concentration, but phenotypic plasticity has been described for microbial development and growth at different substrate stiffness (Rivera-Yoshida et al., 2019; Guégan et al., 2014; Be'er et al., 2009; Box 1). Also, agar plates represent flat and unstructured surfaces, determining properties of microbial aggregates and films such as movement, size and surface tension (Rivera-Yoshida et al., 2018; Persat, 2015). Nutrient supply is constant at optimum concentration rates or at complete scarcity. Genome reduction -also known as genome streamlining- occurs in natural populations when interacting species are metabolically complementary but also in long-term laboratory

conditions (Pande & Kost, 2017; Koskiniemi et al., 2012; Lee et al., 2012). Constant and high nutrient supply are commonly part of experimental scenarios, which could partially explain genome streamlining. Environmental settings are also restricted to small and homogeneous areas, limited by instrumental walls or growing space. Conversely, natural environments involve heterogeneities at different scales, which could in turn drive or restrain collective phenomena. For instance, *M. xanthus* multicellular phenotype can be characterized at the single fruiting body structure, but also at the population level (Rivera-Yoshida et al., 2019; Box 1). The population spatial distribution is determined by the experimental design, however, if larger phenotypic scales are expressed in large and complex media like soil remains unknown.

Abiotic physicochemical factors such as temperature, humidity, pH, pressure, salinity, oxygen concentration, among others, are usually kept constant. However, these are important parameters determining microbial interactions and metabolism (Pham & Kim, 2012). Some of our own unexpected results with *M. xanthus* show how variations in temperature and medium stiffness can strongly affect bacterial multicellular development (Box 1). Indeed, *M. xanthus* fruiting bodies exhibit contrasting phenotypes when developed at different substrate stiffness, so much so that at very low stiffness and standard temperature, no fruiting bodies are formed (Box Figure 1 (c)). At standard stiffness, temperature modification renders subtle phenotypic variation, which could easily lead to the immediate conclusion that this environmental variable does not affect development in a significant way. However, temperature variation reveals drastically different phenotypes at non-standard stiffness conditions, widening the spectrum of phenotypic variation associated to stiffness change (Box Figure 1 (d)-(f)). The joint modification of these two factors renders a phenotypic diversity that could not have been expected from *M. xanthus* being grown at standard conditions, nor from reaction norm experiments considering a single environmental factor (Rivera-Yoshida et al., 2019).

Besides the experimental substrate, other differences between experimental and natural settings can be associated to the management of biological material. Development or growth timing may be different among species, requiring longer or shorter periods to become visible to the experimenter. However, given the high nutrient supply, no more than a few days are given to cultures for their density to increase. Also, population densities are probably much higher than the ones on natural substrates (Pande & Kost, 2017). Regarding *M. xanthus*,

multicellular development of fruiting bodies at nutrient scarcity conditions, is known to happen at high cell densities, around $1x10^4$ cells per fruiting body (Velicer et al., 1998), but actual cell density at natural substrates remains unknown. In the likely case of such densities being much lower than experimental ones, what is known about developmental and quorum sensing mechanisms might be substantially different in natural populations. Additionally, axenic cultures are promoted in experimental designs so that species are intentionally isolated from interspecific interactions. Yet, the importance of dependence, predation and cooperation, among other interactions, for microbial growth and development are largely known (Pande & Kost, 2017; Jacobi et al., 1996). Finally, laboratory populations are mainly composed of clonal populations so that their genetic background lacks the heterogeneity observed in natural populations (Gasch et al., 2015; Eydallin et al., 2013).

The organism-environment interaction is a constantly changing bidirectional process, which also changes with the spatio-temporal scale. In both natural and experimental settings, organisms contribute to the reconstruction of the inter-species niche (Miner et al., 2005; Ryan et al, 2016). For instance, bacterial extracellular matrix secretion is altered by the medium mechanical properties, which in turn are altered by the extracellular matrix secretion (Rivera-Yoshida et al., 2018; Trinschek et al., 2017; Fauvart et al., 2012; Be'er et al., 2009). Thus, dynamics associated to natural and experimental settings can not be fully compared as they follow their own evolutive tempos and paths. Complex ecological interactions are still far from laboratory proxies and efforts improving field protocols or other experimental designs considering environmental complexity are thus necessary.

Final remarks

The microbial world has provided new insights and approaches in the study of organism-environment interactions at both the evolutive and the ecological level (Rivera-Yoshida et al., 2018; O'malley et al., 2015; Love & Travisano, 2013; Jessup et al., 2004). However, developmental mechanisms have been only partially understood since they have been studied through the establishment of experimental designs considering domesticated strains and invariable backgrounds. This approach informs just about a specific and simplified condition from the wide repertoire of environmental settings occuring in nature, in which phenotypic plasticity mechanisms may have obscured. Nevertheless, observations for microbial development highlight the importance of commonly overlooked, yet meaningful

properties of the environment at the microscale. For instance, mechanical factors affecting living and nonliving matter, play a key role determining substrate properties, which in turn, modify organisms' dynamics, such as spreading, moving and developing (Rivera-Yoshida et al., 2018; Persat, 2015). Microbial plastic responses to other ecological factors such as predator presence, interspecies interactions or environment fluctuation remain largely unknown.

The use of laboratory models and conditions like the ones described above respond, at least in part, to the pressure over science to be efficient in terms of time and costs, which in turns favors certain experimental setups and approaches, making standard organisms and experimental conditions to be often the preferred choices (Leonelly & Ankeny, 2013; Ankeny & Leonelli, 2011; Levins & Lewontin, 1985). Subsumed to this scheme, microbial ecological and evolutive processes are probably forced into tempos and conditions that do not match those of natural environments, leaving some open questions. For example, what are the ecologically relevant spatiotemporal scales and variables for microbial development? Is the strength and expression of phenotypic plasticity scale-specific? How plastic are the interspecific interactions? How do different environmental variables interact with each other when they take part in microbial development?

Furthermore, the role of phenotypic plasticity as driver and restrictor in evolutive mechanisms, considered in hypotheses such as "plasticity-first", is probably underestimated since it can not be easily tested in actual experimental designs nor can be compared with phenomena occurring at natural populations (Levis & Pfennig, 2016). For instance, to the best of our knowledge, environmentally-triggered phenotypic novelties and complex interactions among environmental variables have not been explored in microbial systems, not even in paradigmatic long-term evolutionary studies. Additionally, due to their high mutation rate and short generation time, microbial groups could be suitable to the comparison of plasticity versus mutation driven adaptations. Overall, further investigating microbial multicellular development and considering the practical biases underlying its current study can provide invaluable insights to the eco-evo-devo integrative efforts to the understanding of major transitions in evolution.

Table 1. Microbial strains commonly used in laboratory conditions. * No information found.

