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PROCESOS DE DIFERENCIACIÓN CELULAR Y DE FORMACIÓN DE PATRONES DE TIPOS CELULARES DURANTE EL DESARROLLO MULTICELULAR DE *Myxococcus xanthus*: una aproximación de modelos dinámicos

TESIS

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WAIT FOR IT

Resumen

Myxococcus xanthus es una especie de bacteria social y ha surgido como sistema modelo para el estudio del desarrollo multicelular. En condiciones de falta de nutrientes, las células de *M. xanthus* se agregan en estructuras multicelulares llamadas cuerpos fructíferos donde las células experimentan diferenciación celular. Diversos componentes moleculares y ambientales que subyacen el proceso de desarrollo, así como las interacciones entre estos, han sido identificados y caracterizados. Sin embargo, no es claro cómo estos elementos actúan de manera sistémica para especificar los diferentes destinos celulares a partir de una población fenotípicamente homogénea, ni los mecanismos que subyacen su distribución espacial dentro del cuerpo fructífero. En este trabajo se estudian los procesos de determinación de destinos celulares y formación de patrones espaciales de tipos celulares en el desarrollo multicelular de *M. xanthus.* Esto, mediante la elaboración de modelos dinámicos multiescala y su implementación computacional. El modelo sugiere que los componentes identificados experimentalmente integran una red multiestable con el potencial de especificar los diferentes destinos celulares observados en el desarrollo de *M. xanthus*. Además, el modelo sugiere que, dentro de los conglomerados multicelulares, la difusión de señales intercelulares genera de manera dinámica información posicional que determina el destino de las células individuales en función de su posición dentro del conglomerado. Proponemos al modelo como una herramienta para la continua integración de nueva información experimental, así como para responder preguntas relevantes en el campo de la evolución de desarrollo.

Abstract

Myxococcus xanthus is a social bacteria that has emerged as model system for the study of multicellular development. When starved, the individual cells of *M. xanthus* aggregate into multicellular structures called fruiting bodies where cell differentiation takes places. Several molecular components and environmental stimuli, as well as the interactions between them, have been identified and characterized. Nevertheless, it remains unclear how these components integrate to determine the whole range of cell fates from an initially homogeneous cellular population. Here, the processes of cell fate determination and cell-fate patterning in *M. xanthus* are studied across the cellular and population levels through a multiscale approach of dynamic modelling and its computational implementation. The model suggest that the identified components integrate a multistable network that specify the different cell fates observed during *M. xanthus* development. Furthermore, the model suggests that, within multicellular conglomerates, diffusion of intercellular signals dynamically gives rise to positional information that specify the cell fate of individual cells according to its position in the conglomerate. The model is proposed as a tool for the continuous integration of new experimental evidence, as well as, a mean to approach questions relevant in the area of evolutionary developmental biology.

Introducción general

La multicelulularidad es considerada como una de las grandes transiciones de la evolución debido al cambio cualitativo que ocasionó en la forma en la que los sistemas vivos se organizan (Maynard-Smith & Szatmáry, 1997). En función de cómo se defina multicelularidad[1], se estima que esta ha emergido de manera independiente entre 14 a 25 veces durante la evolución (**Figura I1**; Niklas & Newman, 2013). El desarrollo de los organismos multicelulares está caracterizado por procesos de determinación de los destinos celulares y de formación de patrones espaciales de tipos celulares.

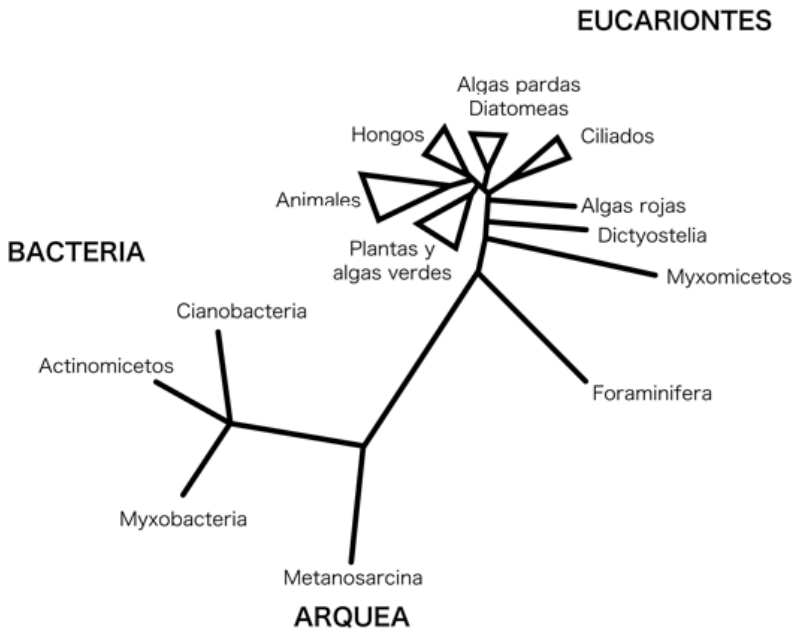

Figura I1. Esquema de un cladograma mostrando la distribución filogenética de algunos de los principales linajes multicelulares identificados en los tres dominios de la vida. Modificada de Bonner (1998).

[1]La definición de "organismo multicelular" se encuentra bajo debate. La mayoría de los autores consideran que los organismos multicelulares se caracterizan por exhibir propiedades de adhesión célula-célula, comunicación intercelular y división del trabajo a través de procesos de diferenciación celular (Mora Van Cauwelaert et al., 2015). Esta descripción puede ser difusa y permite la inclusión de biopelículas bacterianas. Otras características consideradas por algunos autores para caracterizar un organismo multicelular incluyen exhibir individualidad y ser objetos de selección (Michod, 2007). Para algunos, es necesario precisar que el primer conjunto de propiedades define a una *estructura* multicelular mientras que el considerar ambas define a un *organismo* multicelular (Gregory Velicer, comunicación personal). Finalmente, otras discusiones consideran que las formas de organización de los seres vivos forman un rango continúo, y no las categorías discretas de multicelular y unicelular. Por cuestiones operativas, se utiliza el primer conjunto de propiedades para definir a un organismo multicelular reconociendo que una definición precisa va más allá del objetivo de esta tesis.

Respecto a la determinación de destinos celulares, las células de un organismo multicelular, alcanzan diferentes destinos celulares que exhiben fenotipos potencialmente contrastantes, a pesar de tener poca o nula diversidad genética entre ellas. El proceso de diferenciación celular también se ha interpretado en el contexto de redes multiestables (Kauffman, 1969), definidas por las interacciones entre componentes regulatorios y factores ambientales. Se ha postulado que estas redes determinan de manera dinámica estados estacionarios en los cuales las células exhiben propiedades fenotípicas estables que se mantienen en ausencia de perturbaciones y que corresponden a perfiles de expresión génica y de actividad de proteínas característicos.

Durante el desarrollo multicelular, además de surgir los distintos tipos celulares, éstos se organizan en patrones espaciales complejos. Estos patrones ocurren como una consecuencia de la acción de mecanismos de comunicación, mediada por señales químicas o mecánicas, que acoplan la dinámica interna de las células individuales a través de la población (Turing, 1952; Kondo & Miura, 2010). Este acoplamiento resulta en la formación de gradientes que se traducen en información posicional que ocasiona que ciertos destinos celulares se determinen de manera preferencial en ciertas posiciones dentro de un conglomerado de células. La información posicional emerge de manera espontánea como consecuencia del acoplamiento de las células (Turing, 1952). Esto implica que la formación de patrones espaciales puede ocurrir de manera independiente de la acción de factores externos que genere un prepatrón y, por lo tanto, puede generarse generación tras generación como una propiedad inherente del organismo. Si bien este tipo de mecanismos genéricos han sido ampliamente estudiados de forma teórica, desconocemos aún cómo se manifiestan concretamente en ciertos organismos y, en particular, desconocemos cómo operan durante la diferenciación y arreglo celular en la transición a la multicelularidad.

De manera amplia, los organismos multicelulares pueden clasificarse como clonales o agregativos (Grosberg and Strathmann, 2007). En el primer grupo, que incluye los linajes de plantas y animales, el conglomerado multicelular emerge a partir de una sola célula que experimenta múltiples rondas de división celular tras las cuales, las células hijas permanecen adheridas unas a la otras. En el segundo grupo, que incluye grupos bacterianos y amebas eucariontes, el conglomerado se origina como consecuencia de la agregación de células individuales en respuesta a un estímulo ambiental (generalmente, la falta de nutrientes). El estudio del desarrollo multicelular se ha enfocado principalmente en los organismos clonales.

Sin embargo, el estudio el estudio de los organismos multicelulares agregativo podría proveer información sobre los aspectos generales y específicos asociados a la emergencia de la multicelularidad en los diferentes linajes, así como profundizar nuestro entendimiento respecto a los mecanismos que subyacen la evolución de la complejidad y diversidad de los sistemas biológicos.

El orden de myxobacteria (también llamado myxococcales), dentro de la clase de delta-proteobacteria, representa un linaje multicelular agregativo cuya transición ocurrió de manera independiente a la de plantas, animales y hongos (Dworkin, 2007). Este linaje ha surgido como sistema modelo para el estudio del desarrollo multicelular y de su evolución. Dentro de las myxobacteria, *Myxococcus xanthus* es la especie en dónde se ha realizado la mayor parte de la investigación respecto a los mecanismos de desarrollo observados en este grupo. *M. xanthus*, al igual que la mayoría de las myxobacteria identificadas, es una bacteria Gramnegativa frecuentemente denominada como una bacteria social debido a que a través de todo su ciclo de vida exhibe comportamientos que son dependiente de las interacciones entre las células individuales y de la presencia de un alto número de células (Yang & Higgs, 2014). *M. xanthus* exhibe un ciclo de vida bifásico dependiente de la cantidad de nutrientes disponibles. La fase vegetativa se caracteriza por la disponibilidad de nutrientes y en esta, las células exhiben un comportamiento de dispersión en el cual la colonia se expande. Una vez que los nutrientes han sido consumidos, las células entran en una fase de desarrollo en la cual las células las células se agregan en estructuras multicelulares tipo montículo denominadas cuerpos fructíferos donde la diferenciación celular se lleva a cabo (**Figura I2**). Para completar el desarrollo, las células requieren de una superficie sólida sobre la cual desplazarse y de un alto número de células (Yang & Higgs, 2014).

Durante el desarrollo, la población de células alcanza al menos tres destinos celulares: células periféricas, esporas o células que sufren muerte celular programada (Yang & Higgs, 2014). Dentro del cuerpo fructífero, las células periféricas y las esporas se distribuyen en anillos concéntricos con las primeras localizadas preferencialmente en el anillo externo y las segundas en el anillo interno (Sager & Kaiser, 1993; Julien et al., 2000). Respecto a las células que experimental muerte celular programada, su localización dentro del cuerpo fructífero no se ha documentado claramente de manera experimental. A pesar de que diversos componentes moleculares (genes, proteínas y metabolitos) involucrados en el desarrollo de *M. xanthus*, así como las interacciones regulatorias entre estos, han sido identificados y caracterizados; los

mecanismos mediante los cuales estos elementos actúan de manera integral y sistémica para determinar el destino celular de las células, así como su distribución espacial dentro del cuerpo fructífero continúan por definir.

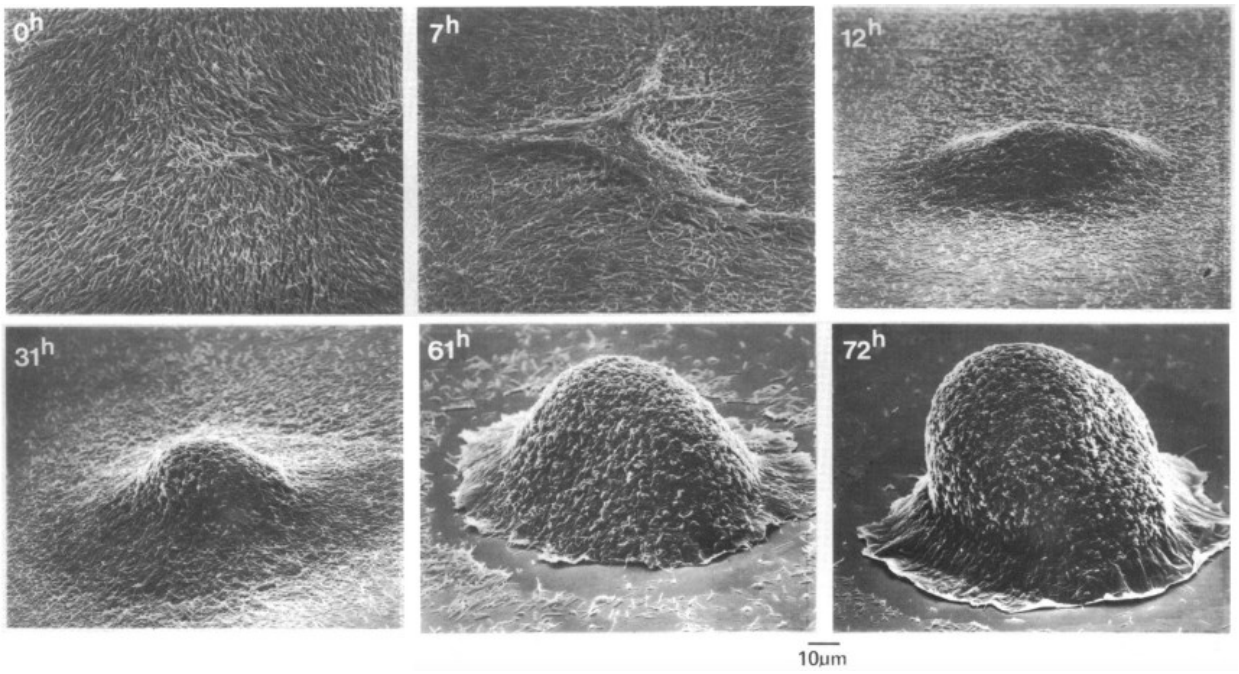

Figura I2. Serie de tiempo del desarrollo de un cuerpo fructífero de *Myxococcus xanthus* DK1622 bajo condiciones experimentales. Los números anotados en la esquina superior izquierda de cada panel indican el tiempo (en horas) después de iniciado el desarrollo. Tomada de Kuner & Kaiser (1982).

Los procesos del desarrollo, incluyendo aquellos observados en *M. xanthus*, tienden a exhibir un comportamiento no lineal que resulta en propiedades emergentes no intuitivas y que no pueden rastrearse a los componentes individuales tratados como entidades aisladas. En este contexto, los modelos matemáticos dinámicos y su implementación computacional han emergido como una estrategia complementaria al trabajo experimental en el estudio de procesos biológicos Von Dassow et al., 2000; Espinosa-Soto et al., 2004; Tomlin & Axelrod, 2010). Un modelo puede entenderse como una abstracción o representación de un sistema real a través del un lenguaje matemático formal. Los modelos dinámicos proveen una herramienta mediante la cual es posible integrar información experimental de diferentes fuentes para estudiar la emergencia de propiedades y procesos como resultado de la interacción entre distintos factores. Suelen ser utilizados para poner a prueba la hipótesis de que un conjunto de propiedades son suficientes o necesarias para explicar un conjunto de observaciones realizadas en el sistema real. Además, los modelos dinámicos pueden ser implementados para

hacer prueba de conceptos al permitir estudiar escenarios que sería difíciles, o incluso imposibles, de estudiar de manera experimental. Finalmente, los modelos dinámicos pueden ser empleados para generar hipótesis que puedan dirigir de manera informada el trabajo experimental y generando así, una retroalimentación entre el trabajo experimental y el teórico.

Justificación

El linaje de myxobacteria representa un potencial sistema modelo para profundizar en nuestro entendimiento respecto a los procesos de desarrollo, su emergencia, mantenimiento y diversificación. Entre los rasgos de posible interés se encuentra que al constituir un linaje cuya transición a la multicelularidad ocurrió de manera independiente a la de otros grupos más ampliamente estudiados, tales como plantas, hongos y animales; su estudio podría proveer información sobre los rasgos comunes y específicos asociados a los organismos multicelulares y su evolución. Dado que su desarrollo ocurre en la microescala, es probable que este ocurra en condiciones similares a aquellas en las que se desarrollaron los primeros organismos multicelulares por lo que su estudio podría proveer información relevante sobre la transición a la multicelularidad en si misma. Dada su tendencia a exhibir una menor diversidad morfológica y de tipos celulares en contraste al observado en plantas y animales, su estudio podría informar sobre los mecanismos que subyacen la evolución de la complejidad y de la diversidad biológica. Finalmente, como organismos bacterianos, representan un excelente sistema experimental para el estudio de la evolución del desarrollo debido a la facilidad para manipularlas (fisiológica y genéticamente) así como exhibir ciclos de vida cortos.

A pesar de que *M. xanthus*, como especie modelo dentro del linaje de myxobacteria, ha sido ampliamente estudiado a través de una variedad de enfoques experimentales y teóricos; no es del todo claro como los diferentes elementos asociados con el desarrollo son integrados para propiciar la emergencia de rasgos característicos de la escala multicelular, particularmente, en lo referente a la diferenciación celular y de formación de patrones espaciales de tipos celulares dentro de la estructura multicelular. El entendimiento del desarrollo como proceso dinámico es crucial como punto de partida para la resolución de preguntas de interés evolutivo.