Species	Natural habitat	Laboratory strain phenotype	Wild strain phenotype	Research focus	Laboratory strain limitations	References
Bacillus subtilis	Plant roots Soil Animal intestinal tracts	Simple macroscopic architecture Thin, fragile, smooth biofilm	Structurally complex Thick and rough biofilm	Molecular mechanisms of colony morphogenesis DNA mediated transformation	Lack of surfactin production: no spreading behavior Inability to form resistant spore structures: multicellularity can not be fully studied Loss of genes or mutations	McLoon et al., 2011 Hong et al., 2009 Aguilar et al., 2007 Branda et al., 2001
Escherichia coli	Soil Water Plant tissues Animal gut	Smooth biofilm	Structurally complex Rough phenotype	Pharmaceutical production Genetic engineering Biotechnology industry	Changes in biofilm structure: loss against predators Changes in metabolic properties	DePas et al., 2014 van Elsas et al., 2011 Blount et al., 2008 Tao et al., 1999 Mikkola & Kurland, 1992

Bartonella henselae	Animal blood and endothelium	*	Fimbriae presence Population genetic variation	Medical research	Mutations and genomic rearrangement during laboratory passaging Decreased genetic variability Loss fimbriae Lower virulence	Arvand et al., 2006 Vhelo et al., 2002
Staphylococcus aureus	Human skin, bones, blood and mucous membrane	*	Increased growth yield Greater ROS production Fitness increase	Medical research	Mutations and genomic rearrangement during lab passaging Lower virulence Alteration in cell density sensing Alteration on the surfactant production	Periasamy et al., 2012 Somerville et al., 2011
Pasteurella multocida	Animal lungs, bloodstream and mucous membrane	*		Medical research	Loss of capsule production: critical for its virulence and antibiotics	Steen et al., 2010 Harper et al., 2006

					resistance	Heddleston et al., 1964
Salmonella enterica	Birds eggs Plant tissues Water Animals intestinal tract	Smooth biofilm	Structurally complex biofilm Dry and rough biofilm	Infectious diseases studies	Diversification of genotypes Altered biosynthesis of cellulose and polysaccharides: loss of spatial phenotype morphology Resistant to desiccation	Davidson et al., 2008 White & Surette, 2006 Barak et al., 2005
Saccharomyces cerevisiae	Oak bark and other trees and plants Human microbiota Insects Soil Bread Beer and wine	Smooth colonies Change in cells shape	Structurally complex biofilm Highly glycosylated extracellular matrix Resistant to antioxidative stress Phenotypic heterogeneity Sporulates	Genetic engineering Biotechnological industry	Loss of extracellular matrix Gene expression reprogramming	Šťovíček et al., 2014 Piccirillo & Honigberg, 2010 Diezmann & Dietrich, 2009 Palková et al., 2004 Kuthan et al., 2003

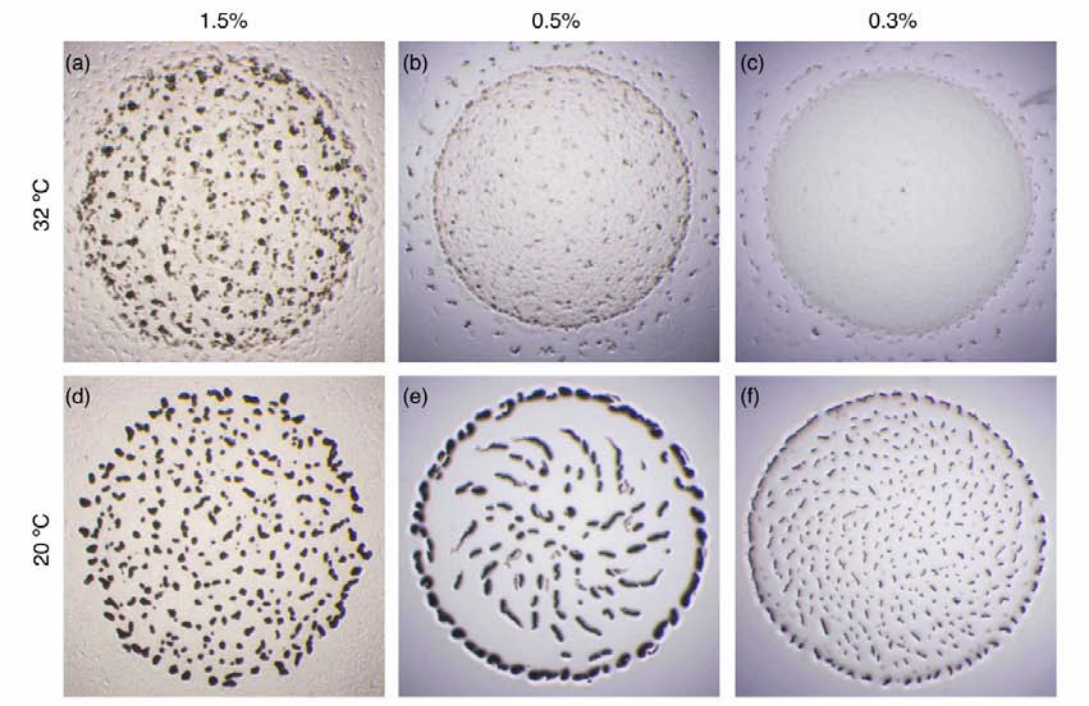
			on a wider range of carbon sources			
Myxococcus xanthus	Soil	Smooth colonies	Large genetic heterogeneity Large variation in phenotypic and developmental traits	Multicellularity Cell differentiation Cell motility	Loss of social behavior: multicellularity can not be fully studied	Kraemer et al., 2010 Velicer et al., 1998

Box 1. Organism-environment interactions shaping multicellular development in *Myxococcus xanthus*

M. xanthus is a widespread soil bacterium with a multicellular developmental stage. It rich substrates, cells are in a vegetative stage and swarm expanding the colony outwards. Conversely, when nutrients are depleted, they glide inwards, developing multicellular structures called fruiting bodies (FBs), where eventually, some cells differentiate into resistant spores (Yang & Higgs, 2014). The standard protocol for multicellular development consists in the deposition of a liquid culture drop over a nutrient depleted agar plate -commonly prepared with 1.5% of agar-. After the drop dries, the plate is stored at 32 ºC avoiding light for about 96h until FBs have developed.

M. xanthus cells sense and respond to the structural and mechanical properties of the substrate over which they move. For example, they realign perpendicularly to mechanical compression applied to the agar mesh (Lemon et al., 2017; Fontes & Kaiser, 1999). Additionally, *M. xanthus* development has revealed two scales of phenotypic expression: the single FB scale and the population scale (consisting on the collection of FBs within a

drop), both of which present phenotypic plasticity when substrate stiffness is modified (Rivera-Yoshida et al., 2019). The effect of other variables such as temperature, has not been widely or systematically tested. Furthermore, substrate stiffness is modified varying agar concentration. However, substrate mechanical properties might be the result of the interaction of more than this single variable. For instance, substrates with the same agar concentration but different temperature, could differ in stiffness.



Box Figure 1. Phenotypic plasticity of *Myxococcus xanthus* multicellular structures. Micrographs of completely matured FBs populations developed over TPM agar plates. Black structures correspond to FBs. DZF1 standard laboratory strain was tested modifying temperature and agar concentration: (a) standard protocol condition: 32 ºC and 1.5% agar concentration. (b) 32 ºC, 0.5% (c) 32 ºC, 0.3% (d) 20 ºC, 1.5% (e) 20 ºC, 0.5% and (f) 20 ºC, 0.3%. Micrographs of each drop were taken at 370.8 pixels/mm using a LEICA m50 stereomicroscope with an ACHRO 0.63x objective lens and a Canon-EOS Rebel T3i camera. Apart from variation in temperature and agar percentage, *M. xanthus* were grown and developed as described in Yang and Higgs, 2014.

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5. Conclusiones y perspectivas

En este trabajo de tesis se integra evidencia conceptual y experimental, donde se muestra que la variación fenotípica en el desarrollo de los microorganismos implica la contribución de los factores genéticos, de los ambientales, y de su interacción. En particular, integra y genera evidencia respecto a la importancia del ambiente físico en el que se desarrollan los microorganismos multicelulares. A través de la revisión bibliográfica del papel de las fuerzas mecánicas en el desarrollo de los microorganismos, se diseñó un experimento de norma de reacción, en el cual el organismo modelo *Myxococcus xanthus* fue desarrollado sobre substratos con rigidez variable. Mediante este experimento, se cuantificó y se analizó la variación fenotípica en el desarrollo de esta bacteria, sugiriendo que la contribución del genotipo, del ambiente y de su interacción, es dependiente de la escala y de los rasgos del fenotipo. Por ejemplo, el substrato parece tener un mayor papel en ciertos rangos de rigidez, reflejándose en ciertos rasgos de manera independiente al genotipo. Estas observaciones permiten repensar sobre el significado del fenotipo y del ambiente cuando se consideran escalas diferentes, como la microescala.