Objetivo general

Estudiar de manera integral, mediante una estrategia de modelado matemático y computacional, los procesos de diferenciación celular y de formación de patrones espaciales de tipos celulares durante el desarrollo multicelular de *Myxococcus xanthus*.

Objetivos particulares

1. Identificar los componentes moleculares (genes, proteínas y metabolitos) y sus relaciones regulatorias relevantes para el desarrollo multicelular de *M. xanthus* a partir de una revisión intensiva en la literatura científica disponible (Capítulo 1).
2. Estudiar las propiedades dinámicas de la red molecular de regulación respecto al proceso de diferenciación celular durante el desarrollo de *M. xanthus* mediante la proposición de un modelo Booleano y su implementación computacional (Capítulo 2).
3. Estudiar las consecuencias de una dinámica a nivel poblacional en los procesos de diferenciación celular y de formación de patrones espaciales de tipos celulares durante el desarrollo multicelular de *M. xanthus,* mediante la proposición de un modelo multiescala y su implementación computacional (Capítulo 3).
4. Realizar, mediante el análisis de los resultados, predicciones de interés biológico las cuales puedan ser puestas a prueba de manera experimental en trabajos futuros (Capítulo 3).
5. Discutir diversos aspectos del desarrollo multicelular de *M. xanthus* a partir del marco de la biología evolutiva del desarrollo (Capítulos 1-3 y Discusión general).

Capítulo 1

El desarrollo multicelular de myxobacteria dentro del marco teórico de la biología evolutiva del desarrollo

La biología evolutiva del desarrollo (Evo-Devo) es un área de investigación enfocada en entender la evolución de los procesos de desarrollo (Muller, 2007). Al mismo tiempo, busca entender el efecto del desarrollo sobre la evolución a través del establecimiento de sesgos del desarrollo (no solo limitantes sino también potenciadores) (Maynard-Smith et al., 1985). Además, este marco enfatiza la contribución de otros factores, además del genético, como fuente de variación fenotípica (West-Eberhard, 2003). Es decir, se reconoce que la variación, y por lo tanto la evolución, puede ocurrir en ausencia de variación genética. Esto es en contraste con las ideas propuestas en la Síntesis Moderna, en la cual, la variación a nivel génica es considera como la principal fuente de variación durante la evolución (Muller, 2007).

El grupo de myxobacteria comprende un linaje de organismos multicelulares caracterizado por un proceso de desarrollo mediante la agregación celular en respuesta a la falta de nutrientes. Si bien se ha sugerido que el desarrollo por agregación conlleva limitaciones importantes en la complejidad de los organismos, en términos de número de tipos celulares y diversidad de planes corporales (Grosberg & Strathmann, 2007), el mecanismo de agregación también parece facilitar la emergencia de paralelismos respecto a sus morfologías dentro y entre linajes de organismos multicelulares (Bonner, 1982; Kaiser, 1986). Además, diversas observaciones sugieren que desarrollo de myxobacteria es influenciado por factores no genéticos incluyendo la superficie sobre la cual se desplazan, la ausencia o presencia de luz y de organismos simbiontes (Huntley et al., 2014; Rivera-Yoshida et al., 2019). Estas observaciones sugieren que el estudio de las myxobacteria puede proveer información importante sobre el papel de factores no genéticos en la evolución del desarrollo y de los mecanismos que sesgan la diversificación de los seres vivos.

En este capítulo se discute la evolución del desarrollo multicelular de myxobacteria en el marco de la biología evolutiva del desarrollo. Esta revisión busca enfatizar la importancia de conceptos y procesos claves en el Evo-Devo para el entendimiento del desarrollo y su evolución, los

cuales, tradicionalmente, se ha centrado en el estudio de la variación genética como único sustrato durante la evolución. Los elementos por discutir son 1) las implicaciones evolutivas de la agregación como mecanismo de desarrollo multicelular, 2) la relevancia en el ensamblado del proceso de desarrollo de la cooptación de mecanismos moleculares potencialmente presentes en el ancestro unicelular de las myxobacteria, 3) el fenómeno de la deriva de los sistemas de desarrollo y la emergencia de paralelismos y 4) la contribución de aspectos no genéticos de el desarrollo multicelular de estos organismo.

REVIEW

An Evo-Devo Perspective on Multicellular Development of Myxobacteria

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ABSTRACT

The transition to multicellularity, recognized as one the major transitions in evolution, has occurred independently several times. While multicellular development has been extensively studied in zygotic organisms including plant and animal groups, just a few aggregative multicellular organisms have been employed as model organisms for the study of multicellularity. Studying different evolutionary origins and modes of multicellularity enables comparative analyses that can help identifying lineage-specific aspects of multicellular evolution and generic factors and mechanisms involved in the transition to multicellularity. Among aggregative multicellular organisms, myxobacteria are a valuable system to explore the particularities that aggregation confers to the evolution of multicellularity and mechanisms shared with clonal organisms. Moreover, myxobacteria species develop fruiting bodies displaying a range of morphological diversity. In this review, we aim to synthesize diverse lines of evidence regarding myxobacteria development and discuss them in the context of Evo-Devo concepts and approaches. First, we briefly describe the developmental processes in myxobacteria, present an updated comparative analysis of the genes involved in their developmental processes and discuss these and other lines of evidence in terms of co-option and developmental system drift, two concepts key to Evo-Devo studies. Next, as has

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been suggested from Evo-Devo approaches, we discuss how broad comparative studies and integration of diverse genetic, physicochemical, and environmental factors into experimental and theoretical models can further our understanding of myxobacterial development, phenotypic variation, and evolution. *J. Exp. Zool. (Mol. Dev. Evol.) 328B:165–178, 2017.* © 2017 Wiley Periodicals, Inc.

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MYXOBACTERIA IN THE INTEGRATIVE STUDY OF AGGREGATIVE MULTICELLULARITY

The emergence of multicellular organisms exhibiting cell differentiation and stereotypic spatial arrangements has been recognized as one of the major transitions in evolution (Maynard-Smith and Szathmary, 2000), and it is estimated to have evolved independently at least 25 times (Grosberg and Strathmann, 2007). The appearance of multicellular organisms allowed an extraordinary increase in the complexity of living systems and the study of the developmental mechanisms and selective forces leading to its emergence, maintenance, and variation is an active research area (e.g., Niklas et al., 2016). In broad terms, multicellular organisms can be classified either as aggregative (also called sorocarpic) or clonal (zygotic), according to the mechanism by which the multicellular conglomerates arise (Grosberg and Strathmann, 2007; Du et al., 2015). In the first case, all the cells in the organism are the offspring of a single cell and remain attached to each other after cell division. For the case of aggregative multicellularity, organisms develop through the gathering of several individual cells that potentially belong to different genetic lineages in natural populations (Bonner, '98; Fortunato et al., 2003; Grosberg and Strathmann, 2007).

The study of different groups of multicellular organisms, both clonal and aggregative, has provided important contributions to our understanding of multicellular evolution. Specifically, studies across different clades and comparisons within and between groups that display multicellular development, and with their unicellular relatives, have pointed out some common developmental aspects. For example, the co-option or reuse of processes and regulatory modules already present in the unicellular ancestors (e.g., pathways for intercellular communication, cell-to-cell adhesion, and multistable intracellular gene regulatory networks) seems to take place recurrently in the transition to multicellularity, as well as in the evolution of development in general (Furusawa and Kaneko, 2002, 2003; True and Carroll, 2002; Sanetra et al., 2005; Newman and Bhat, 2008, 2009; Rokas, 2008; Hernández-Hernández et al., 2012). In line with this idea, the notion of "developmental system drift" (DSD) (True and Haag, 2001) refers to the proposal that development of homologous traits in related species may not be mediated by homologous genetic factors. DSD suggests that genetic networks underlying phenotypes are quite flexible and that differences between regulatory networks in related species arise by elimination or recruitment of new elements, erasing in this way tractable signals of the common ancestry at the genetic level. Model systems in which DSD has been proposed as an explanatory mechanism include the vulva development of *Caenorhabditis* spp., cardiopharyngeal development in ascidians, mating-type system in *Saccharomyces* spp. and related species, and winged polyphenism in ants (Rokas, 2006; Tsong et al., 2006; Kiontke et al., 2007; Nahmad et al., 2008; Shbailat and Abouheif, 2013; Sommer, 2012; Stolfi et al., 2014). The mechanisms triggering divergence in the regulatory networks in DSD can involve both cis- and trans-regulatory changes, and the degree of change can vary from one system to another (Sommer, 2012; Stolfi et al., 2014).

Comparative studies have also highlighted the importance of nongenetic ecological and physicochemical processes, besides the better-studied genetic ones, in the origination and diversification of multicellular phenotypes and in the maintenance of multicellularity (Boraas et al., '98; Shapiro, '98; Kirk, 2005; Grosberg and Strathmann, 2007; Newman and Bhat, 2008, 2009; Ispolatov et al., 2012). Nevertheless, it is still necessary to understand how this diversity of factors interacts during multicellular development, and how they can lead to phenotypic variation. Evolution experiments starting from unicellular cells have shown that it is relatively easy for multicellularity to emerge under certain conditions (Boraas, '98; Fiegna et al., 2006; Ratcliff et al., 2012, 2013), suggesting that there might be generic processes facilitating this transition (Newman and Bhat, 2008, 2009). However, there are also significant differences among groups and it will be important to characterize the factors that have driven diversification within lineages.

In this context, studying different evolutionary origins and modes of multicellularity enables comparative analyses that in turn might help to identify lineage-specific aspects of multicellular evolution, as well as potential generic factors and mechanisms involved in different instances of the transition to multicellularity. Aggregative multicellular organisms are capable of generating complex structures with different cellular types and arrangements but, apart from a few model species (e.g., *Dyctiostelum discoideum* and *Myxococcus xanthus*; see Bonner, '98; Nanjundiah and Sathe, 2011; Sunderland, 2011), the

development of aggregative organisms has not been as extensively explored as zygotic multicellular development (Du et al., 2015). We will thus focus in the discussion of this type of multicellularity, which has emerged multiple times among eukaryotes and at least once in prokaryotes (Parfrey and Lahr, 2013; Sebé-Pedrós et al., 2013; Du et al., 2015). In the case of prokaryotes, aggregative multicellularity has been identified in myxobacteria (also called Myxococcales) (Whitworth, 2008; Yang and Higgs, 2014). In these clades, aggregative multicellular organisms often start development when starving, culminating with the formation of multicellular structures called fruiting bodies (FB). FB range from simple mound-like to branching structures. Interestingly, from an Evo-Devo perspective, FB in different clades exhibit striking resemblance (Du et al., 2015), but it is unclear what are the causes of this similarity. Among aggregative multicellular organisms, a combination of experimental and modeling approaches has been employed to investigate the development of *D. discoideum* and *M. xanthus* (Romeralo et al., 2013; Yang and Higgs, 2014). The parallelism between some of the structures displayed by these organisms, together with the differences in the genetic networks underlying species-specific development means that they are excellent candidates to study the interaction between genetic and nongenetic factors in development and its evolution.

Myxobacteria are an order of the delta-proteobacteria class and form, according to 16S sequences, a monophyletic group characterized by social behavior, aerobic metabolism, large genome size (>9 Mb in comparison to ~4.5 Mb in closely related unicellular species), the ability to synthesize a large number of secondary metabolites, and the ability to develop into very diverse multicellular structures (Huntley et al., 2011; Wrótniak et al., 2016). Estimates of myxobacteria divergence from other delta-proteobacteria range from 800 million to 2 billion years ago (Dworkin, 2007) and, owing to the recurrent presence of multicellular development among myxobacteria, it has been suggested that their common ancestor was able to develop FB (Dworkin, 2007). Early studies show that myxobacteria are mainly associated with superficial soil across almost every ecosystem, but recent sequencing projects have identified myxobacteria in aquatic environments (Reichenbach, '99; Brinkhoff et al., 2012). Perhaps, the most prominent trait of myxobacteria, and of special relevance to this review, is their multicellular development. When nutrients are depleted from the media, groups of myxobacteria cells start a developmental process that culminates with the formation of multicellular structures in which different cell types coexist (Kaiser, 2003; Whitworth, 2008). Cells commit to one of three possible cell types: myxospores, peripheral cells, or cells that eventually undergo programmed cell lysis. At least for myxospores and peripheral cells, the relative position of cells to each other is specified by intercellular signals providing positional information (Julien et al., 2000; Holmes et al., 2010). When nutrients become available again, myxospores germinate and vegetative growth resumes (Whitworth, 2008).

Myxobacteria research has been methodologically and conceptually diverse, resulting in a good understanding of their development. Extensive myxobacteria research has largely disentangled the molecular and genetic components of development and collective behavior, and excellent reviews and books in regard of these aspects of myxobacteria biology have been published (Reichenbach, '99; Kaiser, 2004; Travisano and Velicer, 2004; Kroos, 2007; Whitworth 2008; Velicer and Vos, 2009; Yang and Higgs, 2014; Wrótniak-Drzewiecka et al., 2016; Bretl and Kirby, 2016; Muñoz-Dorado et al., 2016). Furthermore, important advances have been made regarding the evolution and ecology of myxobacteria (Kraemer et al., 2010; Huntley et al., 2014; Velicer et al., 2014). These features, along with the vast information available for the group and the ease of manipulation of some myxobacteria species in the laboratory, make it a great model to study the development and evolution of aggregative multicellularity.

In this review, we aim to integrate both classical and recent evidence regarding myxobacteria multicellular development and discuss it using key conceptual tools from the Evo-Devo framework. First, we briefly review the evidence regarding the origin of the developmental processes in myxobacteria, perform an updated gene-conservation analysis, and discuss it in terms of co-option and DSD. Next, we discuss how genetic and nongenetic factors may contribute to the emergence of the diversity observed across myxobacteria FB and briefly discuss how the different morphologies can be developmentally related. Finally, we highlight the importance of adopting integrative approaches to the myxobacteria research as well to include other myxobacteria species in future studies.

CO-OPTION IN THE EVOLUTION OF MYXOBACTERIAL DEVELOPMENT

Cell-to-cell adhesion, intercellular communication, and cellular differentiation have been proposed as some of the traits required for multicellularity to emerge (Furusawa and Kaneko, 2002, 2003; Abedin and King, 2008; Suga et al., 2013; Mora Van Cauwelaert et al., 2015). As multicellular organisms, myxobacteria exhibit these traits (Kuspa et al., '92; Russo-Marie et al., '93; Thony-Meyer and Kaiser, '93; Julien et al., 2000; Lobedanz and Søgaard-Andersen, 2003). Although it is in fact not known, by genomic comparisons, whether these traits predate the emergence of multicelluarity in myxobacteria or not (Huntley et al., 2011, 2014), we review and present some evidence suggesting that these features could have indeed been co-opted from processes and regulatory systems already present in unicellular ancestors of multicellular myxobacteria.

At the molecular level, cell differentiation during *M. xanthus* development may be accounted for, at least in part, by

the stringent response which is involved in the early stages of development by sensing starvation. Stringent response involves the action of the enzyme (p)ppGpp synthetase I, RelA which rises the intracellular levels of the secondary messengers guanosine penta- and tetraphosphate [(p)ppGpp] (see Box 1), which in turn is associated with large transcriptional changes (Manoil and Kaiser, '80a,'80b). The stringent response is conserved across bacteria, where it is involved in the response to different stresses as nutrient deprivation in *Escherichia coli* (Boutte and Crosson, 2013) and persistence against antibiotics in different organisms including *E. coli* (Maisonneuve et al., 2013), *Pseudomonas aeruginosa* (Khakimova et al., 2013), *Staphylococcus aureus* (Geiger et al., 2010), and *Mycobacteria tuberculosis* (Primm et al., 2000), where this response induces dormancy and is crucial for organism survival by mediating different transcription profiles. Owing to its broad phylogenetic distribution in bacteria, stringent response is likely to have been already present in the unicellular ancestor of myxobacteria where it was co-opted for development during the transition to multicellularity as a mediating mechanism for cellular differentiation.

In *E. coli*, where the stringent response has been extensively studied, this pathway has been shown to additionally involve the protease Lon and a toxin-antitoxin system composed by MazF-MazG proteins (Maisonneuve et al., 2013). Previous comparative studies and the one we performed (Fig. 1) show that all these proteins have identifiable homologous genes across myxobacteria. For example, in *Anaeromyxobacter dehalogenans,* where the components of the stringent response are present, they are likely to mediate cell differentiation under starvation (Fig. 1; Huntley et al., 2011, 2014). In addition to *M. xanthus*, a role of stringent response in cell differentiation and development has been demonstrated in *Sorangium cellulosum* where mutations in the *rel* gene (homologous to *M. xanthus relA*) impairs cell differentiation and results in the inability to survive in nutrient-limited conditions (Knauber et al., 2008). Moreover, there is evidence that in some members of myxobacteria cell differentiation is not necessarily associated with multicellular structures. *A. dehalogenans*, one of the few characterized species to be unable to develop multicellularly, is still able to differentiate into cysts in old cultures (likely in response to the depletion of nutrients) (Sanford et al., 2002). *M. xanthus*, when grown in glycerol liquid media, exhibits an almost complete transformation of the whole population into spheroplasts, a cell type resembling myxospores (Crawford and Shimkets, '99, Sanford et al., 2002). This suggests that cell-fate differentiation occurring in single cells in different times could have been co-opted in the context of multicellular development. As a final line of evidence for this possibility, cell differentiation has been identified in *Bdellovibrio bacteriovorus*, a deltaproteobacteria phylogenetically close to myxobacteria (Ruby et al., '83; Gray et al., '90; Rotem et al., 2015). The observation of development-independent cell differentiation across myxobacteria and in related species in response to diverse conditions, together with the conservation of the stringent response, support the hypothesis that the unicellular ancestor of myxobacteria was able of alternating cell fates over time and that the transition to multicellularity was enabled by the spatiotemporal coexistence of different cell types.