En esta tesis se presenta, hasta donde sabemos, uno de los primeros experimentos de normas de reacción utilizando bacterias como modelo de estudio. Esta aproximación, acompañada de los análisis multivariados, permitieron notar que la variación fenotípica es, en varios casos, mayor en los extremos del rango de rigidez probado. Esta observación hace referencia a estudios teóricos previos en los que se propone que los cambios drásticos en el ambiente pueden descubrir variación que no había sido expresada (Schmalhausen, 1949).

Sin embargo, a pesar de las contribuciones que pueden hacerse mediante los métodos analíticos presentados aquí, los resultados se restringen a la interpretación de la relación pareada entre un rasgo y una variable ambiental para un momento específico del desarrollo. En este sentido, el estudio y el mejor entendimiento de la interacción organismo-ambiente requiere nuevos enfoques que consideren la interacción dinámica entre múltiples rasgos fenotípicos y variables ambientales a lo largo del proceso de desarrollo.

La integración del trabajo de revisión y los resultados experimentales, muestran contundentemente que la variabilidad ambiental contribuye a la variación fenotípica de manera robusta, como se le considera comúnmente a la contribución genética, y no de manera contingente. Este resultado permitió reflexionar sobre la importancia de la elección

de variables y rangos con relevancia ecológica en el diseño de experimentos que pretendan estudiar la relación organismo-ambiente. Permitió, también, discutir sobre la restricción a la variación fenotípica que puede resultar de contextos ambientales en donde una sola variable sea modificada dentro de un contexto constante, o del uso de cepas de laboratorio que han sido adaptadas a un contexto ambiental específico por largo tiempo.

En conjunto, esta tesis destaca la importancia del ambiente, específicamente de sus propiedades físicas y mecánicas, en el desarrollo de estructuras multicelulares microbianas. Además, muestra que en la interacción organismo-ambiente, tanto la contribución genética, como la ambiental, como su interacción, pueden ser fuentes de variación fenotípica, lo cual puede ser relevante en la escala y el contexto en los cuales ocurrió la transición a la multicelularidad. En este sentido, este trabajo se integra al marco conceptual que sugiere que la plasticidad, por sí misma, puede contribuir como causa y no sólo como consecuencia del desarrollo y de las transiciones fenotípicas en la evolución (West Eberhard, 2003; Jablonka & Lamb, 2014; Laland *et al*., 2015).

Finalmente, esta tesis aborda la interacción organismo-ambiente de manera unidireccional, enfocándose en el papel del ambiente en el desarrollo del organismo. Sin embargo, es interesante resaltar que esta interacción representa una compleja dinámica bidireccional, es decir, que los organismos no sólo son sensibles a su entorno, sino que también se encuentran contribuyendo de manera constante a su construcción (Miner *et al*, 2005; Sultan, 2015). Además, quedan por descifrarse los mecanismos mediante los cuales se moldea la interacción de los organismos con su ambiente en este camino de ida y de vuelta.

6. Apéndice

Plastic multicellular development of *Myxococcus xanthus*: genotype-environment interactions in a physical gradient

Natsuko Rivera-Yoshida[1,2,3], Alejandro V. Arzola[4], Juan A. Arias Del Angel[1,2,3], Alessio Franci[5], Michael Travisano[6], Ana E. Escalante[1]*, Mariana Benítez[1,2]*

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Supplementary Information

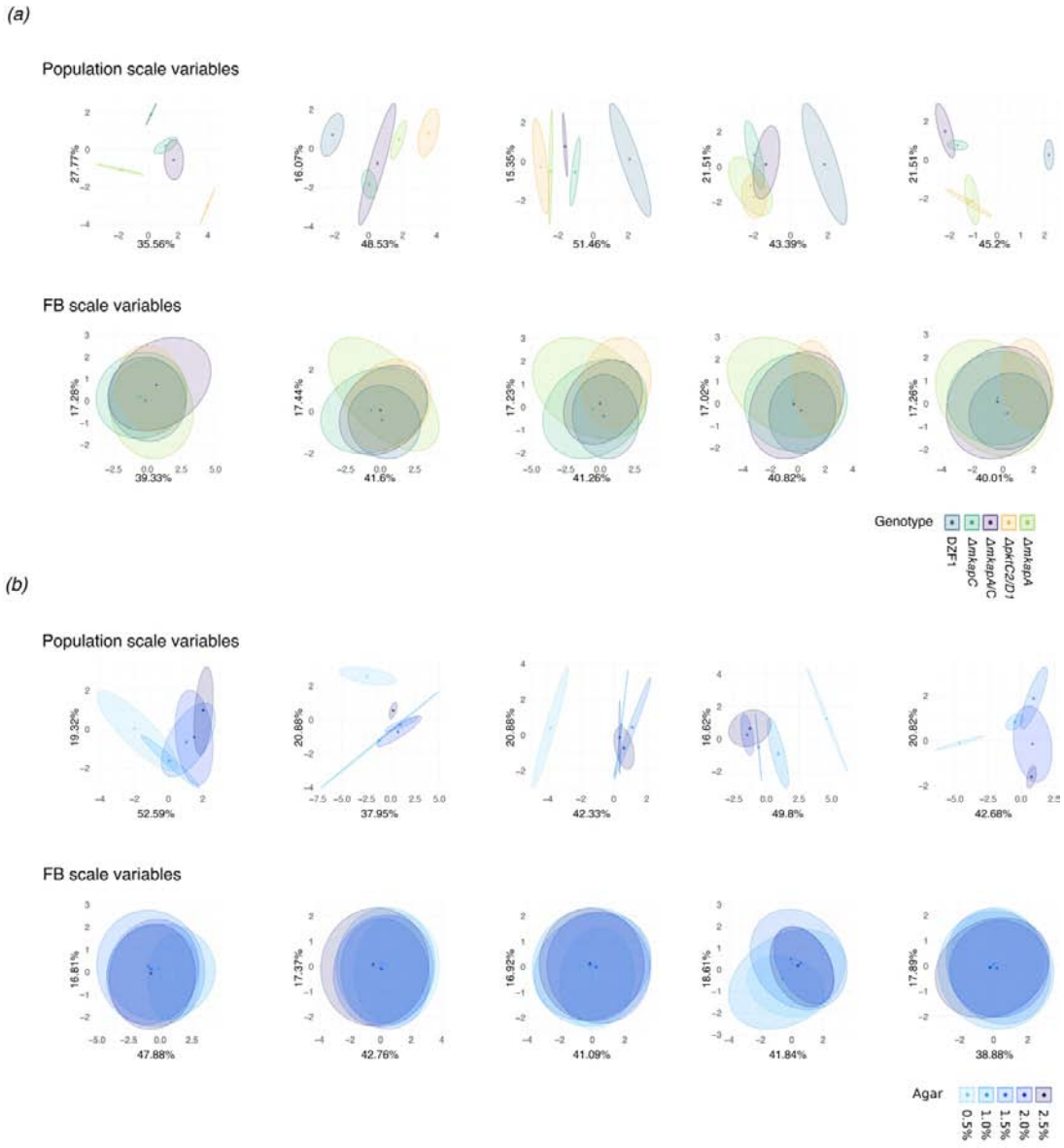


Figure S1. FAMD analysis based on data grouped by phenotypic expression scale (FB scale, population scale) per *(a)* substrate agar concentration and *(b)* genotype. 95% confidence interval ellipses enclose data centroids.