As for cell–cell communication and adhesion, the evidence for co-option is less clear. In *M. xanthus*, the so-called C-signal is proposed to be regulated by cellular contact (Box 1). Comparative analyses suggest that such signal is not conserved across myxobacteria (Fig. 1). Similarly, genes involved in the regulation of A-signal, a mixture of amino acids and short peptides mediating cellular communication, does not appear to be conserved in myxobacteria (Fig. 1). However, it is likely that other molecules involved in cellular communication and adhesion among cells and between cells and the medium are more widely observed in myxobacteria and were present in their unicellular ancestor. Among them might be carbohydrates and proteins that have been characterized in the slime and extracellular matrix of *M. xanthus* and that could be ubiquitous among bacterial organisms (Konovalova et al., 2010; Hu et al., 2016).

While it seems likely that the transition to multicellularity might have largely relied on the co-option of preexisting elements and processes, not necessarily requiring the gain of genetic material (Newman and Bhat, 2008, 2009; Niklas and Newman, 2013), there is evidence suggesting that gene duplication, horizontal gene transfer, and recombination could also have been involved in the diversification of multicellular lineages, or in the canalization of certain phenotypes involved in the transition to multicellularity. In the case of myxobacteria, the available genomic sequences from multiple species and from some of their closer unicellular relatives within the deltaproteobacteria have been used to explore how genomic change could have impacted the evolution of myxobacteria development (Goldman et al., 2006, 2007; Evans and Whitworth, 2010; Schlüter et al., 2011). For example, by analyzing a set of genes involved in the development of *M. xanthus*, Goldman and coworkers (2007) estimated that 22% of them were likely acquired via horizontal gene transfer. Among them are genes involved in the synthesis of exopolysaccharides and lipopolysaccharides, which are associated with the phenotypic changes occurring in myxospores (Lu et al., 2005; Konovalova et al., 2010). However, genes coding for key components in signal transduction and gene regulatory pathways, which may be involved in cell-fate determination, are likely to predate the divergence from the other deltaproteobacteria and to be vertically transmitted (Goldman et al., 2007). This evidence suggests that horizontal gene transfer could have shaped specialization of the functions of cell types or made the differentiation process more robust, but did not necessarily have a role in the origin of cell differentiation.

Additionally, some of the putative gene donors during the events of gene transfer were identified to be actinobacteria and cyanobacteria, both groups of aerobic bacteria including

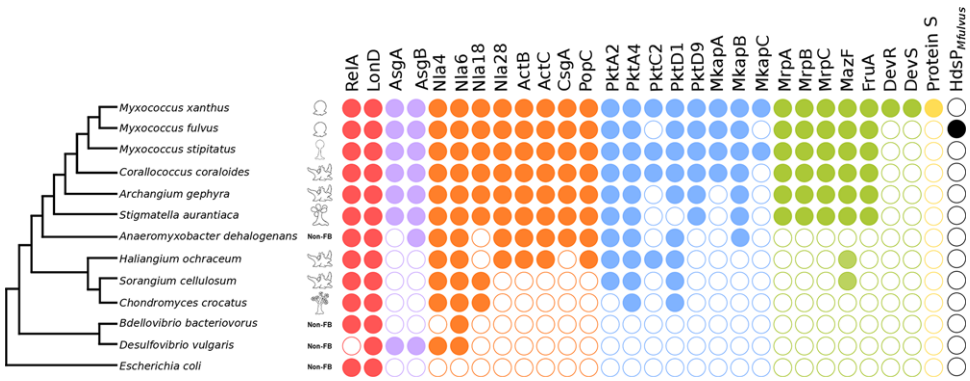


Figure 1. Comparative analysis of genes involved in the multicellular development of myxobacteria. (Left) A cladogram showing the phylogenetic relationships among myxobacteria (based on Huntley et al., 2011). (Middle) Representations of the FB developed by the listed myxobacteria species. (Right) The absence/presence of detectable homologous of genes involved in *M. xanthus* development in other myxobacteria species. Filled or empty circles represent the presence or absence of homologous elements, respectively. Columns represent genes listed at the top and rows represent species listed in the cladogram. Detection was performed using pBLAST reciprocal best hit (BBH) over coding-sequences for each myxobacteria genome and employing *M. xanthus* protein sequences as query. The analysis depicted in this figure stems from that presented by Huntley and co-workers (2011, 2014). We used the same BLAST (e-value, query coverage) parameters as these authors, but included more genomes and genes, considering also genes that are clearly involved in developmental stage of Myxococcales, but that can also act during the vegetative stage. HdsP, which has been described for *M. fulvus* development is also included (Pan et al., 2010). [Color figure can be viewed at wileyonlinelibrary.com]

multicellular members (Shapiro, '88; Bonner, '98). This may point out that aerobic metabolism is somehow associated with the emergence or maintenance of multicellularity. In fact, it has been observed that the radiation of multicellular metazoa came after the great oxidation of the atmosphere as a consequence of photooxygenic metabolism (Donoghue and Antcliffe, 2010; Schirrmeister et al., 2013). While the mechanisms behind this association are still unknown, it could be speculated that aerobic metabolism could result in oxygen gradients within cellular masses relevant in terms of positional information and cell differentiation (e.g., Bonner et al., '95).

Genome expansion through gene duplication is a feature characteristic of multicellular myxobacteria, and it is suggested to be somehow related to the capacity to develop FB. However, this association is not perfect. While all the fruiting species have been reported to have large genome size (Huntley et al., 2011, 2014), a nonfruiting myxobacteria, *Labilithrix luteola*, has been recently reported to have a large genome size (~12 Mb) (Yamamoto et al., 2014). This suggest that genome expansion does not trigger the transition to multicellularity but may be instead one of the mechanisms leading to multicellular organism canalization, specialization, and diversification.

HOMOLOGY AND DSD IN MYXOBACTERIA DEVELOPMENT

Based on the observation that multicellular development is a widely distributed trait across the members of myxobacteria (Dworkin, 2007; Huntley et al., 2011), an early hypothesis suggested that their common ancestor was able to form FB (i.e., homologous development across myxobacteria) (Dworkin, 2007). Currently, this scenario is under debate due to the evidence obtained through the sequencing of the genomes of different myxobacteria species (Huntley et al., 2011, 2014). The comparative analysis of available genomes for myxobacteria species found that genes considered as essential for *M. xanthus* development are absent in other myxobacteria. Based on these results, the homology of myxobacteria FB has been questioned and the possibility of multiple independent emergence of multicellularity within myxobacteria has been put forward.

It is in this context that the notion of developmental systems drift might become handy, as it suggests that the development of homologous traits can be mediated by nonhomologous pathways (Wagner, '89, 2007). Under this view, ancestral pathways rewire along evolution and it is during this process that previous elements are eliminated or replaced for new, likely unrelated, components. In this scenario, descendants inherit the undisrupted trait already present in the ancestor whereas new mechanisms arise underlying its development.

The genetic elements associated with multicellular development in *M. xanthus* have been shown to be able to sustain the formation of FB in the face of diverse genetic mutations. Furthermore, subsequent mutations can restore development almost to wild-type levels, suggesting that the underlying

genetic circuit is flexible and capable of accommodating genetic variation without elimination of the phenotype. Strikingly, some of the genes considered as crucial for the development (e.g., *csgA* and *asgA*) are included in these cases (Rhie and Shmikets, '89, Yang and Kaplan, '97; Hager et al., 2001; Cusik et al., 2015; Rajagopalan et al., 2015). Similarly, MazF, which is part of the gene network associated with *M. xanthus* development (Box 1), seems to be dispensable in some *M. xanthus* strains as mutations in this gene have a modest impact over development (Lee et al., 2012; Boynton et al., 2013). This further suggests that rewiring occurring in the gene regulatory networks along evolution has made developmental processes in this group considerably robust.

It is worth to note that the plausibility of DSD largely depends on the clear definition of the scale and phenotype over which DSD may be acting. When the capacity to develop into FB is the phenotype under consideration, then DSD may account for the observed patterns of gene content. Nevertheless, when the morphology of the FB is considered, it is hard to claim that DSD may explain the lack of conservation, since genetic variation may mirror the morphological variation across species.

Finally, time-lapse analyses of *C. crocatus* development have shown that structures at intermediary stages resemble mature FBs of other myxobacteria stages, which has led some authors to suggest that the differences observed between the *M. xanthus* and *S. aurantiaca* fruiting bodies could be accounted by heterochrony, the change of relative timing of events during development (Crawford and Shimkets, '99). In the same context, Escalante and collaborators (2012) proposed that heterochrony may arise from mutations affecting developmental timing yet not disrupting the process and thus being likely to induce relevant changes in morphology. In addition, a similar scenario has been proposed by Bonner ('82) for the case of *Dictyostelium* fruiting bodies. Nevertheless, the validity of these hypotheses has not been tested and further information coming from diverse myxobacteria species is still required. In addition, different genes have been shown to induce timing changes during *M. xanthus* development without apparent changes in fruiting body morphology, indicating that timing change is not enough to induce morphological variation and thus other mechanisms may be involved, such as developmental constrains and the abovementioned sources of phenotypic variation. Exploring these factors in the context of myxobacteria potentially may provide important insights about the mechanisms behind organism phenotypic diversification and innovation evolution.

DIVERSITY IN THE CAUSES BEHIND MYXOBACTERIAL DEVELOPMENT

In the previous sections, we have focused on the genetic evidence behind the origin and diversification of multicellular development in *M. xanthus* and other myxobacteria. Indeed FB morphology and variation is partially determined by genetic factors (Spröer et al., '99; Yan et al., 2003). However, as Evo-Devo studies have pointed out for diverse developmental systems (Pigliucci and Müller, 2010), other sources of phenotypic variation may potentially contribute to the origin of multicellular development in myxobacteria and its diversification.

While *M. xanthus* is the most studied species among myxobacteria, it is also the one with the most basic FB morphology, consisting of a spore-filled sphere and a short stalk composed of peripheral cells that attaches to the substrate (Dawid, 2000; Zusman et al., 2007). In contrast, other myxobacteria species display more complex morphologies developing branched structures (Fig. 2). Beyond the FB morphology, myxobacteria display differences in the relative size and arrangement of vegetative cells and myxospores (Dawid, 2000; Huntley et al., 2014). The variation observed across myxobacteria FBs is striking, but the sources and mechanisms leading to this variation are still to be well characterized. In this section, we review studies suggesting that nongenetic factors, such as ecological and physicochemical factors, could be associated with this variation and constitute an integral part of the developmental systems themselves (Gilbert and Epel, 2009).

Ecological Factors

Myxobacteria are broadly distributed, and it has been suggested that local organism–environment interactions may have contributed to the diversification of development (Reichenbach, '99; Dawid, 2000; Kraemer et al., 2010). In *M. xanthus* and other myxobacteria species, the main event triggering development is depletion of nutrients. However, the development in *Stigmatella aurantiaca* also requires the presence of light as opposite to *M. xanthus* where development is inhibited by light (Qualls et al., '78; Inouye et al., '80; Stephens and White, '80, Elías-Arnanz, 2008). When *S. aurantiaca* cultures are grown in darkness, populations do not undergo development, even when starving (Qualls et al., '78). Since myxobacteria live on the soil surface (Dawid, 2000), light is likely to exert a considerable influence over these organisms and the co-option of light-perceiving pathways into development as a mean to integrate and respond to starvation and light in *S. aurantiaca* may have been be favored (van der Horst et al., 2007). Yet, the exact pathway that enables the sensing of light has not been identified. In *M. xanthus* cells, light is perceived through carotenoids whose synthesis is under the transcriptional control of CarD–CarG signaling pathway (Whitworth and Hodgson, 2001; Browning et al., 2003; Elías-Arnanz et al., 2008; Galbis-Martínez et al., 2012; Abellón-Ruiíz et al., 2014) and are responsible for the characteristic bright color of *M. xanthus* colonies and associated with oxidative protection against environmental oxygen (Galbis-Martiínez et al., 2012).

In addition to the effect of abiotic factors over development, other ecological interactions such as symbiotic relationships are widespread among living organisms and have been put forward as one of the principal evolutionary forces (Gilbert et al., 2015, 2016). Among myxobacteria, *Chondromyces crocatus* is not able

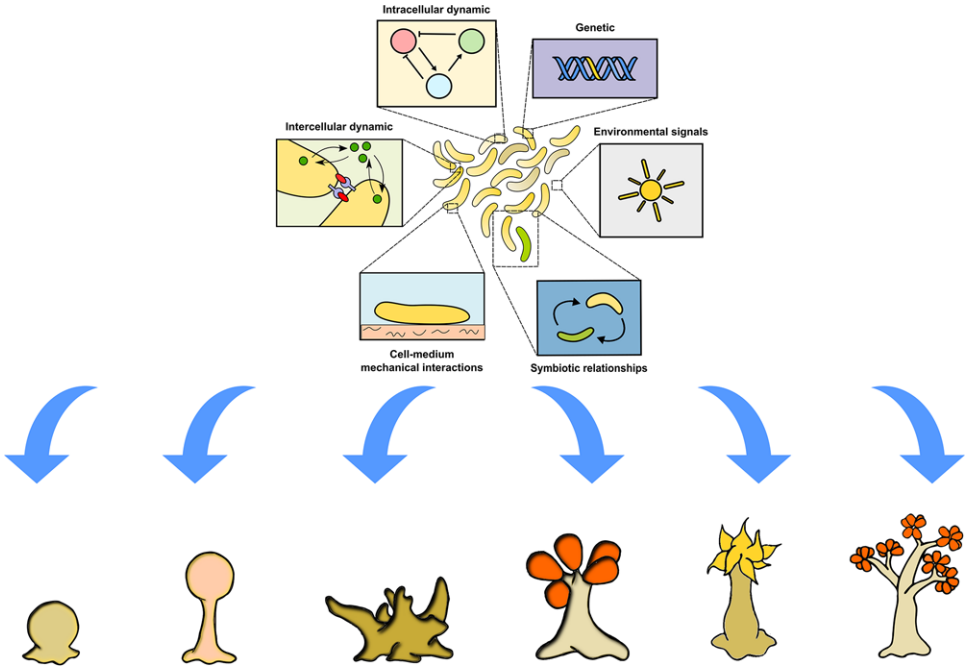


Figure 2. Myxobacterial development as a model system for integrative studies. A diversity of molecular, environmental, and physicochemical factors interact in complex ways in the multicellular development of myxobacteria species. One of the major challenges in the study of this group is to understand the mechanisms that articulate these diverse factors and their relative role in the origin and variation of FB. [Color figure can be viewed at wileyonlinelibrary.com]

to fully develop in pure culture. Its successful culture is only possible when cocultured with members of the Sphingobacter genus, which in turn can only be cultured with *C. crocatus* (Jacobi et al., '96; Reichenbach, '99). The co-occurrence of both species is transgenerationally assured as Sphingobacter is incorporated in the sporangioles of the FB during *C. crocatus* development (Jacobi et al., '96; Reichenbach, '99). *C. crocatus* develops into the most morphologically complex FB among myxobacteria, and the absence of the symbiont produces a delay of development that may extend for a week (Pinoy, '13; Jacobi et al., '96, '97). The collected evidence shows that *C. crocatus*–Sphingobacter interaction is necessary for both vegetative and developmental stages (Reichenbach, '99). In addition, a similar case of symbiotic relationship has also been reported for *D. discoideum* (Brock et al., 2011). The exact effect of symbiosis on morphology in aggregative organisms remains to be elucidated, and further research is required to find out if the association *C. crocatus*–Sphingobacter affects the formation of the morphologically complex structures displayed by *C. crocatus* fruiting bodies. However, the fact that these interactions are reproduced with the organism's life cycle and that they are needed for stereotypic development to occur, makes it necessary to consider such ecological interactions not only as inputs or as contingent conditions, but as additional causes behind the development of multicellular structures and their evolution (Gilbert et al., 2015).

Physicochemical Factors

Developmental processes are both driven and constrained by generic—as they affect both living and nonliving matter—physicochemical factors, such as the action of mechanical forces, chemical excitability, reaction–diffusion processes, among others. In fact, while physicochemical factors are usually considered as limiting conditions in development, they can also enable ecological and evolutionary relevant phenotypic variation (Müller and Newman, 2003; Newman, 2012). The combination of both experimental and computational approaches has started to disentangle the effect of factors such as mechanical forces during myxobacteria development (Harvey et al., 2012; Czerwinsky and Shaevitz, 2014).

During aggregative development, motion and organization of individual cells are affected by different physicochemical aspects such as medium viscosity, cell–medium adhesion, diffusion of intercellular signals, and hydrodynamic forces (Persat et al., 2015). In fact, the movement of myxobacteria during the aggregation stage has been empirically compared to the behavior of liquids in a thin layer, following the so-called Ostwald

dynamics (Bahar et al., 2014). Under these dynamics, particles, and arguably aggregating cells, tend to minimize the surface area contacting the media by reducing the number of aggregates and increasing the aggregate volume. With this in mind, Bahar and collaborators (2014) were able to predict the position and fate of nascent aggregates during the early stages of *M. xanthus* development. However, this model fails to predict the collective dynamics for later stages possibly due to the fact that their study did not consider changes occurring as a consequence of development, like cell differentiation. Since cells stop moving when they become spores, this could affect the distribution and shape of the resulting FB as considered by other theoretical studies (Holmes et al., 2010). In another work, researchers motivated by studies of collective behavior in self-propelled rods showed that *M. xanthus* cells can align and form clusters through mechanical interactions among cells and between cells and substrate (Balagam and Igoshim, 2015). Moreover, their computational explorations suggest that mechanical cell alignment combined with slime-trail-following is sufficient to explain the clustering behaviors in wild-type and mutant *M. xanthus* strains. It would be interesting to test these ideas by growing *M. xanthus* in media with different mechanical properties and exploring to what extent these properties and the cell–medium interaction affect the size and shape of FB.

The formation of gradients and concentration patterns via generic processes of diffusion and reaction–diffusion is central for cell differentiation and arrangement during plant and animal development (Kondo and Miura, 2010), as originally proposed by Turing ('52). These mechanisms also seem to be crucial in the early organization and patterning of multicellular aggregates. Moreover, preexisting co-opted traits (see above) and the action of generic multistable dynamics in regulatory networks, suggests that, as explored in some theoretical studies, single-cell differentiation may have preceded the appearance of multicellular development in myxobacteria, and that stereotypical patterns of cell differentiation could have appeared in the transition to multicellularity as cells stayed together via cell-to-cell adhesion mechanisms and became coupled via diffusion and reaction–diffusion mechanisms (Furusawa and Kaneko, 2002; Newman and Bhat 2009; Mora Van Cauwelaert et al., 2015). Indeed, the robustness and ubiquitousness of generic physicochemical processes may help explain how multicellular phenotypes can be reproduced generation after generation, even if the associated regulatory networks diverge in the evolutionary scale.

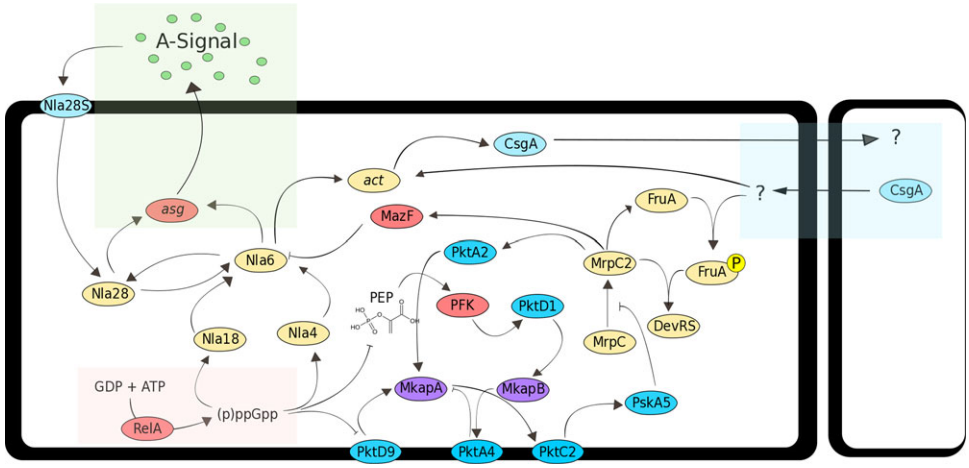
The examples in this section illustrate the complex feedbacks between physicochemical and biochemical process that take place during the development of myxobacteria aggregates. Given such complexity, computational and mathematical models have proven to be valuable tools to articulate such processes, as well as to integrate empirical and theoretical approaches to the study of myxobacterial development (e.g., Sozinova et al., 2006; Holmes et al., 2010; Hendrata et al., 2011; Balagam et al., 2014; Balagam and Igoshin, 2015).

FINAL REMARKS

Myxobacteria have called the attention of researchers over the years due to the remarkable traits of social behavior and diverse multicellular development. Importantly, new technologies and complementary approaches, such as whole genome sequencing and mathematical modeling, came to complement the extensive results obtained by experimental and comparative procedures. As a consequence, diverse aspects of their biology have started to be elucidated, yet it is still necessary to understand the mechanisms that integrate environmental, physicochemical, and molecular aspects of myxobaterial development, what the relative role of each factor is and how they contribute to the generation of variation at the ecological and evolutionary scales. Evo-Devo conceptual and experimental tools, such as reaction norm and diverse comparative approaches, just to name a couple, can help tackle these questions. Indeed, focusing into myxobacteria multicellularity, we pointed out to the importance of pursuing the discussion of myxobacterial development in the context of Evo-Devo theory as it has been started by some authors (Huntley et al., 2011; Velicer et al., 2014; Wall et al., 2014). Evo-Devo has provided a powerful framework to study and discuss multicellular development and its evolution in the plants and animals. Particularly, we discussed the concept of DSD to understand the scenario of divergence at the genetic developmental program in myxobacteria as well as the potential contribution of ecological and physicochemical factors which in combination with genetic may account for FB formation. We consider that by incorporating different species, jointly with the tools currently available (whole genome sequences, experimental and theoretical approaches), will render important insights about the origin, maintenance, and diversification of multicellular organisms in general.

Box 1. Regulation of development in *M. xanthus*

M. xanthus has been employed as a model organism for the myxobacteria and therefore, much of our current knowledge of the mechanisms underlying development came from extensive studies on *M. xanthus* (Whitworth, 2008, Yang and Higgs, 2014). Through diverse genetic, biochemical, and cellular studies, it has been shown that *M. xanthus* development involves the action of transcription factors, enzymes (particularly, protein kinases), scaffolds, membrane proteins, metabolites, and other small molecules (Kroos, 2007; Rajagopalan et al., 2014; Bretl and Kirby, 2016). Briefly, development starts as a response to starvation after sensing the lack of amino acids and other nutrients in the medium (Dworkin, 2007). Once starvation is sensed, ribosomes stall and the enzyme RelA, which is conserved across bacteria, increases the intracellular concentration of the alarmone [(p)ppGpp] (Manoil and Kaiser, '80a,'80b).



Box Figure 1. *Regulatory network associated to cell-fate determination in M. xanthus.* The network includes transcription factors (yellow), membrane proteins (sky blue), protein kinases (dark blue), scaffold proteins (purple), enzymes (red), and their associated reaction products. PEP stands for phosphoenolpyruvate. Arrows represent positive interactions, and flat ends correspond to negative ones. [Color figure can be viewed at wileyonlinelibrary.com]

(p)ppGpp induces the stringent response, which in turn arrests cellular growth and enables development (Chatterji and Ojha, 2001; Boutte and Crosson, 2013).

After (p)ppGpp accumulates, the A-signal is released to the medium, where it mediates a quorum-sensing mechanism that in turn enables a coordinated population-level response to starvation (Kuspa et al., '92; Shimkets, '99). Subsequently, transcription of *csgA* and *mrpC* genes is activated. CsgA, whose regulation relies on a cascade of NtrC-like elements, encodes a short-chain alcohol dehydrogenase, which is involved in the C-signaling pathway (Gronewold and Kaiser, 2001, 2007; Giglio et al., 2011, 2015). The specific mechanisms by which CsgA mediates intercellular communication are still unknown, but it has been suggested that CsgA is a membrane-associated protein that mediates communication via the recognition of a cognate receptor in neighbor cells. Recent evidence suggests that CsgA is not an outer membrane protein, but that it possess phospholipase activity (Boynton and Shimkets, 2015). However, it is not clear yet how the enzymatic activity is related to the mediation of intercellular communication. Regardless of the specific model, evidence shows that CsgA is involved in cell-to-cell adhesion and movement coordination during development (Søgaard-Andersen et al., 2003; Lobedanz and Søgaard-Andersen, 2003). Owing to its role in signal coordination and transduction, CsgA is considered a key element during development (Julien et al., 2000; Holmes et al., 2010). In addition to A- and C-signaling, at least other three signals, named B-, D-, and E-signal, have been found to allow intercellular communication and coordination of individual cells during development, but the mechanisms of action are not as well understood as those associated with the A- and C-signals (Kaiser, 2004; Bretl and Kirby, 2016).

Moreover, the transcriptional factors MrpC and FruA are involved in the activation of the operon *devRS* (Viswanathan et al., 2007; Campbell et al., 2015). On the one hand, MrpC is regulated at the posttranslational level (Nariya and Inouye, 2005a, 2006). During vegetative growth, MrpC is phosphorylated by the PktC2-PskA5 phosphotransfer chain (Nariya and Inouye, 2005a). When phosphorylated, MrpC displays reduced affinity for its binding sites and it is unable to efficiently act as transcription factor (Nariya and Inouye, 2006). In addition, PktC2-PskA5 indirectly interacts with at least other four kinases (PktA2, PktA4, PktD1, and PktD9) through protein–protein interactions with the scaffold proteins MkapA, MkapB, and MkapC (Nariya and Inouye, 2005a,b). These six kinases and Mkaps proteins conform the so-called STPK cascade. During development, the STPK cascade appears to integrate signals that inactivate PskA5 and release MrpC from phosphorylation (Inouye et al., 2008). The components of STPK cascade act in a cell-autonomous fashion and are involved in regulation of development timing and spore maturation (Escalante et al., 2012). The activity of MrpC as a transcription factor is further enhanced by proteolysis mediated by LonD (Nariya and Inouye, 2006). On the other hand, FruA transcription is under the regulation of MrpC (Ueki and Inouye, 2003; Nariya and Inouye, 2006) and transcription of MrpC has been suggested to be under the control of MrpA–MrpB, a two-component system which activates after receiving a yet unidentified

external stimulus (Sun and Shi, 2001a,b). Elements downstream of MrpC y FruA are likely to participate in the phenotypic changes associated with cell differentiation such as cytoskeleton rearrangement and biosynthesis of the spore cell wall (Yang and Higgs, 2014).

Finally, MrpC is also responsible for the activation of MazF, an endonuclease whose activity is associated with programmed cell death (PCD), at least in some *M. xanthus* strains (Nariya and Inouye et al., 2008; Lee et al., 2012; Boynton et al., 2013). MrpC and MazF have been suggested to form a toxin–antitoxin system (Nariya and Inouye, 2008). In this system, MazF activity is reduced when associated with MrpC but enhanced once dissociated from it after LonD-dependent proteolysis of MrpC. In this way, MrpC activation is simultaneously involved in trigger both PCD fate and myxospore differentiation (see a graphical representation of the regulatory network involved in *M. xanthus* development in Box Fig. 1).

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Capítulo 2

Determinación del destino celular en el desarrollo de *M. xanthus*

Uno de los rasgos característicos de los organismos multicelulares es la diferenciación celular, mediante la cual las células con pocas o nulas diferencias genéticas alcanzan fenotipos diferentes. Durante el desarrollo multicelular de *Myxococcus xanthus*, las células de una población inicialmente homogénea alcanzan uno de al menos tres destinos celulares: células periféricas, esporas o sufren muerte celular programada (Yang & Higgs, 2014). A pesar de la identificación de diversos componentes moleculares asociados al desarrollo de este organismo, así como las interacciones regulatorias entre estos, no es claro cómo emergen comportamientos sistémicos que especifican el espectro de tipos celulares observados experimentalmente.

Los procesos de determinación del destino celular durante el desarrollo multicelular han sido abordados ampliamente mediante los modelos de redes dinámicas (Kauffman, 1969; Von Dassow et al., 2000; Espinosa-Soto et al., 2004; Benítez et al., 2008). Los modelos Booleanos, inspirados inicialmente en circuitos electrónicos, son un formalismo para el estudio de las redes dinámicas en las cuales, los genes, proteínas y los metabolitos que constituyen el sistema de interés son representados como variables Booleanas para las cuales se hace referencia de manera cualitativa a su nivel de expresión o de actividad a través de los descriptores de “encendido o arriba de un umbral” (1) y “apagado o abajo de un umbral” (0). La dependencia del estado de los componentes es especificada mediante enunciados lógicos construidos a través de la combinación de los operados lógicos de conjunción, disyunción y negación. En estos modelos, la combinatoria de la regulación (es decir, el efecto de la acción simultanea por parte de múltiples reguladores sobre un mismo componente de la red) y las asas de retroalimentación son de particular interés ya que estas subyacen la emergencia de comportamientos no lineales.

En este capítulo se estudia el proceso de determinación del destino celular en la escala de una sola célula durante el desarrollo de *M. xanthus* mediante un modelo dinámico empleando el formalismo Booleano que describe de manera cualitativa los niveles de expresión o actividad

de los componentes involucrados. El modelo es desarrollado mediante la integración de la evidencia experimental disponible en la literatura científica y su formalización a través de un modelo dinámico provee información relevante respecto a los mecanismos que canalizan al sistema hacia un número limitado de estados estables los cuales, con base en los perfiles de expresión y actividad, corresponden a los destinos celulares observados de manera experimental en *M. xanthus*. Esta observación demuestra la utilidad de los estudios teóricos para realizar observaciones relevantes respecto a la emergencia de las propiedades dinámicas de los sistemas multicelulares.

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ORIGINAL ARTICLE

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Cell-fate determination in *Myxococcus xanthus* development: Network dynamics and novel predictions

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Myxococcus xanthus is a myxobacterium that exhibits aggregation and cellular differentiation during the formation of fruiting bodies. Therefore, it has become a valuable model system to study the transition to multicellularity via cell aggregation. Although there is a vast set of experimental information for the development on *M. xanthus*, the dynamics behind cell-fate determination in this organism's development remain unclear. We integrate the currently available evidence in a mathematical network model that allows to test the set of molecular elements and regulatory interactions that are sufficient to account for the specification of the cell types that are observed in fruiting body formation. Besides providing a dynamic mechanism for cell-fate determination in the transition to multicellular aggregates of *M. xanthus*, this model enables the postulation of specific mechanisms behind some experimental observations for which no explanations have been provided, as well as new regulatory interactions that can be experimentally tested. Finally, this model constitutes a formal basis on which the continuously emerging data for this system can be integrated and interpreted.

KEYWORDS
dynamic model, fruiting body, multicellularity, *Myxococcus xanthus*, regulatory network

1 | INTRODUCTION

The appearance of cellular aggregates where multiple cell types can coexist has been recognized as one of the major transitions in evolution (Maynard-Smith & Szathmary, 2000). In multicellular organisms, cell differentiation enables the coexistence of otherwise metabolically incompatible functions in unicellular organisms, which achieve such functions through alternation of cellular types or functions over time. At least in principle, all cells conforming multicellular organisms possess an intracellular genetic regulatory network with steady expression states (attractors) that can be associated to specific cell types (Espinosa-Soto, Padilla-Longoria, & Alvarez-Buylla, 2004; Furusawa & Kaneko, 2002; Kauffman, 1969; Laurent & Kellershohn, 1999).

Regulatory networks are conformed by components of diverse nature such as transcription factors, intra- and intercellular signaling proteins and other biochemical factors, each being affected and affecting the activity of other components in the network. The nonlinear interactions among the network components can result in the emergence of systemic properties that cannot be predicted by analyzing the individual elements (Saadatpour et al., 2010).

A formalism that has enabled the study of systemic properties arising in regulatory networks is the Boolean network modeling, which has been fruitfully used in the study of developmental and other biological processes (Albert & Othmer, 2003; Benítez, Espinosa-Soto, Padilla-Longoria, & Alvarez-Buylla, 2008; Espinosa-Soto et al., 2004; Kauffman, 1969; Li, Assmann, & Albert, 2006; Martinez-Sanchez, Mendoza, Villarreal, & Alvarez-Buylla, 2015; Ortiz-Gutiérrez et al., 2015; Thomas, 1973). Moreover, dynamic models provide a tool for identifying common properties and particularities in the developmental processes in different systems. In particular, they can provide clues about generic aspects of cell-fate determination in the transition to multicellularity (Arias Del Angel et al., 2016; Duran-Nebreda,

Montañez, Bonforti, & Solé, 2016; Furusawa & Kaneko, 2002; Mora Van Cauwelaert, Del Angel, Antonio, Benítez, & Azpeitia, 2015).

Implementation of dynamic models for the study of cellular differentiation has been mostly limited to eukaryotic model organisms developing through a zygotic or "staying-together" mechanism (Albert & Othmer, 2003; Benítez et al., 2008; Jaeger & Reinitz, 2012). However, much less is known about the dynamic properties underlying cell-fate determination in the development of cellular aggregates, such as the fruiting bodies of *Dictyostelium discoideum* (Marée & Hogeweg, 2001; Nanjundiah & Sathe, 2013) and myxobacteria (Bretl & Kirby, 2016; Kroos, 2017).