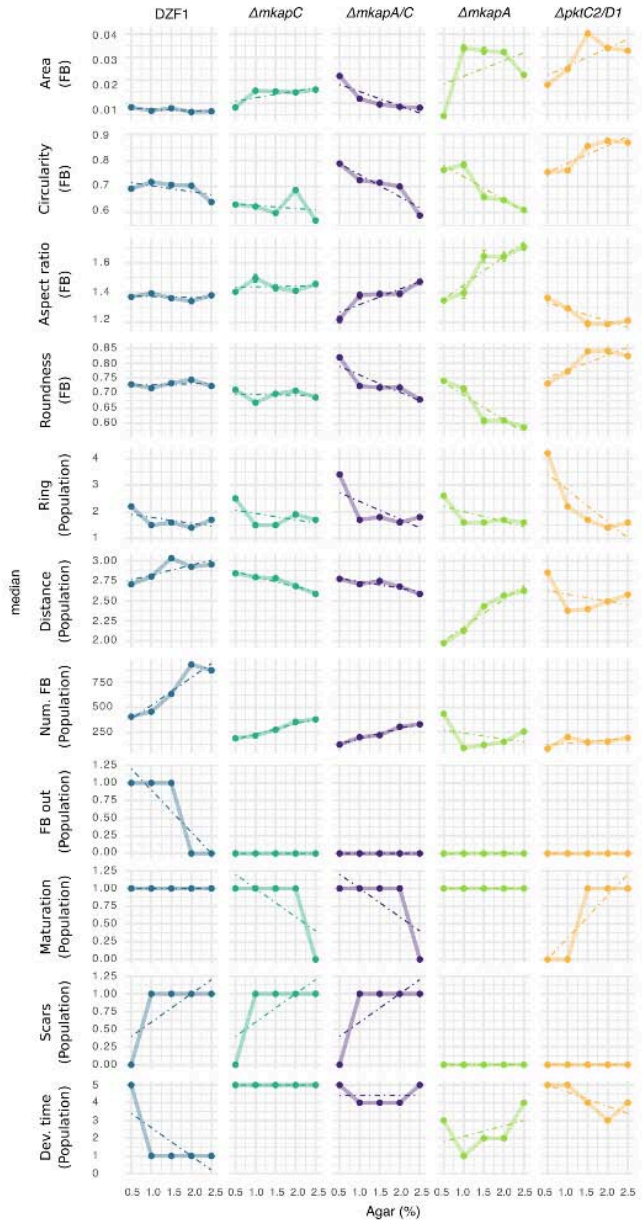


Figure S2. Reaction norms of all considered phenotypic traits among *Myxococcus xanthus* parental and mutant genotypes in response to substrate agar concentration. Each line represents the reaction norm of a single genotype (columns) based on the median ± standard error of single phenotypic traits (rows). Linear regression fit with a dotted line, although p-values are not significant in all cases.

Phenotypic trait (short name)	Phenotypic trait	Formula / Symbol	Phenotypic scale
Area	Area	mm^2	FB
Circularity	Circularity (Shape descriptor)	$4\pi * [Area]/[Perimeter]^2$ Ranges from 0 (infinitely elongated polygon) to 1 (perfect circle) Dimensionless	FB
Aspect ratio	Aspect ratio (Shape descriptor)	[Major axis] / [Minor axis] Dimensionless	FB
Roundness	Roundness (Shape descriptor)	$4 * [Area] / \pi \times [Major axis]$ Dimensionless	FB
Ring	Ring formation at the edge of the drop	max[g(r)] / mean[g(r)] Where g is the density of gray values, in the inverted image, as a function of the radial position r. mm^{-2}	Population
Distance	Standard distance between FBs	$\sqrt{[\Sigma((coordsX - mcX)^2 + (coordsY - mcY)^2)]}$ / Num. FB Where coordsX, coordsY = FB coordinates on X and Y axis, respectively mcX, mcY = mean center coordinate on X and Y axis respectively mm	Population
Num. FB	Number of FB	Count of FB	Population
FB out	FB outside the edge of the drop	0 (No) 1 (Yes)	Population
Maturation	Complete maturation (fully developed/complete darkening) of FB at 96h	0 (No) 1 (Yes)	Population
Scars	Vestiges of not clearly defined FB	0 (No) 1 (Yes)	Population
Dev. time	Developmental time (full development from 0h to 96h)	Range from 1 (fast development) 5 (slow development)	Population

Table S1. Phenotypic traits, formulas and scales.

Strain: DZF1	Dim.1 (33.97%)	Dim.2 (19.46%)	Dim.3 (11.77%)	Dim.4 (8.99%)	Dim.5 (8.46%)
Area	0.36	0.03	2.42	69.13	1.59
Circularity	0.00	**22.16**	0.40	3.22	0.00
Aspect ratio	0.00	**38.62**	0.42	1.09	0.03
Roundness	0.01	**38.41**	0.40	1.18	0.10
Ring	**18.43**	0.03	9.76	0.09	0.71
Distance	15.64	0.00	0.46	1.75	1.82
Number FB	14.69	0.01	10.73	1.95	1.20
FB out the drop	6.30	0.15	43.28	0.14	0.59
Complete Maturation	0.00	0.00	0.00	0.00	0.00
Scars	**22.24**	0.02	2.12	0.19	0.72
Dev. time	**22.24**	0.02	2.12	0.19	0.72
Replica	0.09	0.55	27.89	21.06	92.53

Strain: *ΔmkapC*	Dim.1 (26.40%)	Dim.2 (22.32%)	Dim.3 (13.70%)	Dim.4 (9.33%)	Dim.5 (9.02%)
Area	1.98	1.96	5.73	4.38	1.58
Circularity	3.11	**22.33**	1.03	0.11	0.02
Aspect ratio	2.31	**33.42**	0.02	0.00	0.03
Roundness	2.13	**34.20**	0.31	0.02	0.01
Ring	11.93	0.00	35.57	2.07	0.30
Distance	**18.34**	3.21	15.64	0.77	0.00
Number FB	**21.99**	2.96	4.64	1.83	0.03

FB out the drop	0.00	0.00	0.00	0.00	0.00
Complete Maturation	17.17	1.23	12.09	3.38	1.01
Scars	**20.26**	0.46	21.50	0.03	0.76
Dev. time	0.00	0.00	0.00	0.00	0.00
Replica	0.78	0.24	3.46	87.41	96.26

Strain: *ΔmkapA/ΔmkapC*	**Dim.1 (27.01%)**	**Dim.2 (18.57%)**	**Dim.3 (15.97%)**	**Dim.4 (10.43%)**	**Dim.5 (9.92%)**
Area	2.28	2.03	2.85	0.03	6.88
Circularity	13.40	9.06	0.98	0.11	0.59
Aspect ratio	12.68	11.32	11.90	0.60	0.02
Roundness	**13.81**	10.15	11.75	0.48	0.06
Ring	11.15	**19.07**	1.60	6.88	2.85
Distance	6.15	0.97	11.82	19.59	3.08
Number FB	**16.48**	3.83	9.97	0.02	8.33
FB out the drop	0.00	0.00	0.00	0.00	0.00
Complete Maturation	7.68	4.29	29.14	4.35	0.46
Scars	**15.85**	**17.57**	0.03	1.43	1.17
Dev. time	0.05	**21.24**	18.08	10.05	0.25
Replica	0.48	0.46	1.87	56.46	76.29

Strain: *ΔmkapA*	**Dim.1 (38.71%)**	**Dim.2 (16.42%)**	**Dim.3 (11.46%)**	**Dim.4 (10.01%)**	**Dim.5 (7.12%)**
Area	14.82	3.29	0.50	5.10	1.42

Circularity	**15.21**	8.35	0.92	0.47	0.03
Aspect ratio	**14.86**	15.29	3.94	1.64	0.43
Roundness	14.84	**15.50**	3.57	0.74	0.61
Ring	**16.17**	11.32	1.82	9.75	0.30
Distance	11.28	0.62	7.66	35.77	0.41
Number FB	12.03	**17.65**	5.36	6.98	0.00
FB out the drop	0.00	0.00	0.00	0.00	0.00
Complete Maturation	0.00	0.00	0.00	0.00	0.00
Scars	0.00	0.00	0.00	0.00	0.00
Dev. time	0.52	**26.22**	28.43	9.50	0.00
Replica	0.27	1.76	47.80	30.05	96.81

Strain: *ΔpktC2/ΔpktD1*	**Dim.1 (30.54%)**	**Dim.2 (20.81%)**	**Dim.3 (15.06%)**	**Dim.4 (9.69%)**	**Dim.5 (9.11%)**
Area	3.85	8.17	9.86	1.16	1.02
Circularity	15.24	8.37	0.05	0.57	0.16
Aspect ratio	10.38	**18.87**	8.65	0.34	0.04
Roundness	11.52	**16.94**	9.36	0.52	0.03
Ring	**17.72**	13.90	1.74	0.20	0.46
Distance	2.48	**18.13**	14.20	7.87	1.06
Number FB	0.84	12.80	29.77	1.60	0.00

FB out the drop	0.00	0.00	0.00	0.00	0.00
Complete Maturation	**19.06**	1.30	12.21	2.37	0.11
Scars	0.00	0.00	0.00	0.00	0.00
Dev. time	**18.68**	0.56	12.26	0.35	1.79
Replica	0.23	0.97	1.90	85.03	95.32

Agar: 0.5%	**Dim.1 (25.19%)**	**Dim.2 (21.39%)**	**Dim.3 (17.53%)**	**Dim.4 (8.50%)**	**Dim.5 (8.23%)**
Area	6.50	2.18	0.07	1.08	2.39
Circularity	1.66	**19.47**	2.86	0.41	0.17
Aspect ratio	0.04	**17.41**	22.81	0.00	0.06
Roundness	0.02	**17.91**	22.10	0.00	0.01
Ring	8.04	14.78	14.06	0.61	0.01
Distance	**20.44**	3.63	5.40	0.35	3.58
Number FB	**26.53**	0.99	0.35	0.32	1.82
FB out the drop	0.14	13.26	18.58	0.38	2.85
Complete Maturation	11.39	5.78	5.73	0.04	4.05
Dev. time	**22.53**	4.33	7.15	0.81	0.08
Replica	2.70	0.26	0.89	96.01	85.00

Agar: 1.0%	**Dim.1 (35.71%)**	**Dim.2 (19.91%)**	**Dim.3 (10.73%)**	**Dim.4 (9.56%)**	**Dim.5 (7.81%)**
Area	6.82	0.12	2.07	17.66	2.64
Circularity	0.16	**25.90**	0.28	0.00	0.08

Aspect ratio	0.03	**33.13**	2.55	0.84	0.10
Roundness	0.13	**32.72**	3.08	0.87	0.03
Ring	13.86	0.45	13.28	5.10	0.07
Distance	12.41	0.40	6.88	12.84	0.90
Number FB	**13.94**	1.98	12.28	0.00	0.91
FB out the drop	**14.88**	1.88	11.40	1.56	0.97
Complete Maturation	12.38	0.85	5.72	1.21	2.28
Scars	**15.32**	0.48	5.36	8.52	0.01
Dev. time	9.69	1.61	13.36	20.04	5.32
Replica	0.37	0.48	23.74	31.37	86.70

Agar: 1.5%	**Dim.1 (37.74%)**	**Dim.2 (20.44%)**	**Dim.3 (11.61%)**	**Dim.4 (8.96%)**	**Dim.5 (8.19%)**
Area	9.31	0.16	9.20	8.86	0.11
Circularity	0.13	**27.59**	0.05	0.48	0.21
Aspect ratio	1.24	**34.02**	0.00	1.44	0.07
Roundness	0.77	**34.77**	0.02	1.30	0.03
Ring	6.11	1.32	25.46	4.70	0.01
Distance	**19.25**	0.53	3.27	1.17	0.00
Number FB	**20.96**	0.10	0.32	1.74	0.11
FB out the drop	**19.06**	0.03	0.45	8.84	1.07
Complete Maturation	0.00	0.00	0.00	0.00	0.00
Dev. time	12.45	0.01	2.70	28.11	2.82

Replica	0.67	0.12	45.53	22.37	94.79

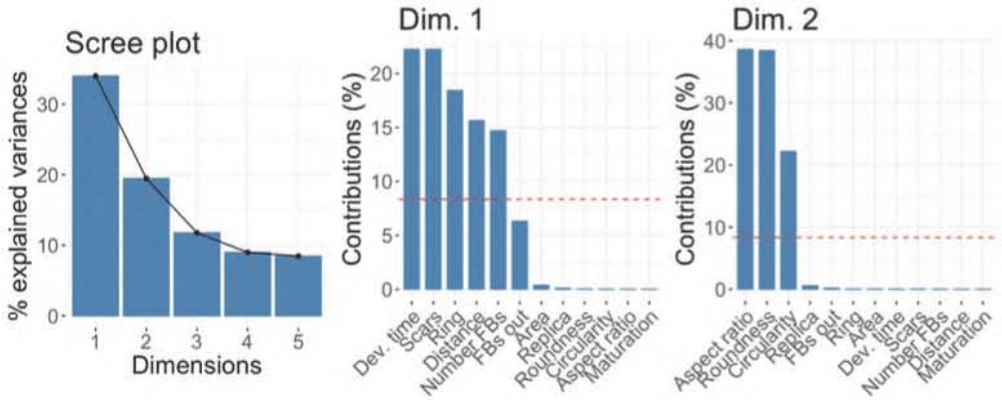
Agar: 2.0%	**Dim.1 (32.87%)**	**Dim.2 (20.27%)**	**Dim.3 (14.37%)**	**Dim.4 (10.41%)**	**Dim.5 (6.41%)**
Area	11.01	1.76	4.21	4.69	1.25
Circularity	0.71	**25.92**	0.41	0.00	0.17
Aspect ratio	2.97	**29.21**	2.94	1.64	0.39
Roundness	2.24	**30.56**	2.68	1.38	0.46
Ring	9.64	1.78	13.52	15.37	0.00
Distance	**19.78**	3.28	4.38	0.77	0.07
Number FB	**22.73**	1.89	0.60	0.15	0.73
FB out the drop	6.63	0.01	23.56	13.07	0.01
Complete Maturation	0.00	0.00	0.00	0.00	0.00
Scars	6.98	5.31	9.78	16.07	10.56
Dev. time	**16.79**	0.04	2.99	8.86	13.50
Replica	0.54	0.22	34.94	38.00	72.84

Agar: 2.5%	**Dim.1 (33.24%)**	**Dim.2 (19.83%)**	**Dim.3 (15.30%)**	**Dim.4 (8.48%)**	**Dim.5 (8.40%)**
Area	7.31	4.68	5.28	0.24	1.70
Circularity	1.49	**24.48**	0.06	0.67	0.04
Aspect ratio	2.77	**26.31**	9.54	0.05	0.15
Roundness	2.36	**27.92**	8.66	0.08	0.06
Ring	3.74	1.38	20.06	1.94	0.00