Among myxobacteria, *Myxococcus xanthus* is the most well-studied species, for which transcription factors and signaling pathways involved in its development have been described (Rajagopalan, Sarwar, Garza, & Kroos, 2014; Bretl & Kirby 2016; Kroos, 2017). *M. xanthus* exists as individual vegetative cells that nonetheless exhibit social behavior (Yang & Higgs, 2014). However when nutrients are depleted from the medium, *M. xanthus* starts a developmental process in which about 10^5–10^6 individual cells aggregate culminating in the formation of spore-filled multicellular structures called fruiting bodies (FBs) (Kaiser et al., 2010). During FB development, cells may reach one of at least three possible cellular fates: programmed cellular death (PCD), peripheral cell, or myxospore. Most of the cells in the population undergo PCD (Lee, Holkenbrink, Treuner-Lange, & Higgs, 2012; Nariya & Inouye, 2008), about 15% differentiates into peripheral cells which resemble vegetative cells and are mainly located in the base of the fruiting body (Higgs, Hartzell, Holkenbrink, & Hoiczyk, 2014; O'Connor & Zusman, 1991a,b), and the remaining of the population differentiates into myxospores, a cell type that is metabolically quiescent and easily dispersible (O'Connor & Zusman, 1991a). When nutrients become available in the medium, myxospores germinate and restart the vegetative life stage (Otani, Inouye, & Inouye, 1995; Shimkets & Seale, 1975). Under other stress conditions *M. xanthus* may form spores independently of the development of FBs (Dworkin & Gibson, 1964; Higgs et al., 2014; Müller, Treuner-Lange, Heider, Huntley, & Higgs, 2010).

At the molecular level, *M. xanthus* development starts with the stringent response triggered by the intracellular accumulation of the molecule (p)ppGpp (Harris, Kaiser, & Singer, 1998). This molecule regulates downstream genes such as the ones coding for the transcription factors Nla's and the *actABCD* operon (Giglio, Caberoy, Suen, Kaiser, & Garza, 2011; Gronewold & Kaiser, 2001), which in turn allow the expression of at least five signals involved in the coordination and intercellular communication that are crucial to the progression of FB development (Kaiser, 2004; Arias Del Angel et al., 2017; Kroos, 2017). Later in the developmental regulatory process, MrpC, a key transcription factor involved in cellular differentiation towards myxospore, is expressed (Robinson, Son, Kroos, & Kroos, 2014; Sun & Shi, 2001). In at least some strains, the PCD cell fate is achieved due to the action of the mRNA interferase, MazF which is co-transcribed with MrpC (Higgs et al., 2014; Lee et al., 2012; Nariya & Inouye, 2008). Additionally, MrpC activity is post-translationally regulated by the Serine/Threonine protein kinase (STPK) cascade, a set of eukaryotic-like kinases and scaffold proteins (named multiple kinase associated proteins, Mkaps) (Nariya & Inouye, 2005a,b). In the vegetative state, PskA5, a member of the STPK cascade, phosphorylates to MrpC and reduces its affinity for its target genes (Nariya & Inouye, 2005b, 2006). Once development starts, PskA5 is inhibited and MrpC is released to act as transcription factor. Downstream of MrpC, the expression *fruA* and the *devTRS* operon is stimulated (Campbell et al., 2015; Ueki & Inouye, 2003). These in turn regulate effector genes directly involved in the phenotypic changes associated to cellular differentiation (Higgs et al., 2014; Müller et al., 2010; Rajagopalan et al., 2014).

Previous models have been proposed to explain certain aspects of the *M. xanthus* development, however they have almost exclusively focused in the role of C-signal during FB formation, particularly in the aggregation phase, without paying attention to the additional components reported to be involved in the regulatory network (Bahar, Pratt-Szeliga, Angus, Guo, & Welch, 2014; Balagam & Igoshin, 2015; Igoshin, Goldbeter, Kaiser, & Oster, 2004; Janulevicius, van Loosdrecht, & Picioreanu, 2015; Sozinova, Jiang, Kaiser, & Alber, 2005, 2006). Here we integrate the available information about cell fate determination during development of *M. xanthus* FBs into a dynamic Boolean network model. The model presented here is able to reproduce attractors that correspond to the reported cell fates, providing a robust mechanistic explanation for cellular determination in *M. xanthus* FB development. The model also reproduces mutant phenotypes and helps understand the dynamics behind the formation of spheroplasts, a spore-like cell type, that is independent of FB development. Also, the model was used to study the delay in myxospore formation observed in strains carrying mutations in the STPK cascade components. Finally, we use the model to postulate mechanisms behind some experimental observations for which no explanations have been provided, as well as new regulatory interactions that could be experimentally tested.

2 | METHODS

2.1 | Network reconstruction and Boolean model

The regulatory network is grounded on experimental information available until 2016 for the development of *Myxococcus xanthus*. In the network, nodes represent the components involved in development (genes, proteins and metabolites) and edges represent regulatory interactions among such components. In the network model, each node can have one of two possible states: the state of the node is 0 if the node is not expressed or below a certain threshold, and 1 when the node is expressed or above a certain threshold. For each node, a logical function is assigned to establish the dependence between the state of the node and the state of its regulatory nodes. To facilitate further analyses, the complete network was reduced by elimination of the nodes with no input or output interactions and by condensing nodes acting in a linear cascade. Network specification details and logical functions are available in Tables S1 and S2, respectively.

2.2 | Estimation of missing logical functions

For those functions for which no sufficient experimental detail was available, we implemented a modified version of the algorithm originally proposed by Azpeitia, Weinstein, Benítez, Mendoza, and Alvarez-Buylla (2013). This algorithm allows to test one-by-one the set of possible single missing interactions (i.e., those not currently reported in the literature) and test their effect on the network attractors. The algorithm considers a set of principles that reduce the set of possible logical functions to those that are biologically meaningful and consistent with experimental evidence considered in the network.

We only had protein-to-protein interaction data for the STPK cascade elements and lacked further information to define the regulatory interactions among members of the STPK cascade. Thus we used the algorithm proposed by Azpeitia et al. (2013) to elucidate the subset of interactions which were most likely to be involved in the process under study, this is, the subset of interactions among STPK cascade elements that were consistent with the rest of the data and with the expected attractors.

2.3 | Network dynamics

In the network simulations, the state of the nodes is allowed to change over time (measured as iterations) according to:

$$X_n(t+1) = F_n(X_{n1}(t), X_{n2}(t), \ldots, X_{nk}(t)), \quad (1)$$

where $X_n(t+1)$ is the state of the node X_n a time $t+1$. Such state is determined by a logical function *Fn* that depends on the state of the regulators X_{n1}, ,X_{n2}, ..., X_{nk} at time t. The network was updated using a synchronous scheme in which the state of all the nodes is updated simultaneously at each iteration. We followed all the possible initial conditions to find the attractors of the network.

2.4 | Regulatory network model robustness

Robustness of the network was assessed by using the bit-flip method that changes the output of logical functions one-by-one or the state of a node at a given time point. Then, we assessed the network tendency to maintain its properties in the face of these perturbations, as compared to a population of 10,000 randomized versions of the network. We also tested the effect of an asynchronous updating scheme on the number and identity of the network attractors.

2.5 | Temporal dynamics analysis

We estimated the number of iterations required for the wild-type network model and multiple single in silico mutations to reach the attractor corresponding to myxospores. Mutants were modeled by fixing the state of a given node to 0 throughout the whole realization. In these simulations, the systems (original and mutants) were initialized in the same condition representing the setting of starvation. The order in the number of iterations taken by wild-type and mutant models to reach spore formation is referred to as temporal sequence.

To explore the robustness of such temporal sequence, temporal dynamics were also analyzed under stochastic perturbations, in each iteration one node obeys one alternative logical function with probability p. Alternative logical functions were generated through one of four different methods: (i) The node state is fixed to 0, (ii) the node state is fixed to 1, (iii) the alternative logical function is generated by reorganizing the bits of the original truth table, or (iv) the alternative logical function is selected from the set of possible logical functions for N regulators; where N is the number of regulators of a given node. Three error probabilities p (.5, .1 and .05) were tested for each of the four methods described above.

2.6 | Continuous model

Boolean models are known to recover fixed points of continuous systems (attractors) but the trajectories may be artifacts of the specific formalism (Ortiz-Gutiérrez et al., 2015; Saadatpour, Albert, & Albert, 2010). To test if the trajectories we studied were an artifact, we mapped our Boolean model to a continuous model by adjusting the discrete behavior of the Boolean rules to a continuous sigmoidal function. The Boolean model was converted into a continuous one by adjusting the Boolean functions into continuous functions with the form (Villarreal, Padilla-Longoria, & Alvarez-Buylla, 2012):

$$\frac{d[X_i]}{dt} = \frac{1}{1+\exp[-2b(w_i-h)]} - \gamma_i[X_i], \quad (2)$$

where $[X_i]$ is a continuous value between 0 and $1/\gamma_i$ representing the relative activity of the variables in the model. w_i is the Fuzzy logic version of the classic Boolean function in which the conjunction ($A \wedge B$), disjunction ($A \vee B$) and negation ($\rightarrow A$) operators are converted into min(A, B), max(A, B), and 1 – A, respectively. b and h are parameters controlling the shape of the function and γ_i is the decay rate for the X_i variable in the model. The values for b = 10 and h = 0.5 were selected to recover a sigmoidal behavior. All the decay rates are set to 1.

Estimation of missing logical functions was performed by using a modified version of the original scripts provided by E. Azpeitia (Azpeitia et al., 2013) and implemented in Perl v5.16.2. Network model analyses were performed using the R package BoolNet v2.1.1 pre-built functions and our own customized scripts. The code used to perform the reported analysis is available at https://github.com/laparcela/mxan_regulatory_network_model.

3 | RESULTS

3.1 | The regulatory network model recovers attractors associated with cell types observed during *M. xanthus* life cycle

We propose a regulatory network that integrates the experimental evidence related to the development of *M. xanthus* (Table S1 and S2; Figure 1). The nodes of this network represent diverse elements including transcription factors, enzymes (particularly, protein kinases), metabolites, membrane proteins, scaffold proteins and diffusible

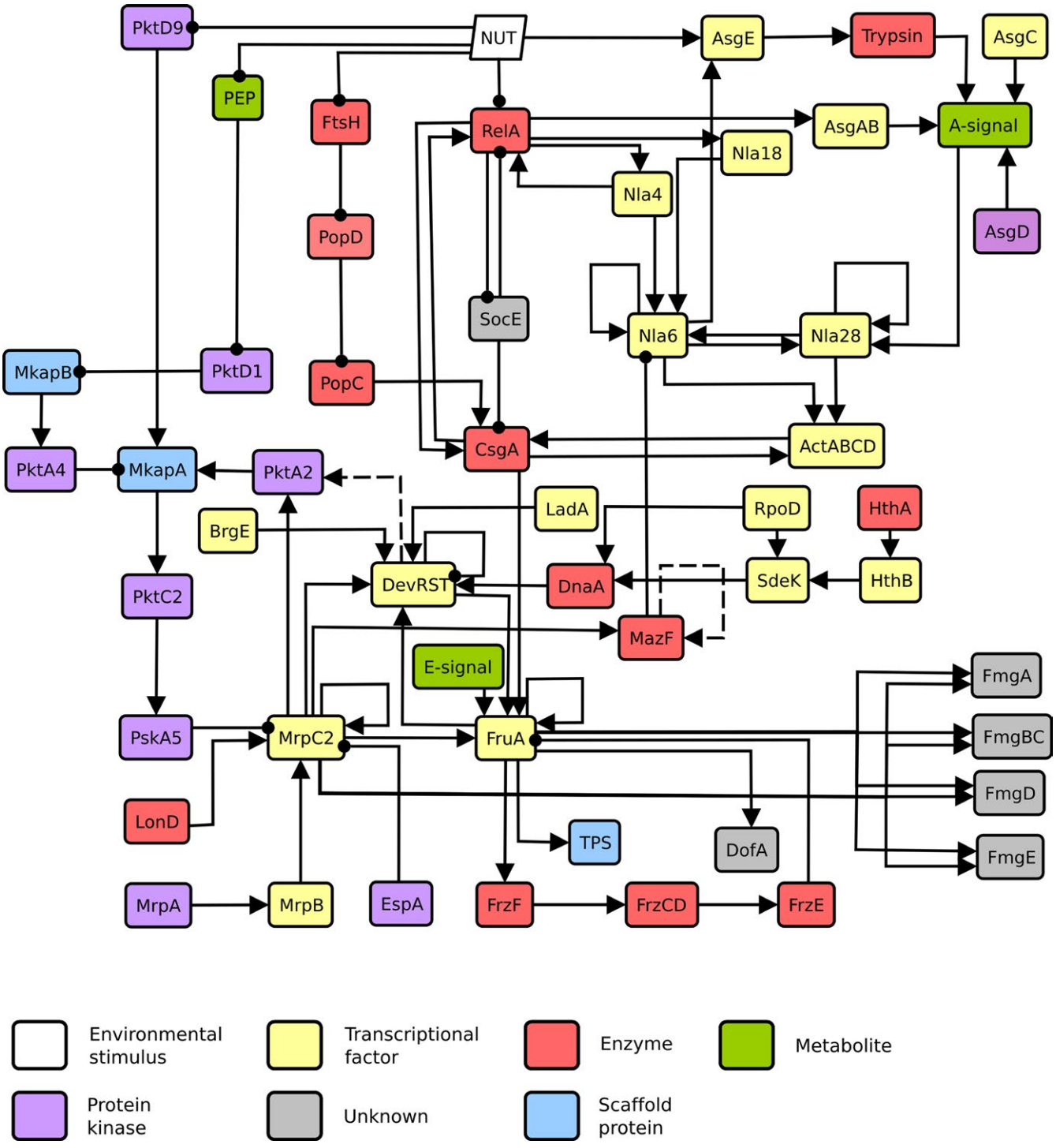

FIGURE 1 Regulatory network associated to *Myxococcus xanthus* development. The nodes in the network represent transcription factors, enzymes, scaffold proteins, metabolites or environmental stimuli. Edges represent interactions among the nodes. Edges ending in arrows and circles represent positive and negative regulation, respectively. Solid lines are interactions based on experimental evidence and dashed lines represent hypothetical interactions

molecules that interact with each other through different regulatory levels (transcriptional, phosphorylation, protein–protein interactions and enzymatic catalysis). For the case of the STPK cascade, there is vast information suggesting protein–protein interactions identified using the yeast-two-hybrid (YTH) method (Nariya & Inouye, 2005a,b; Nariya & Inouye, 2006). However, from this data it is impossible to infer direct interactions and logical rules. Moreover, from all the interactions suggested by YTH experiments, only some could be taking place in vivo in *M. xanthus*. Therefore, we followed the procedures implemented by Azpeitia et al. (2013) to explore all the possible interactions among the elements of the STPK cascade and selected those biologically plausible and consistent with experimental data (Figure 1; Figure S1).

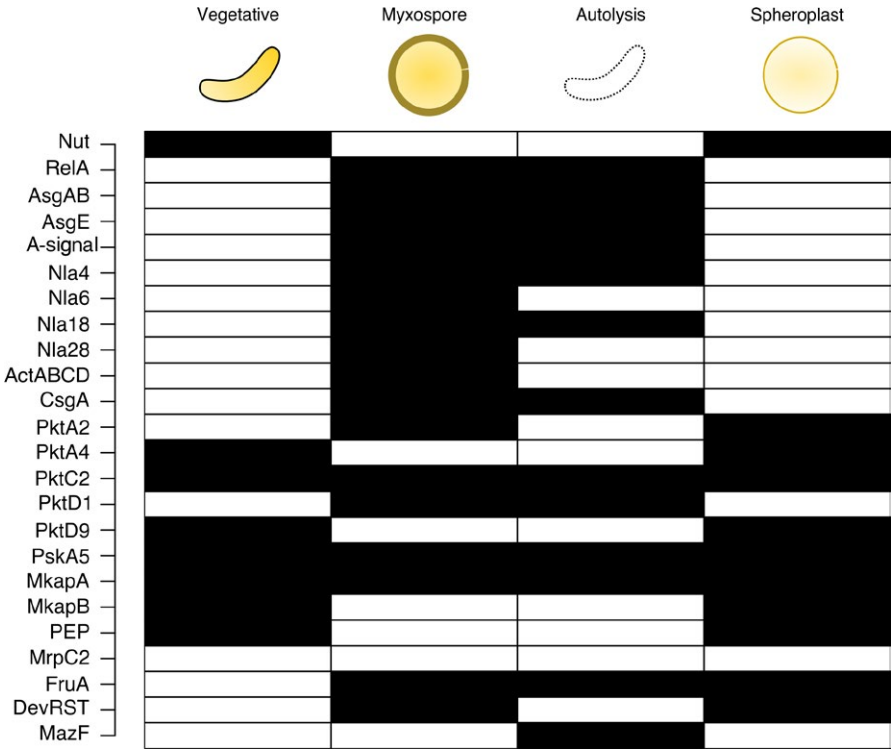

FIGURE 2 Attractors recovered by the dynamic Boolean model. Rows represent the state of the nodes in the network model and columns represent attractors recovered by the model. For each attractor, black or white represent the state of the corresponding node, 1 or 0, respectively. Each attractor is named based on the profile of cell types occurring during the *M. xanthus* life cycle. At the top, schematic representations of the cell types of *M. xanthus* associated to each attractor

We then explored the network dynamics by Boolean regulatory network simulations and found that it was unable to generate the expected attractors, i.e., those corresponding to the expression profiles reported for *M. xanthus* cell types. Based on indirect evidence, we thus postulated two additional interactions, namely, a self-regulatory interaction for MazF and a positive interaction from DevTRS to PktA2 (Table S1). With the resulting network the model was capable of reproducing four fixed point attractors that can be interpreted as four cell types observed during the life cycle of *M. xanthus* (Figure 2). Nodes used to identify attractors with cell types included the well studied proteins MrpC2, FruA, A- and C-Signal, DevTRS operon, RelA, MazF, PktA2, PktD9, PktC2 and PktD1, as well as the presence or absence of nutrients in the medium represented by the node "NUT" (nutrient availability).

Myxospores have been shown to express most of our diagnostic genes, being MrpC2 and DevTRS and the dephosphorylation of PktC2 and PskA5 key indicators of cell type. Both peripheral and vegetative cells display similar morphological traits and expression profiles, so it is possible to discriminate between both of them and myxospores, but not between them. Thus, we represent peripheral and vegetative cells

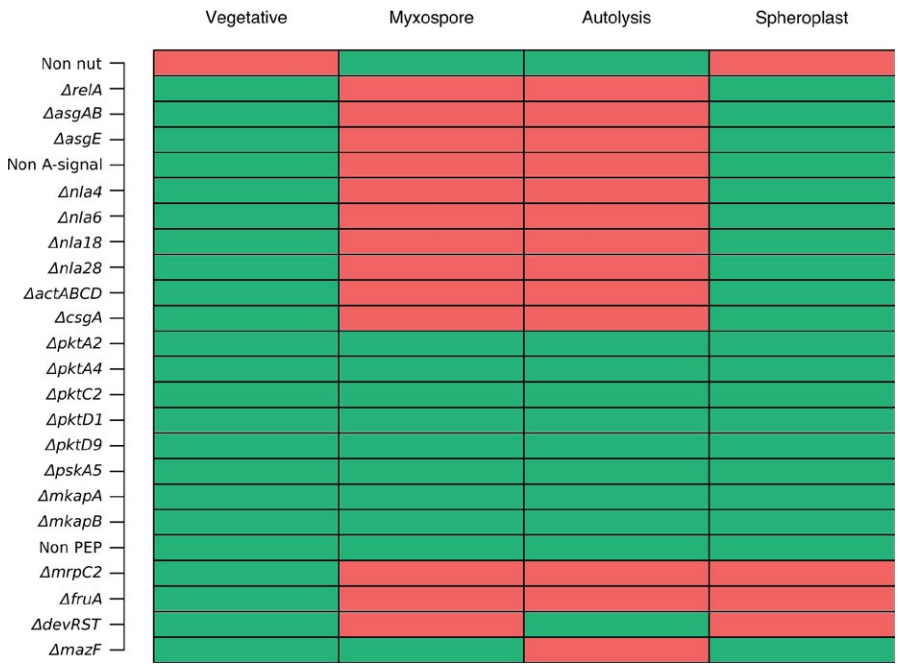

FIGURE 3 Validation of the network using in silico mutants. In the table, rows represent an in silico mutant and columns represent the attractors recovered by the network. Cells are colored in green or red if the attractor is recovered or not in the corresponding in silico mutant

as a single attractor of the regulatory network (Figure 2). A third cell fate, corresponding to programed cell death, is identified by the activation of MazF and the absence of Nla6.

It is worth noting that the model predicted an unexpected attractor that, when contrasted with available data, can be associated with spheroplasts. This cell type is phenotypically and morphologically similar to myxospores but lacks some proteins occurring in the myxospores and may occur independently of FB formation. Spheroplast have been induced by a treatment of glycerol at high concentrations in liquid media or in the presence of antibiotics (Müller et al., 2010) (Figure 2).

To validate the capacity of the model to recover biologically relevant evidence, we simulated lost-of-function mutations by setting the state of the individual nodes to 0 independently of their regulators. The results of these simulations are congruent with available experimental data (Figure 3). (Figure S2).

3.2 | The regulatory network model recovers the transitory activation of MrpC2 during *M. xanthus* development

We were interested in analyzing the system trajectory when initialized in a condition that simulates the setting of starvation. We assumed that the network state at such initial condition is similar to the vegetative attractor, with the difference that the NUT node is set to 0. In congruence with available evidence, and as validation to our model, setting NUT's value to 0 in the vegetative attractor leads the system to the myxospore attractor.

A closer inspection of the trajectory with such an initial condition showed a transitory activation of MrpC2 that is consistent with previous reports (Lee et al., 2012) (Figure 4). Indeed, MrpC2 has been shown to peak around 24–36 hr after the establishment of starvation and then it is reduced later during development (Lee et al., 2012). However, the mechanisms behind this transitory activation are still unknown. To study potential mechanisms, we repeated our simulation using in silico mutants and found that the null mutant *ΔpktA2* reaches a myxospore attractor but the MrpC2 state remains constant once activated (Figure 3), suggesting that PktA2 and its regulation by MrpC and DevTRS is involved in the re-activation of the STPK cascade and the concomitant inhibition of MrpC2 later in development. Since the trajectories of a Boolean system are particularly sensitive to the discrete implementation of the model, we verified this result by using a continuous approximation of the system (Villarreal et al., 2012), and found that the transitory behavior is conserved (Figure S5).

3.3 | The regulatory network model recovers the developmental time delays observed in mutant strains

Mutations of kinases of the STPK cascade have been shown to produce timing changes of the aggregation and cell differentiation towards spores, but do not arrest development (Escalante et al., 2012). We investigated if the dynamic properties emerging from the interactions between the network components explain the timing alterations observed experimentally. In order to test this, we used the in silico null mutants for each of the kinases included in the STPK cascade and simulated the transition starting from the setting of starvation to the myxospore formation. Each of the perturbed networks reaches an attractor that can be associated to myxospore formation, but the number of time steps required for each network to reach the attractor is different (Figure 4).

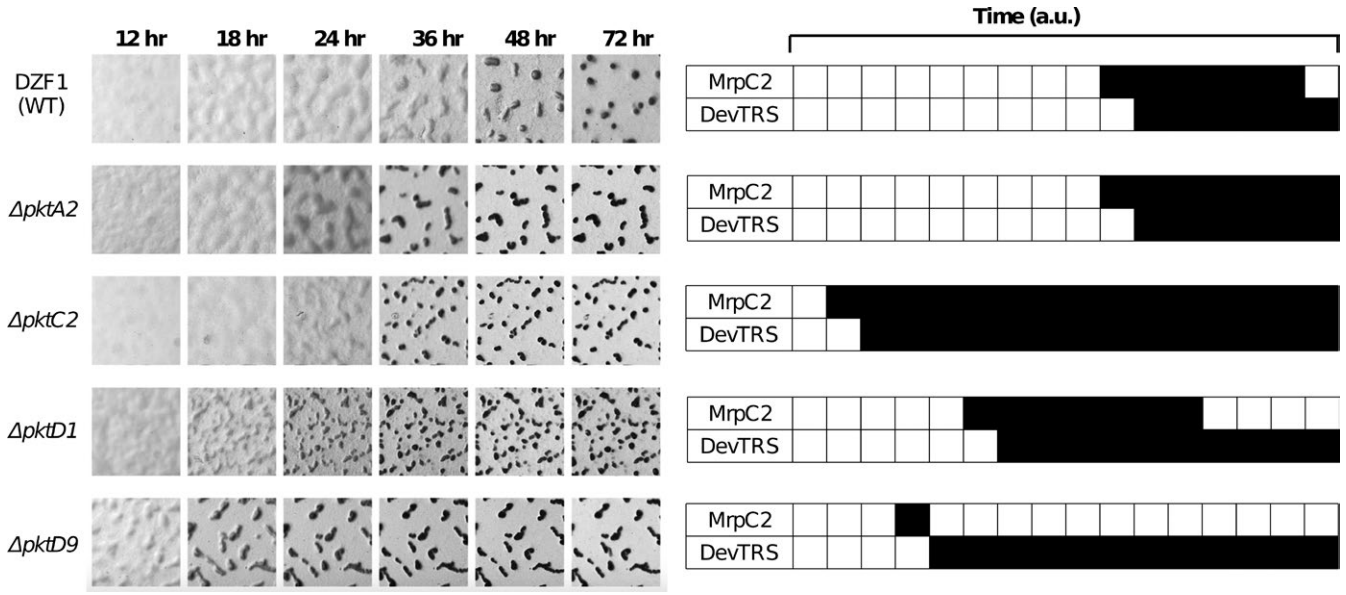

FIGURE 4. Temporal sequence of sporulation across M. xanthus strains. Comparison between experimental and model simulations of the temporal sequence of sporulation in Myxococcus xanthus. (Left) Rows represent strains (wild-type or knock-out mutants) which are allowed to develop in low-nutrient medium. Columns represent time-points during the developmental process. Image taken from Escalante et al., 2012 (Right) System dynamic recovered by the model starting from a initial condition representing the set of starvation. For each in silico mutant, the trajectory of MrpC2 and DevTRS is shown. It is assumed that cell differentiation toward myxospore occur when the states of both MrpC2 and DevTRS are 1

The results for the simulated sporulation timing (*ΔpktC2* > *ΔpktD9* > *ΔpktD1* > *ΔptkA2* = DZF1) are in good agreement with the experimental evidence (*ΔpktD9* > *ΔpktD1* > *ΔpktC2* > *ΔpktA2* > *DZF1*). However, for *ΔpktC2* the predicted sporulation timing occurs earlier that reported (Escalante et al., 2012). To test the robustness of these results, we conducted similar simulations with either an asynchronous or an stochastic updating schemes, finding that these results are not an artifact of the deterministic synchronous updating scheme (Figure S3 and S4).

4 | DISCUSSION

Previous experimental work and dynamic models for *M. xanthus* have approached cell-fate determination as the result of the differential expression of single genes associated to *M. xanthus* development (e.g., C-signal; Sozinova et al., 2005, 2006; Holmes, Kalvala, & Whitworth, 2010). However, some integrative efforts have highlighted the importance of understanding development as the result of interactions among diverse factors (Arias Del Angel et al., 2017; Bretl & Kirby, 2016; Kroos, 2017; Rajagopalan et al., 2014). Nevertheless, these efforts still have not captured the complex dynamics underlying *M. xanthus* development.

The model proposed here integrates the available information for *M. xanthus* cell-fate determination into a dynamic framework. This model reproduces attractors that emerge from the dynamic interactions in the network and that can be associated to the cell types reported for fruiting body development in this species. Since the network components represent known genes and molecules, the model provides us with a mechanistic explanation of the process and with the possibility to test the importance of certain nodes or interactions and to generate predictions that can feedback experimental work. In particular, our model predicts that an hypothetical interaction between *devTRS* operon and PktA2 is important to reproduce the transitory activation of MrpC/MrpC2, which has been reported but not yet understood at a mechanistic level (Lee et al., 2012). Among the attractors recovered by the model, the one associated with spheroplast was not expected. Thus, our model suggests that the same regulatory network involved with cell differentiation during fruiting body development is also associated with development-independent cell differentiation in *M. xanthus*. Moreover, this partially validates the model, as it can reproduce experimental results that were not considered for the model specification.

While the model provides us with an explanation for cell-fate determination in *M. xanthus* development, it can not fully recover some reported behaviors. Specifically, when we analyzed the delay series across the in silico mutants for the components of the STPK cascade, the model results did not match the experimental observations regarding the sporulation timing of the *ΔpktC2* strain under either synchronous, asynchronous or stochastic updating schemes. This inconsistency suggests that there is still some information missing in the regulatory network (in particular regarding the regulation from or to PktC2) or that the specification of the Boolean model is insufficient to explain some behaviors of the system. In particular, one of the main challenges of Boolean models is to recover transient dynamics, and it is actually when we look at the transitory states of our system that we find more inconsistencies with empirical data. Also, multiple or continuous levels of activity are Boolean models and it is possible that some of the behaviors not recovered by our model may be associated to the assumption of Boolean states. In particular, some of the components included in the models are suggested to have three activity levels (low, intermediate and high). For example, at intermediate levels FruA promotes certain aggregative behaviors (Jelsbak & Søgaard-Andersen, 2000), but these are impossible to consider in our model since we are not including spatial dynamics. However, for single cells, the two levels assumed for FruA activity seem to be sufficient to recover the different expected cell types. It is also worth noting that our model only considers intracellular events, while *M. xanthus* development involves multiple cell-to-cell interactions in a spatial scale that is not explicitly considered in the present model. A deeper understanding of this system, which represents one of the model species for the study of the transition to multicellularity, might be obtained from the development of spatial models that consider intra- and intercellular dynamics.

The model presented here contributes with a formal articulation of the available empirical data and provides a basis on which further experimental results can be integrated, and which may complement other modeling efforts focusing on the aggregative dynamics of *M. xanthus* (Holmes et al., 2010; Sozinova et al., 2006). Moreover, it constitutes a necessary step to explore the developmental dimension of cooperation and conflict dynamics (Mora Van Cauwelaert et al., 2015). Finally, this integrative and dynamic model enables further comparative studies aiming to identify the common and specific elements and interactions that have been important in the evolution of multicellularity.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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Capítulo 3

Formación de patrones espaciales de tipos celulares durante el desarrollo de *M. xanthus*

Durante el desarrollo multicelular, los diferentes destinos celulares tienden a coexistir temporalmente dentro del conglomerado multicelular y a exhibir patrones espaciales en los cuales estos ocupan posiciones preferenciales dentro del conglomerado o respecto a su vecindario (Turing, 1952; Von Dassow et al., 2000; Furusawa & Kaneko, 2002; Benítez et al., 2008; Tosenberger et al., 2017; Varahan et al., 2019). Esta distribución no aleatoria de los destinos celulares es mediada por la generación de información posicional, la cual puede ser generada de manera dinámica por las células dentro del conglomerado mismo como consecuencia del acoplamiento de la dinámica interna de las células individuales y sesga el destino celular en función su posición en el agregado y de su vecindario (Turing, 1952; Wolpert, 1969; Furusawa & Kaneko, 2002).

A pesar de que los cuerpos fructíferos de *M. xanthus* son estructuras tridimensionales, las células periféricas y esporas forman un patrón bidimensional en el plano perpendicular a la superficie sobre la cual se desarrollan. Dentro de los cuerpos fructíferos de *M. xanthus*, las células periféricas y las esporas se distribuyen en dominios concéntricos con las primeras localizadas preferencialmente en el dominio externo y las segundas en el domino interno (Saeger & Kaiser, 1993; Julien et al., 2000). Para el caso de las células que experimental muerte celular programada su posición no ha sido determinada claramente (Lux et al., 2004).

En este capítulo se estudia el de desarrollo de *M. xanthus* en el contexto de una población de células inicialmente homogéneas con el objetivo de estudiar de manera simultanea los procesos de determinación del tipo celular y de formación de patrones espaciales de tipos celulares. El modelo propuesto aquí toma en consideración comportamientos celulares, la dinámica de la red regulatoria interna y los mecanismos de comunicación intercelular. El modelo provee indicios de los mecanismos que subyacen la formación de patrones espaciales de tipos celulares y que permiten la coexistencia espacial de los diferentes destinos dentro de un mismo agregado. A diferencia del modelo presentado en el Capítulo 2, el modelo presentado aquí es capaz de recuperar el espectro completo de destinos celulares observados en este

organismo. Finalmente, el modelo enfatiza la importancia del tamaño de los agregados y del tipo de comunicación sobre los procesos estudiados aquí.

Role of aggregate size, multistability and communication in determining cell fate and patterning in *M. xanthus*

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1. Abstract

The emergence of multicellular organisms that exhibit cell differentiation and stereotypic spatial arrangements has been recognized as one of the major transitions in evolution. Myxobacteria have emerged as a useful study model to investigate multicellular evolution and development. Here, we propose a multiscale model that considers cellular adhesion and movement, molecular regulatory networks (MRNs), and cell-to-cell communication to study the emergence of cell fate determination and spatial patterning of *Myxococcus xanthus* fruiting bodies. The model provides a dynamic accounting of the roles of MRN multistability, intercellular communication and conglomerate size in determining cell fate and patterning during *M. xanthus* development. It also suggests that for cell fate determination and patterning to occur, the cell aggregate must surpass a minimum size. The model also allows us to contrast alternative scenarios for the C-signal mechanism and provides stronger support for an indirect effect (as a diffusible molecule) than a direct one (as a membrane protein).

2. Introduction

The emergence of multicellular organisms that exhibit cell differentiation and stereotypical spatial arrangements has been recognized as one of the major transitions in evolution (**Maynard-Smith and Szathmáry, 2000**), and it is estimated to have evolved independently about 25 times (**Grosberg and Strathmann, 2007**). While division of labor by cellular differentiation is recognized as a central feature of multicellular organisms, the evolutionary origin of cell fates during the transition to multicellularity remains unclear. Some authors have postulated that multicellular masses appeared first and only later gradually acquired different

cell fates and patterns, thus generating spatial differentiation (referred to as "patterning", **Haeckel, 1874; Arendt, 2008)**. Alternatively, other authors have proposed that even unicellular organisms were capable of differentiation by alternation of cell fates over time, a view derived from a dynamic perspective of development. Thus, as a result of the formation of multicellular masses, organisms spontaneously exhibited the coexistence and patterning of these cell fates. In turn, these cell fates may correspond to stable states enabled by the dynamics of multistable molecular regulatory networks already present in single cells. Under this view, as cells are incorporated into a conglomerate, new local chemical and mechanical microenvironments may bias cells to spontaneously reach different cell fates (**Kauffman, 1969; Furusawa and Kaneko, 2002; Newman et al., 2003; Mora van Cauwelaert et al., 2015**). Importantly, the formation of such microenvironments may require a minimum conglomerate size, and conglomerate size may in turn be constrained by the accumulation of metabolic waste released by the cells (**Asally et al., 2012**) or by mechanical forces acting over the whole conglomerate and the individual cells (**Jacobeen et al., 2018; Rivera-Yoshida et al., 2018**).