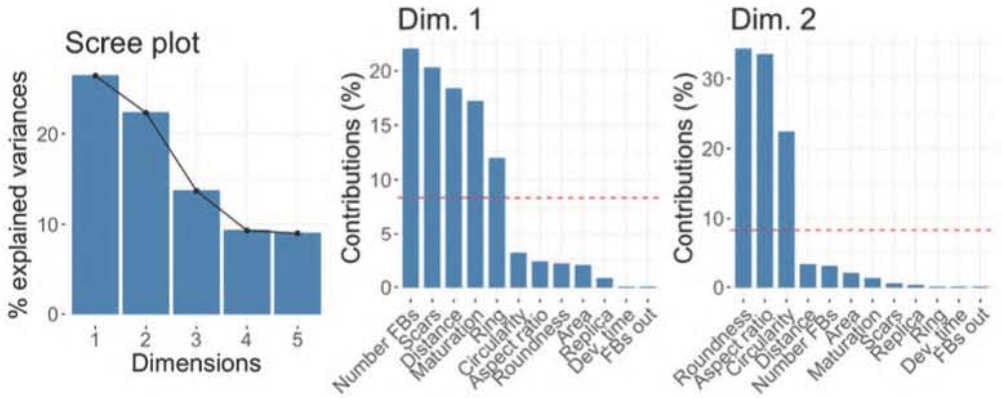
Distance	**22.45**	1.77	0.00	0.14	0.90
Number FB	**21.73**	3.56	0.90	0.00	0.05
FB out the drop	0.00	0.00	0.00	0.00	0.00
Complete Maturation	11.70	1.35	23.17	1.04	0.12
Scars	3.21	8.13	30.48	0.79	0.05
Dev. time	**23.15**	0.37	1.07	0.00	0.01
Replica	0.08	0.05	0.79	95.05	96.93

Table S2. Dimension values of FAMD analysis for each phenotypic trait. Data was grouped by genotype and by agar concentration.

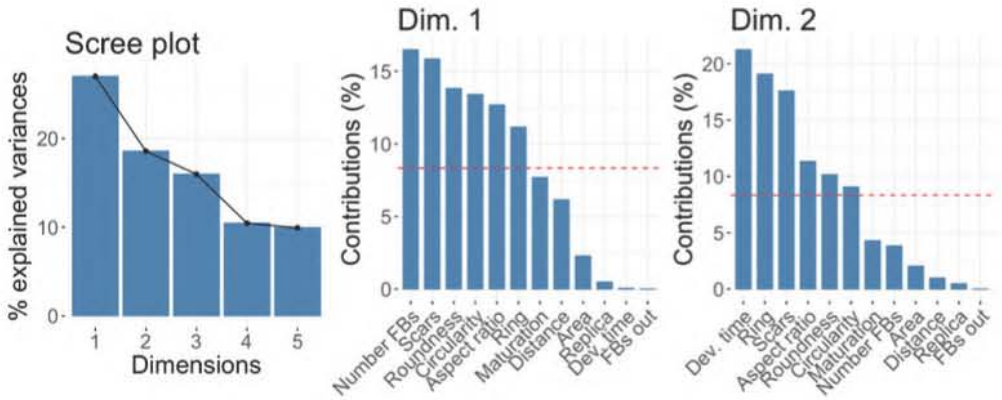
DZF1



ΔmkapC



ΔmkapA/C



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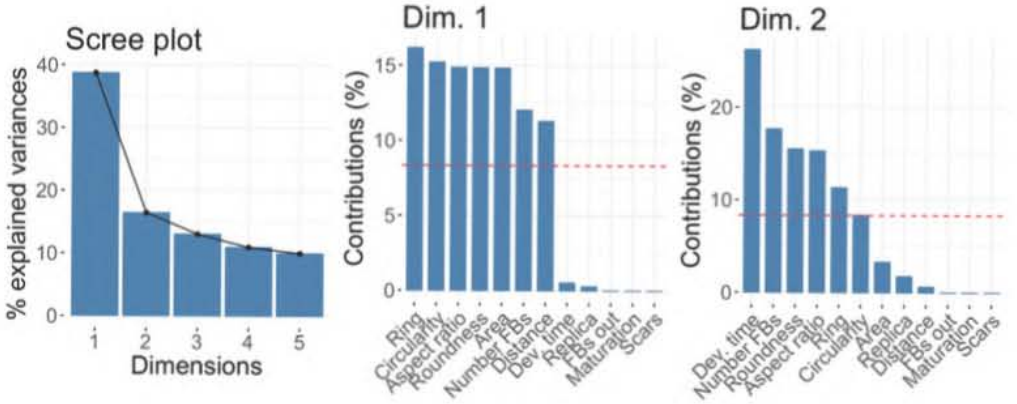
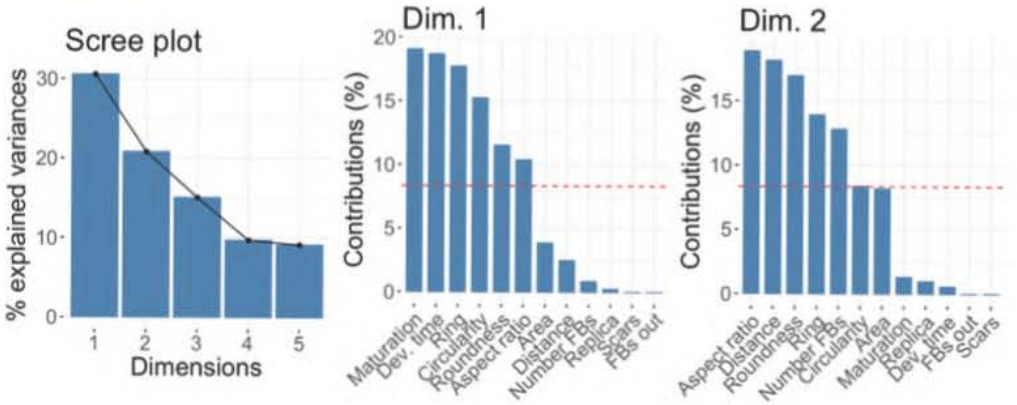


ΔpktC2/D1



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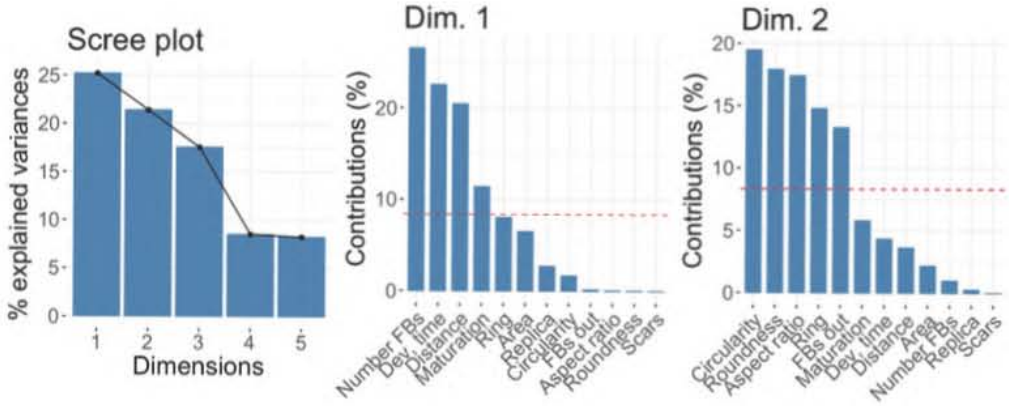

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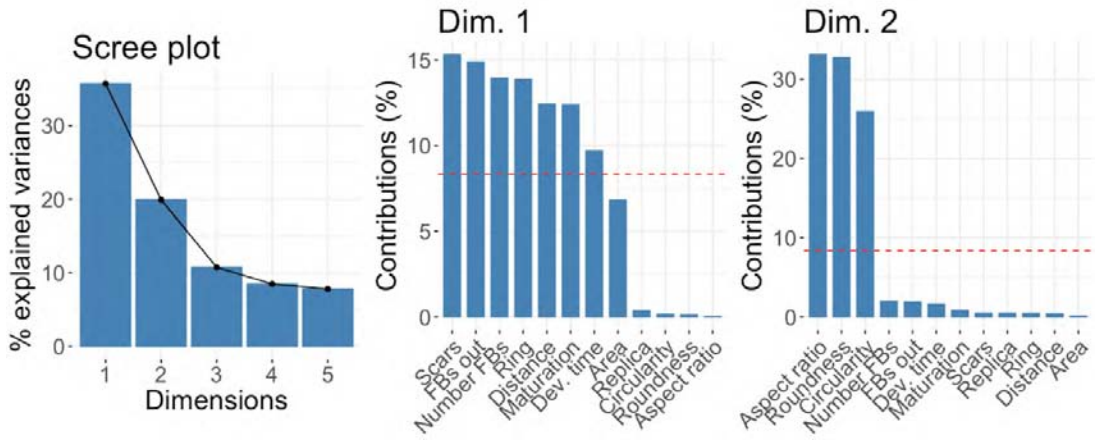
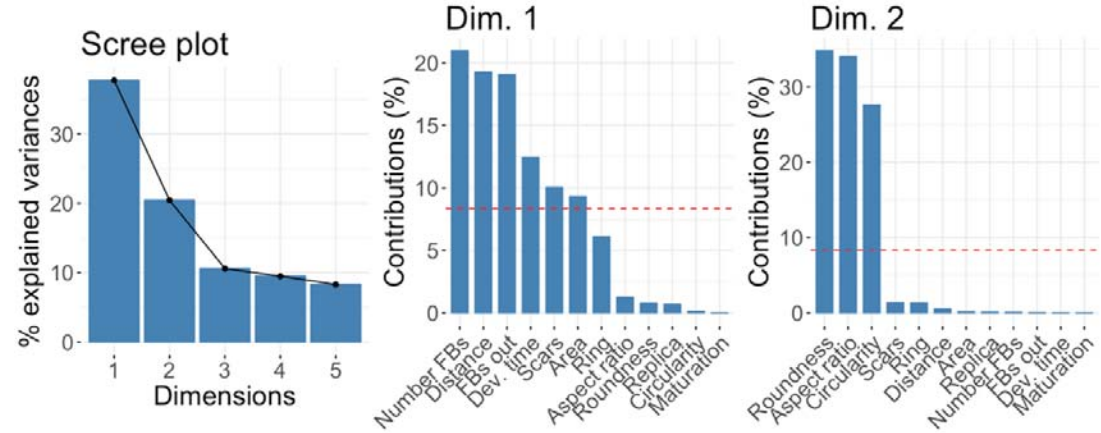

Scree plot
% explained variances
Dimensions
Dim. 1
Contributions (%)
Scars
FBs out
Number FBs
Ring
Distance
Maturation
Dev. time
Area
Replica
Circularity
Roundness
Aspect ratio
Dim. 2
Contributions (%)
Aspect ratio
Roundness
Circularity
Number FBs
FBs out
Dev. time
Maturation
Scars
Replica
Ring
Distance
Area

1.5%


Scree plot
% explained variances
Dimensions
Dim. 1
Contributions (%)
Number FBs
Distance
FBs out
Dev. time
Scars
Area
Ring
Aspect ratio
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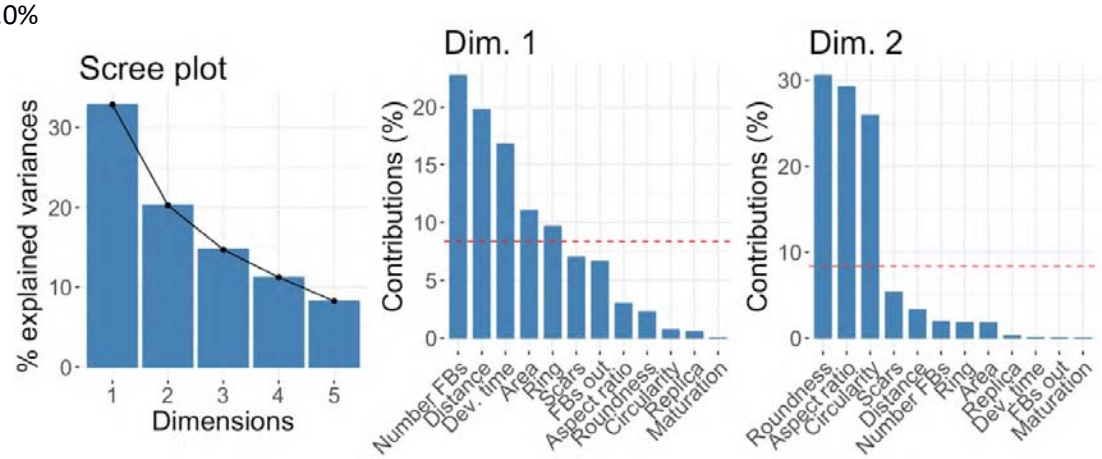
Replica
Circularity
Maturation
Dim. 2
Contributions (%)
Roundness
Aspect ratio
Circularity
Scars
Ring
Distance
Area
Replica
Number FBs
FBs out
Dev. time
Maturation

2.0%


Scree plot
% explained variances
Dimensions
Dim. 1
Contributions (%)
Number FBs
Distance
Dev. time
Area
Ring
Scars
FBs out
Aspect ratio
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Replica
Maturation
Dim. 2
Contributions (%)
Roundness
Aspect ratio
Circularity
Scars
Distance
Number FBs
Ring
Area
Replica
Dev. time
FBs out
Maturation

2.5%

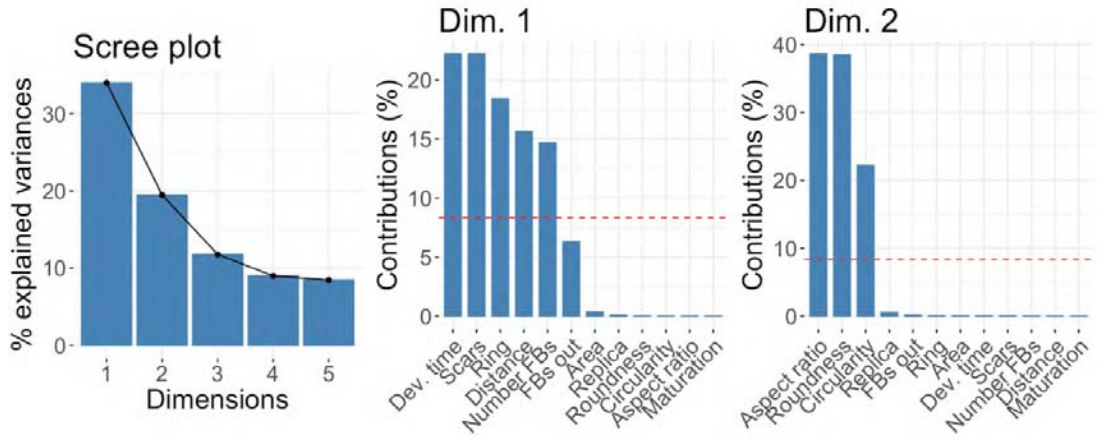

Figure S3. FAMD analysis summary. Data was grouped by genotype and by agar concentration. *(a)* Scree plot. Percentage of explained variances in the first five dimensions. *(b)* Contributions of variables to Dimension 1 and *(c)* contributions of variables to Dimension 2. The red dashed line indicates the expected average value.