In broad terms, multicellular organisms develop through either a clonal ("stay-together") or aggregative ("come-together") mechanism (**Tarnita et al., 2013**). While multicellular development and its evolution have been most extensively studied in organisms in which multicellularity is clonal, such as animals and plants (**Grosberg and Strathmann, 2007**), aggregative multicellular organisms are also capable of generating complex structures with different cellular fates and arrangements (**Bonner 1998; Sunderland, 2011; Nanjundiah and Sathe et al., 2011**). However, aside from a few model species (e.g. *Dictyostelium discoideum*; see **Bonner 1998**), the development of aggregative organisms remains largely unexplored. Studying different evolutionary origins and modes of multicellularity will enable comparative analyses that could help to identify both common and lineage-specific aspects in the evolution of cell fate determination and size regulation in multicellular organisms.

Myxobacteria, an order in the delta-proteobacteria, have emerged as a useful study model for investigating multicellular evolution (**Muñoz-Dorado et al., 2016; Arias del Angel et al., 2017**) and elucidating the dynamics behind cell fate determination and patterning in aggregative multicellular organisms. Among myxobacteria, *Myxococcus xanthus* is the most studied species, and transcription factors and signaling pathways involved in its development have been described (**Bretl and Kirby 2016; Kroos 2017; Arias Del Angel et al., 2017, 2018**). When nutrients in the medium are exhausted, vegetative cells (VEG) in *M. xanthus* aggregate into

mound-like multicellular structures called fruiting bodies (**Kaiser, 2003)**. During fruiting body development, cells may reach one of three possible cell fates: programmed cellular death (PCD), peripheral cell (ROD) or myxospore (SPO). Inside the fruiting body, cell fates are arranged into two concentric domains: myxospores are concentrated in the inner domain, and peripheral rods in the outer domain (**Sager and Kaiser, 1993a, b; Julien et al., 2000**). Cells undergoing PCD appear to have a broader, although not well characterized, distribution across the fruiting body (**Lux et al., 2004**).

At the intracellular level, *M. xanthus* development initiates with the activation of the so-called stringent response; this mechanism, which is conserved across bacteria, is responsible for genome-wide transcriptional change and survival under stress conditions (**Boutte and Crosson, 2013**), as well as with the sensing of extracellular cues via signaling pathways, such as the A- and C-signals (**Bretl and Kirby, 2016; Kroos, 2017**). The A-signal is a mixture of amino acids and small peptides which freely diffuse in the medium and are thus likely involved in long-range intercellular communication. The C-signal was originally proposed as a membrane protein involved in communication via cell-to-cell contact and has more recently been considered a diffusible molecule (or a producer of them) (**Lobedanz and Løtte-Soggard, 2003; Muñoz Dorado et al., 2016**). These signals are coupled with a complex regulatory network that has been previously shown to be able to reach steady states of the expression profiles of spores, rod and PCD cells (**Arias Del Angel et al., 2018; Supplementary Figure 1**).

In this work, we propose a multiscale model that considers cellular adhesion and movement, molecular regulatory networks (MRNs), and cell-to-cell communication to study the emergence of cell fate determination and spatial patterning of *M. xanthus* fruiting bodies. Specifically, we apply mathematical modeling to gain an understanding of the emergence of spatial arrangements in a population of initially homogeneous vegetative cells whose MRN dynamics are connected through signaling pathways. The model provides a dynamic accounting of the roles of MRN multistability, communication via C-signal and conglomerate size in determining cell fate determination and patterning during *M. xanthus* development. It also suggests that for cell fate determination and patterning to occur, cell aggregates must surpass a minimum size. Finally, the model is employed to contrast the alternative scenarios for the C-signal mechanism, providing support for an indirect effect (as a diffusible molecule), over a direct one (as a membrane protein).

3. Methods

3.1 Model description

To study cell fate determination and spatial patterning in a virtual population of *M. xanthus* cells, we specified a Glazier-Graner-Hogeweg (GGH) model that considered cellular adhesion and movement, molecular regulatory interactions and cell-to-cell communication (**Figure 1**). In the model, an intracellular MRN specified the internal state of each cell and determined the fate of that cell, as well as the production of intercellular signals. Communication via the intercellular signals mediated the coupling between the individual MRNs; in this way cells could affect the state of MRNs in neighboring cells.

The GGH formalism is useful for integrating phenomena at the cellular- and subcellular-levels occurring at different timescales while explicitly considering a spatial domain (**Swat et al., 2012**). In this framework, each scale was captured through a different modeling formalism. The model considered processes at three different spatio-temporal scales, which were all coupled to each other: (1) a dynamic hybrid Boolean/continuous model captured cell fate determination occurring at the intracellular level, (2) the Potts formalism was employed for cell-level behaviors, and (3) partial differential equations were employed for diffusion of chemical fields mediating long-range intercellular communication. Further details of the phenomena and formalisms considered at each scale is presented in the following sections.

3.1.1. Sub-cellular level

At the sub-cellular level, each cell contained an MRN based on experimental evidence that was previously employed to study cell fate determination at the single-cell scale (**Arias Del Angel et al., 2018**). In the MRN, the nodes represented genes, proteins, metabolites or environmental stimuli. At this scale, the MRN dynamics led to multiple steady states that, when compared with the reported experimental data, corresponded to VEG, SPO and PCD. In the MRN, each node could have one of two possible states: 0 if the node was not expressed or was below a certain threshold, and 1 if the node was expressed above the threshold. The state of the nodes changed over time (measured as iterations) according to:

$$X_n(t+1) = f_n\left(X_{n1}(t), X_{n2}(t), \dots, X_{nk}(t)\right), \quad (1)$$

where $X_n(t+1)$ is the state of the node X_n at time $t+1$. That state was determined by a logical function f_n, which depends on the state of the regulators $X_{n1}(t), X_{n2}(t), \dots, X_{nk}(t)$ at time t. The network was updated using a synchronous scheme such that all of the nodes were updated simultaneously at each iteration.

The MRN presented here was adapted to explicitly consider the role of cell-to-cell interactions. Specifically, the following modifications were made to the previously reported network model (**Arias Del Angel et al., 2018; Supplementary Figure 1)**: (1) the Boolean variables for NUT, ASG and CSG were replaced by continuous variables. This modification facilitated the coupling between cells and the chemical fields while preserving the stepwise behaviour observed in gene regulatory interactions. (2) It was no longer assumed that the state of the transcriptional factor FruA at time (t) (FRUA(t)) was a function of the elements in the C signaling pathway (CSGA(t)) in the same cell. The modified Boolean function for FRUA(t) considered that FRUA(t) was activated when the corresponding cell was surrounded by at least θ_{CSG} neighbor cells with CSGA(t) = 1. The complete set of functions specifying the GRN is shown in Supplementary Information 1.

3.1.2. Cellular-level

The Cellular Potts formalism was employed to model cellular adhesion and movement. In this scale, the space was discretized into a regular 1000 x 1000 square lattice with periodic boundaries. Cells consisted of non-overlapping sets of sites over the lattice called pixels. Time was discretized as well into arbitrary units called Monte Carlo Steps (MCS), consisting of a single round of the Metropolis algorithm which allowed the simulation of cell movement across the lattice. The temporal dynamic of the Cellular Potts models is determined by a principle of energy minimization, where energy is specified through a Hamiltonian function (H) as specified in equation (2).

$$H = \sum_{i,j\ neighbors} J\left[\left(\tau\left(\sigma(\vec{i})\right)\right),\left(\tau\left(\sigma(\vec{j})\right)\right)\right]\left[1 - \delta\left(\left(\tau\left(\sigma(\vec{i})\right)\right),\left(\tau\left(\sigma(\vec{i})\right)\right)\right] + \sum_{\sigma} \lambda[v(\sigma) - v_0(\sigma)]^2, \quad (2)$$

The first and second terms on the right-hand side of equation (2) are the cell-to-cell/cell-to-medium adhesion energies and the volume conservation energy, respectively. The first term on the right side of equation (2), $J[(\tau(\sigma(\vec{i}))),(\tau(\sigma(\vec{j})))]$ is the boundary energy per unit area between two cells (σ_1, σ_2) of given fates $((\tau(\sigma(\vec{i}))),(\tau(\sigma(\vec{j}))))$ at a contact point. The term $(1 - \delta((\tau(\sigma(\vec{i}))),(\tau(\sigma(\vec{i})))))$ avoids taking into account pixels belonging to the same cell. In the second term on the right side of the equation, $v(\sigma)$ is the actual volume of a cell and $v_0(\sigma)$ is the target volume. λ is a constant determining the constraint length.

Two cells were considered to be neighbors if they shared a boundary of at least one-pixel unit. In this model, the values of matrix *J* were considered to be equal over the cell population and thus differential adhesion was not considered. However, values in the matrix *J* representing cell-to-cell (cohesion) interaction strength were allowed to differ from the cell-to-medium (adhesion) interaction strengths. In some versions of the model, the values in the matrix *J* were modified to explore the role of the balance between adhesion and cohesion. The values employed for adhesion and cohesion were 7, 10 and 13 (arbitrary units) in all possible pairwise combinations.

When moving over the lattice, virtual cells attempted to copy their pixel state to neighbor pixels, thus changing *H*. Pixel copy occurred following a Boltzmann probability distribution according to equation (3).

$$P[\sigma(\vec{i}) \rightarrow \sigma(\vec{j})] = \left\{1 : \Delta H \leq 0;\ e^{\frac{-\Delta H}{T}} : \Delta H > 0\right\}, \quad (3)$$

At this scale, cell death was simulated by setting $V_0(\sigma)$ equal to zero. This change occurred based on the state of the internal GRN for each cell. For further details about Cellular Potts model specification and implementation see **Swat and collaborators (2012)** and **Glazier and collaborators (2007).**

3.1.3. Supra-cellular level

The supra-cellular level considered diffusion of chemical fields, which mediate long-range intercellular communication. Three chemical fields were considered in the model, representing the available nutrients, A-signal and C-signal. The model assumed that all three chemicals freely diffused across the medium. Also, both A- and C-signal fields were considered to be relatively stable in the medium, with no degradation or consumption occurring outside the cells. Both, A- and C-signal could be freely exchanged between cellular boundaries. In the case of the nutrients, they could be incorporated into the cells, but could not be released back to the medium. Dynamics of the diffusion process for both fields was modeled by partial differential equations as shown in equations (4) and (5).

$$\boxed{\frac{\partial[NUT]}{\partial t} = -\rho + D_{NUT}\nabla[NUT]}, \quad (4)$$

$$\boxed{\frac{\partial[ASG]}{\partial t} = D_{ASG}\nabla[ASG]}, \quad (5)$$

3.2. Indexes for assessing spatial organization

To characterize spatial organization, we implemented indexes to quantify the local and global distribution of cell fates in the virtual aggregates. The cellular center of mass, neighborhood and cell fate were recorded for each cell over time. The position of cell fates relative to each other was analyzed for each cell fate by measuring the number of neighbor cells with a given cell fate in order to define the neighborhood preferences, as in **Tosenberger and collaborators, 2017**. The position of the cell fates inside the aggregate was analyzed by calculating the Euclidean distance between the edge of the aggregate (defined as the farthest cellular center of mass from the center of mass of the whole aggregate) and the individual center of mass.

3.3. Software and model robustness to parameter variation

To evaluate the sensitivity of the model to parameter variation, we ran the model after modifying the nominal value of key individual parameters. Robustness was assessed by comparing the

indexes for spatial organization (section 1.2) obtained for the nominal and modified versions of the model. The tested parameters are highlighted in Table S1.

All models were developed and simulated using CompuCell3D software v.3.7.5 (**Swat et al., 2012**). Because of the stochastic behavior of Glazier-Graner-Hogeweg models, all simulations were repeated at least 30 times. Simulations were visualized using the generic output from CompuCell3D or yEd graph editor v3.16 (**yFiles software, Tubingen, Germany**). The Python library NetworkX v2.1 was employed for network analysis (**Hagberg et al., 2008**) and statistical analysis were performed using R v3.2.3 (**R Core Team 2015**). Graphics were generated using the R package ggplot2 v2.2.1 (**Wickham, 2009**). All models and code applied for analysis are freely available at (https://github.com/laparcela/Myxobacteria-CC3D_model/).

4. Results

4.1. Coexistence of cell fates is a result of intercellular coupling

We employed the model to study the spatiotemporal dynamics of the developmental process in *M. xanthus*. The system was initialized in the condition representing vegetative growth (VEG). Cells were homogeneously distributed and were allowed to adhere to neighbor cells and to consume nutrients (see Methods and Table S1). As time proceeded, the state of the internal network changed as a response to cell-to-cell interactions and local concentrations of the nutrients and signals. Because the model implemented incorporated the possibility that different cells were exposed to different conditions, the initially homogeneous population segregated into different subpopulations, each one characterized by a different steady state of the MRN (**Figure 2a**) and levels of diffusible elements (**Figure 2b**). In the model, individual cells could reach one of four steady states, associated with cell fates, three of which were also recovered by the single-cell Boolean model previously reported, and correspond to VEG, SPO and PCD cells (**Arias Del Angel et al., 2018**). In the spatiotemporal model, however, an additional steady state was generated and reached by some cells in the population, which matched the ROD profile (ROD cells are characterized by low levels of both ASG and CSG diffusible elements) (**Figure 2a**). This steady state seemed to be generated as a consequence of relaxing the assumption of self-signaling considered in the previous Boolean model (**Arias Del Angel et al., 2018**).

Under a deterministic updating scheme, each attractor recovered in the Boolean model has its own well-defined and non-overlapping attractor basin (i.e., the set of initial conditions leading to

the attractor), and transitions between attractor basins were not observed in the absence of stochastic perturbations (**Álvarez-Buylla et al., 2008**). In the spatial model, the local conditions and cell-to-cell interactions to which a cell was exposed enabled them to transit from one attractor to another, causing the proportions of cell fates to vary over time (**Figure 2c**). These transitions were not random, but rather follow an ordered sequence constrained by the dynamics of the internal MRN. Cells remained in the VEG state as long as they had access to nutrients; once nutrients were exhausted, the population rapidly differentiated into ROD cells. ROD cells could remain in this state or differentiate into either SPO or PCD cells (**Figure 2a,b**). For cell populations with low NUT, the concentration of A- and C-signals defined a two-dimensional space in which ROD, PCD and SPO can be mapped (**Figure 2b**). In this space, ROD cells were defined by low levels of both A- and C-signals. PCD cells had high levels of A-signal and low levels of C-signal. Finally, SPO cells were defined by high levels of both A-signal and C-signal.

Despite of the stochastic component of the GGH models, the results obtained by the model followed a general trend with low variation across repetitions, indicating that the mechanisms included in the model are sufficient to account for a robust process (**Figure 2c**). This trend held for populations of different sizes above a minimum lattice size of 500x500 (**Figure S2**).

4.2. Differentiation and patterning are dependent on the aggregate size

Cell fate determination in individual cells within an aggregate did not occur at random, but rather depended on the local context of each cell, mainly on the interaction with neighboring cells. Moreover, cell differentiation never occurred below a critical aggregate size of ~50 cells (**Figure 3a**).

Since these transitions between cell fates depended on both the depletion of nutrients and the accumulation of A- and C-signals, large cellular aggregates were more likely to reach relatively high concentrations of these signals and locally accumulate them (**Figure 3a**). Small aggregates (and individual cells) accumulated these signals more slowly because a fraction of the signal diffused away into the medium (**Figure 3a and 3c**). Depletion of nutrients and accumulation of A-signal and C-signal did not occur homogeneously within a single aggregate. Also, nutrient and A-signal levels became more heterogeneous among cells as aggregate size increased (**Figures 3c and 3e**).

Within the aggregates, cells at the periphery were more likely to accumulate high levels of nutrients but low levels of A-signal, while the opposite pattern was observed for cells at the interior of the aggregates (**Figure 4**). Since local concentration of the nutrients and signals biased the state of the internal network, their gradients might generate positional information that trigger cell fate determination. The emerging cell fates displayed a preferential position relative to the center of mass of the aggregates and to the cell fates in their neighborhood (**Figure 5**). This cell fate patterning, as well as the change in proportion of cell fate subpopulations over time (**Figure 1c**), was robust to the specific numerical values of key parameters included in the model. Nevertheless, cell fate determination, proportion and patterning were differentially sensitive to specific parameters (**Figure S3 and S4**). For instance, variations in the diffusion rate of the A- and C- signals seemed to affect the cell fate proportions, but not the presence of all four cell fates.

4.3. Balance between cohesion and adhesion determine aggregate size distribution

The aggregate size distribution was the result of both the strength of cell-to-medium (adhesion) and cell-to-cell (cohesion) affinities. This balance is known as wettability and can be specified at the cellular level through the Potts formalism (Swat et al., 2012). When cohesion was stronger than adhesion, cells tended to form larger aggregates, and vice versa. Different values of wettability resulted in qualitatively different distributions of the aggregate sizes, with a non-linear response to changes in either adhesion or cohesion (**Figure 6**).