(a)

Genotype/Strain	Trait	P value	F stat.	R2	Slope	Intercept	Sqr_err
ΔmkapA	Area	0.000	544.108	0.141	0.009	0.009	0.000
	Circularity	0.000	679.817	0.170	-0.080	0.785	0.022
	Aspect ratio	0.000	417.361	0.112	0.224	1.356	0.284
	Roundness	0.000	499.915	0.131	-0.073	0.755	0.025
	Ring	0.000	6839.785	0.673	-0.540	2.841	0.101
	Distance	0.000	15865.950	0.827	0.296	1.896	0.013
	Num. FB	0.000	1387.754	0.295	-110.439	471.363	20828.100
	FB out	NaN	NaN	NaN	0.000	0.000	0.000
	Maturation	0.307	3323.881	0.500	0.000	1.000	0.000
	Scars	NaN	NaN	NaN	0.000	0.000	0.000
	Dev. time	0.000	341.329	0.093	0.384	2.551	1.022
ΔmkapA/C	Area	0.000	13.073	0.004	-0.002	0.021	0.000
	Circularity	0.000	408.548	0.106	-0.078	0.793	0.023
	Aspect ratio	0.000	66.910	0.019	0.105	1.379	0.251
	Roundness	0.000	101.390	0.029	-0.042	0.764	0.027
	Ring	0.000	1567.481	0.313	-0.411	2.638	0.166
	Distance	0.000	491.914	0.125	-0.072	2.762	0.016
	Num. FB	0.000	4615.038	0.573	94.328	106.963	2967.003
	FB out	NaN	NaN	NaN	0.000	0.000	0.000
	Maturation	0.000	4074.926	0.542	-0.502	1.578	0.095

	Scars	0.000	2307.834	0.401	0.290	0.391	0.056
	Dev. time	0.000	463.223	0.119	0.295	3.836	0.289
ΔmkapC	Area	0.000	132.558	0.030	0.003	0.012	0.000
	Circularity	0.000	25.115	0.006	-0.018	0.635	0.027
	Aspect ratio	0.280	1.165	0.000	0.014	1.589	0.321
	Roundness	0.496	0.463	0.000	-0.003	0.681	0.030
	Ring	0.000	776.066	0.154	-0.207	2.150	0.111
	Distance	0.000	5663.661	0.571	-0.129	2.927	0.006
	Num. FB	0.000	35269.270	0.893	103.746	129.636	616.349
	FB out	NaN	NaN	NaN	0.000	0.000	0.000
	Maturation	0.000	4517.801	0.515	-0.461	1.507	0.095
	Scars	0.000	3816.291	0.473	0.345	0.281	0.063
	Dev. time	0.087	4247.723	0.500	0.000	5.000	0.000
ΔpktC2/D1	Area	0.000	209.507	0.083	0.007	0.021	0.000
	Circularity	0.000	461.348	0.166	0.075	0.675	0.013
	Aspect ratio	0.000	74.236	0.031	-0.096	1.506	0.129
	Roundness	0.000	99.891	0.041	0.044	0.705	0.020
	Ring	0.000	2337.550	0.503	-0.877	3.409	0.344
	Distance	0.002	9.170	0.004	-0.013	2.518	0.018
	Num. FB	0.000	202.778	0.081	16.852	140.398	1463.382
	FB out	NaN	NaN	NaN	0.000	0.000	0.000
	Maturation	0.000	6041.181	0.723	0.611	-0.352	0.065
	Scars	NaN	NaN	NaN	0.000	0.000	0.000
	Dev. time	0.000	3920.039	0.629	-0.946	5.610	0.239

DZF1	Area	0.000	167.262	0.017	-0.001	0.010	0.000
	Circularity	0.000	74.269	0.007	-0.016	0.704	0.015
	Aspect ratio	0.907	0.014	0.000	0.001	1.446	0.115
	Roundness	0.452	0.565	0.000	0.002	0.719	0.019
	Ring	0.000	2448.239	0.197	-0.161	1.897	0.047
	Distance	0.000	4709.355	0.321	0.103	2.727	0.010
	Num. FB	0.000	34552.620	0.776	277.660	259.570	9867.596
	FB out	0.000	16259.910	0.620	-0.588	1.552	0.094
	Maturation	0.069	9947.936	0.500	0.000	1.000	0.000
	Scars	0.000	8337.421	0.456	0.331	0.313	0.058
	Dev. time	0.000	8337.421	0.456	-1.323	3.750	0.929

(b)

Genotype/Strain	**Trait**	**P value**	**F stat.**	**R2**	**Slope**	**Intercept**	**Sqr_err**
ΔmkapA	Dim. 1 FAMD	0.001	18.187	0.583	-2.468	3.702	2.176
ΔmkapA/C		0.000	54.204	0.659	-2.698	4.047	1.880
ΔmkapC		0.000	61.712	0.589	-2.493	3.740	2.165
ΔpktC2/D1		0.000	101.046	0.635	-2.620	3.929	1.970
DZF1		0.000	138.558	0.655	-2.615	3.922	1.801
ΔmkapA	Dim. 2 FAMD	0.000	46.447	0.389	-1.889	2.833	2.804
ΔmkapA/C		0.000	16.390	0.183	-1.184	1.775	3.120
ΔmkapC		0.120	2.478	0.033	-0.470	0.705	3.253
ΔpktC2/D1		0.653	0.204	0.003	0.120	-0.181	2.600
DZF1		0.015	6.179	0.078	0.603	-0.905	2.148

Table S3. Linear regression fit for *(a)* phenotypic traits per genotype and *(b)* Dimension 1 and Dimension 2 of the FAMD multivariate analysis. Statistical information is shown in columns.

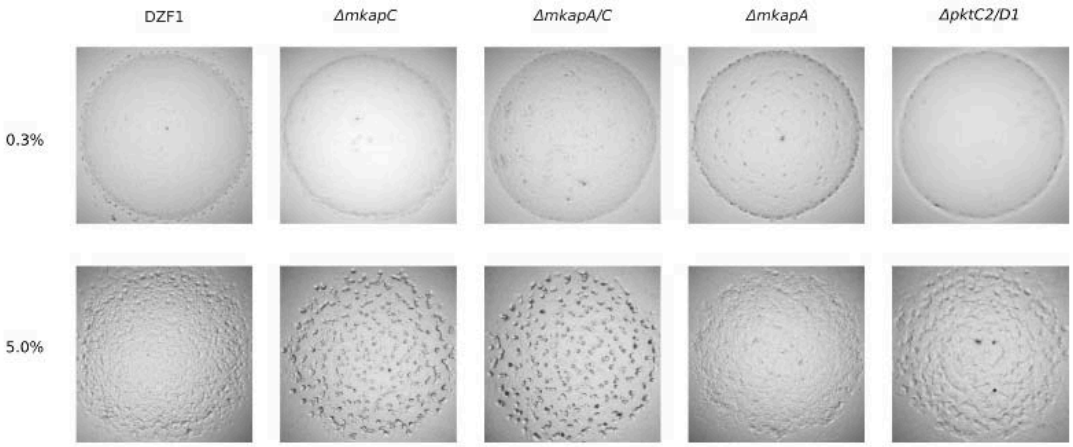

Figure S4. Fruiting bodies do not fully develop at 2.5% and 0.3% agar percentages. Note that even if aggregates are visible for some strains, they do not darken, indicating that spores do not differentiate. All micrographs were taken at 96 h.

7. Referencias

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