4.4. Contrasting scenarios for the molecular nature of the C-signal

Once we were confident that the model could reproduce the key features of cell fate determination and patterning in *M. xanthus*, we employed it to test the plausibility of two contrasting scenarios. C-signal was initially suggested to be a membrane protein mediating direct cell-to-cell communication (**Lobedanz and Løtte-Soggard**), but recent evidence proposes that it is (or gives rise to) a diffusible molecule involved in indirect cell-to-cell communication. We developed two versions of the model that capture these two alternative scenarios. We found that while both versions of the model were capable of recovering most of the tested properties, only the model considering C-signal as a diffusible molecule was able to

recover the appearance of isolated spores (i.e., outside of multicellular aggregates) that has been previously reported (**Higgs et al., 2014**) (**Table 1)**.

5. Discussion

Multicellular development is characterized by cell differentiation and patterning. For *Myxococcus xanthus*, our study system, a previous network model grounded on experimental evidence, accounts only for single-cell processes (**Arias Del Angel et al., 2018**), and it does not capture events at the population or multicellular scales at which multicellular development and spatial patterning occur. In this work, we present results of a dynamic multiscale model based on the Cellular Potts formalism to explicitly consider cell movement, gene regulatory interactions and intercellular communication among individual cells during fruiting body formation in *M. xanthus*. Specifically, our results support a dynamic accounting for the origin of cell fates in the transition to multicellularity, in which coexisting cell fates may arise with the aggregation of cells from the coupling of multistable networks already present in single cells (**Kauffman, 1969; Furusawa and Kaneko, 2002; Newman et al., 2003; Mora van Cauwelaert et al., 2015**). This contrasts with a vision in which multicellular masses appear first, and then gradually acquire different cell fates and spatial differentiation **(Haeckel, 1874; Arendt 2008)**.

When the model is employed to simulate fruiting body development in response to starvation, individual cells aggregate, and the population transits from the state representing vegetative growth to those representing the cell fates that emerge during fruiting body. Even when the population consists of initially homogeneous cells, the differentiated local environment inside the aggregates allow them to reach different steady states corresponding to cell fates. It is worth mentioning that in this spatiotemporal model, an additional steady state representing the peripheral rods, emerged as the result of the interaction between cells and their medium. An outcome of the model is that it predicts the stereotypic sequence of VEG -> ROD -> SPO or PCD cell fate determination that emerges from the heterogeneous accumulation of nutrients and signals and the MRN dynamic (**Figure 2**). These results suggest that during multicellular development in *M. xanthus*, the coupling among cells may be important in generating the whole spectrum of cell fates. Given the genetic homogeneity of the cell population, this supports the idea that cellular differentiation can occur in a multicellular context in the absence of genetic variation (**Turing 1952; Von Dassow et al., 2001; Benítez et al., 2008; Mora van Cauwelaert et al., 2015**). Hence, the change of scale during aggregation may on its own constitute an

important source of developmental innovation, and changes in the cell-to-cell interactions may render developmental variation.

In addition to cell fate determination, positional information is also generated as a consequence of the formation of gradients of nutrients, A- and C-signals, which translate into cellular patterning with non-random preferential positions independent of any pre-pattern. The cell pattern exhibited qualitative robustness against the specific parameter values employed in the model. The position of SPO relative to ROD recovered by the model is in agreement with experimental evidence that has shown that fruiting bodies are patterned into two concentric domains, with myxospores preferentially located in the inner domain and peripheral rods in the outer domain (**Sager & Kaiser, 1993a, b; Julien et al., 2000**). To the best of our knowledge, the position of PCD cells has not been clearly determined experimentally, but evidence from **Lux and co-workers (2004)** suggests that they occur broadly over the entire fruiting body. Our model suggests that PCD tend to occur in an intermediate ring (**Figure 4**). Because of the limited evidence, this result provides a prediction for future experimental work.

The proposed model suggests that a threshold aggregate size must be surpassed to trigger cell fate determination and patterning. In relatively small aggregates, cells are unable to reach high enough levels of diffusible substances (A- and C-signal) because a significant portion of these signals is lost to the medium through diffusion. In large aggregates, self-activation feedback loops compensate for the proportion of the signals lost to cell-to-medium diffusion and intracellular levels of these signals reach high enough values to trigger downstream effects in the MRNs. In fact, previous studies have considered conglomerate size as a key factor favoring and constraining complexity during development and evolution of multicellular organisms (**Bonner, 1998a**). Moreover, other authors have argued that as cells are incorporated into a conglomerate, new local chemical and mechanical microenvironments may give rise to cues biasing cells toward certain steady states (**Furusawa & Kaneko, 2002**). Overall, our results support the idea that aggregate size may be a cue for development on the basis of a data-grounded model for *M. xanthus* as a model organism.

As previously mentioned, the nature of C-signal has been recently discussed in light of new experimental research. Specifically, the C-signal was originally suggested to be a membrane protein capable of mediating direct intercellular communication (**Lobedanz and Søggard-Anderson, 2003**). In contrast, recent evidence supports an alternative scenario where the C-

signal is instead a diffusible molecule (or perhaps involved in the synthesis of such a molecule), mediating indirect cell-to-cell communication (**Muñoz Dorado et al., 2016**). Here, we employed the dynamic model to test the plausibility of these two contrasting scenarios and found more support for the scenario of a diffusible signal mediating indirect cell-to-cell communication. The proposal of a diffusible element is also supported by bioinformatic analyses suggesting that *csgA*, the gene encoding for C-signal, does not contain any putative membrane-anchoring sequence (**Lee et al., 1995**).

Additionally, our model provides insights into the role of MazF, the only reporter marker for PCD. Evidence for the role of MazF has been controversial because its effect has been validated only in a strain where its action might be mediated by interactions with the specific genetic background (**Boynton et al., 2013; Müller et al., 2013**). (**Nariya & Inouye, 2008; Lee et al., 2012; Boynton et al., 2013**). We were aware of these limitations when we included this element in the model; we conclude that, regardless of the specific molecular identify of MazF, PCD-inducing factors acting in other strains may be under direct or indirect regulation of MrpC, as assumed in our model for MazF. Overall, the model as presented renders precise predictions that may ultimately inform experimental work.

In summary, we provide a dynamic accounting of cell fate determination and patterning in *M. xanthus*, which generates several testable predictions. As ongoing research reveals further details of the developmental mechanisms in other myxobacteria, models like the one proposed here may enable comparative studies of developmental processes and dynamics (**Nahmad et al., 2008; Arias Del Angel et al., 2017; Benítez et al., 2018**), thus shedding light on the generic and particular aspects of different fates and instances of multicellularity.

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8. Figures

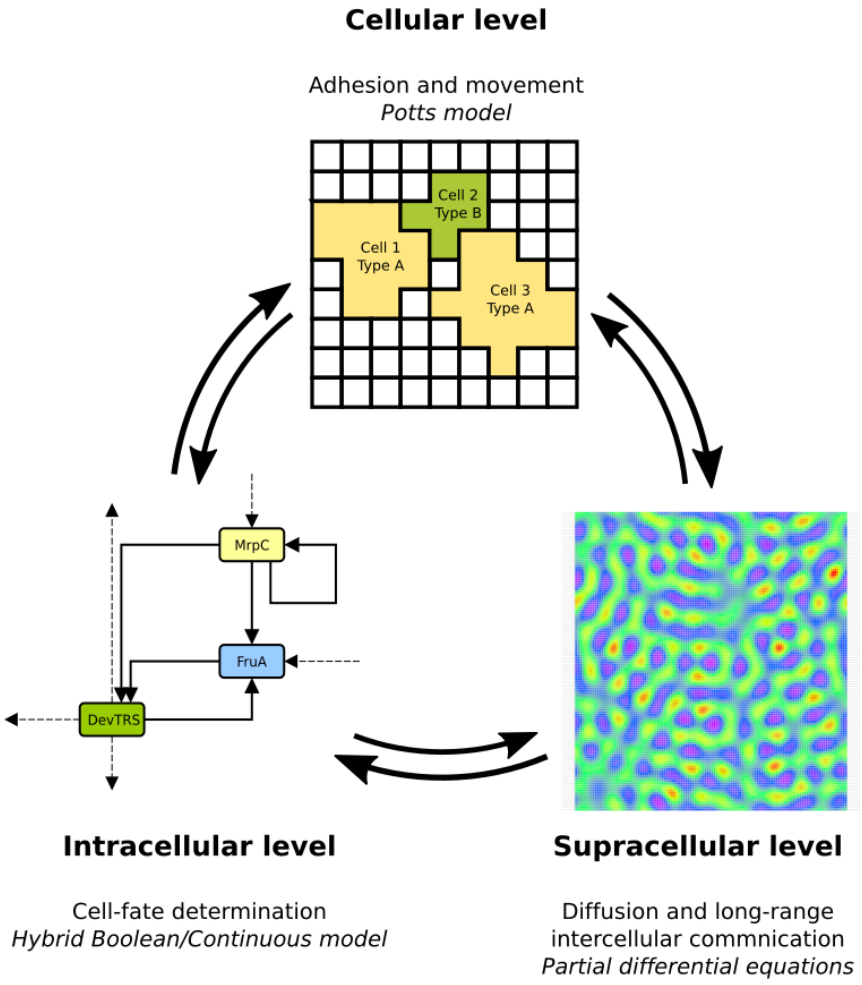

Figure 1. Schematic representation of the Cellular Potts model for cell fate patterning during multicellular development in *M. xanthus*. A Cellular Potts model considering phenomena at the cellular, intracellular and supracellular level is presented. Each level (bold font) captures characteristic phenomena through a different modeling formalism (italic font) and feedback with each other.

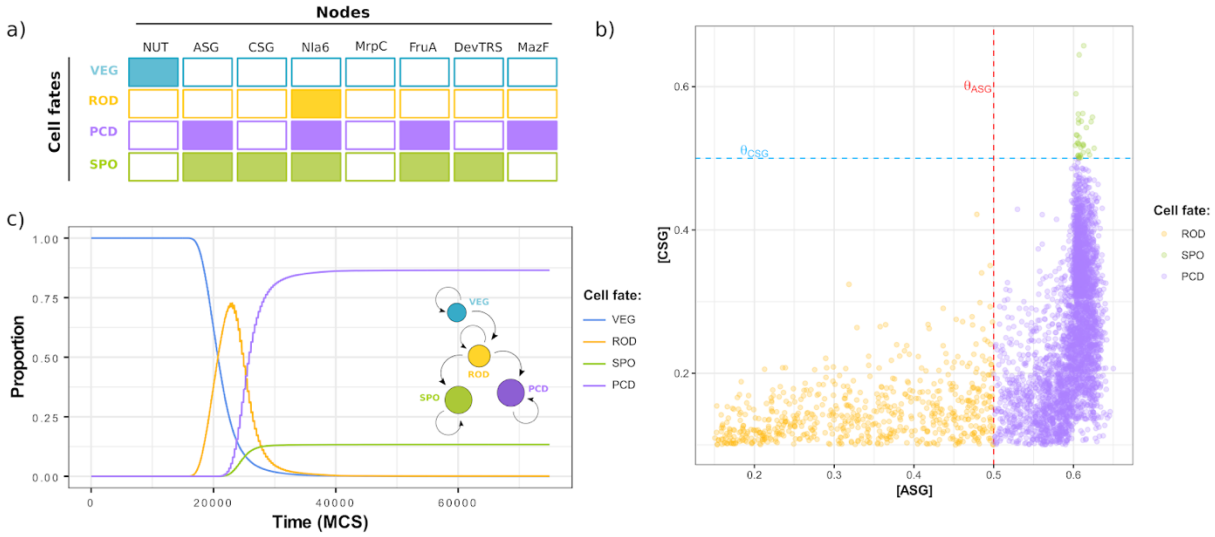

Figure 2. Dynamics for cell fate determination across a population of *M. xanthus* virtual cells. Individual cells in the population reach one of four attractors which are interpreted as cell fates. Cell fates are defined by the state of the internal network **(a)** and relative values of variables representing intercellular communication pathways **(b).** Panel **(a)** shows a subset of the nodes considered in the GRN, which are diagnostic variables that specify cell fates. In **(b)**, dotted lines represent threshold values for ASG and CSG activation. **(c)** Cell fate proportions change over time as a consequence of cell-to-cell interactions. A schematic diagram of the transitions between cell fates is shown inset in the plot (c).

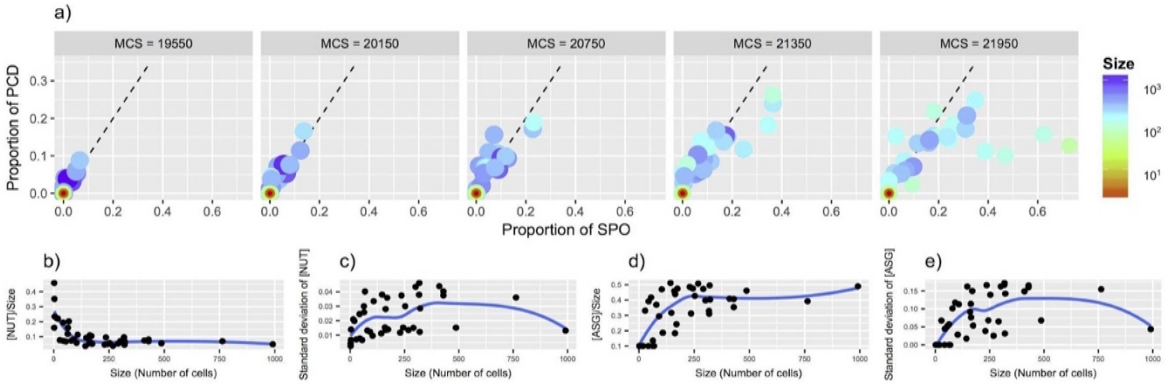

Figure 3. Correlation between cell fate determination and aggregate size. (a) Change over time (increasing MCS in panels from left to right) of SPO and PCD proportions at different aggregate sizes. Dotted lines represent equal proportion of SPO and PCD. Colors represent the aggregate size (measured as number of cells in the aggregate). **(b)** Change in the expected level of NUT (nutrient) per cell as a function of aggregate size. **(c)** Change of the standard deviation in NUT (nutrient) levels across the cells inside a single aggregate as a function of aggregate size. **(d)** Change of the expected level of ASG (A-signal) per cell as function of aggregate size. **(e)** Change of the standard deviation in ASG levels across the cells inside a single aggregate as function of aggregate size. In **(b-e)** the blue line represents a polynomial function adjusted to describe the general behaviour of the data.

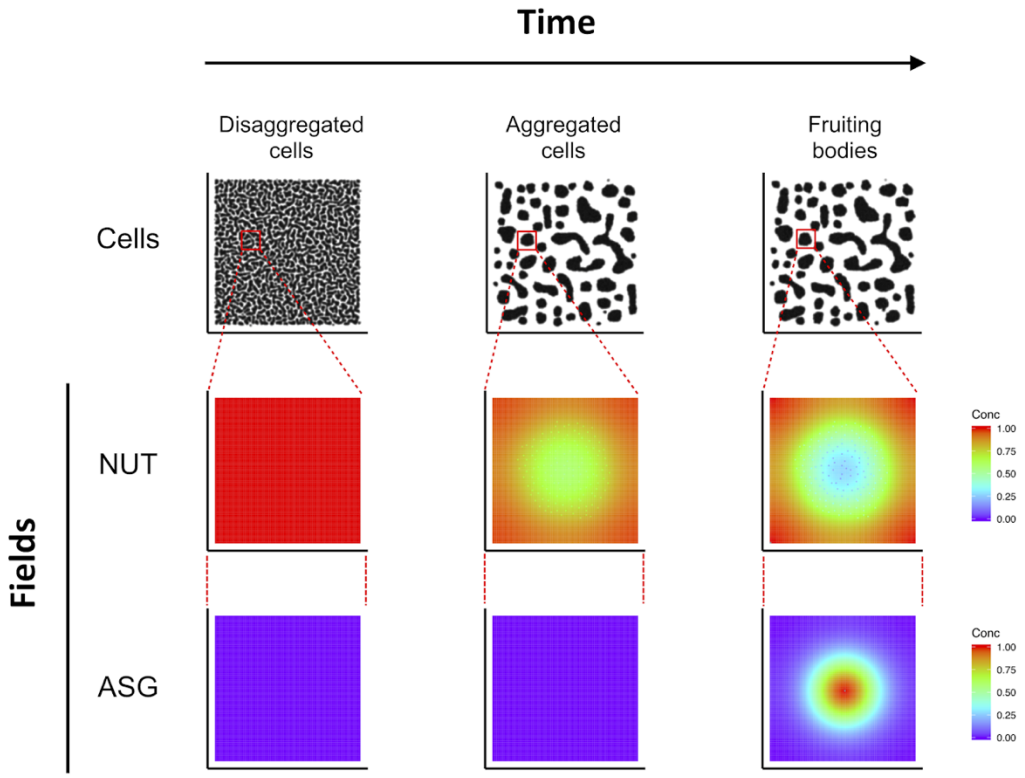

Figure 4. Spatial dynamics recovered by the model for *M. xanthus* FB development. Each row represents a different level and each column a representative time point for the main events recovered by the model (movement of disaggregated cells, aggregation of the individual cells and differentiated fruiting bodies). For the NUT (nutrient) and ASG (A-signal) gradients, only the portions delimited by the red circle in the cellular field are shown and amplified.

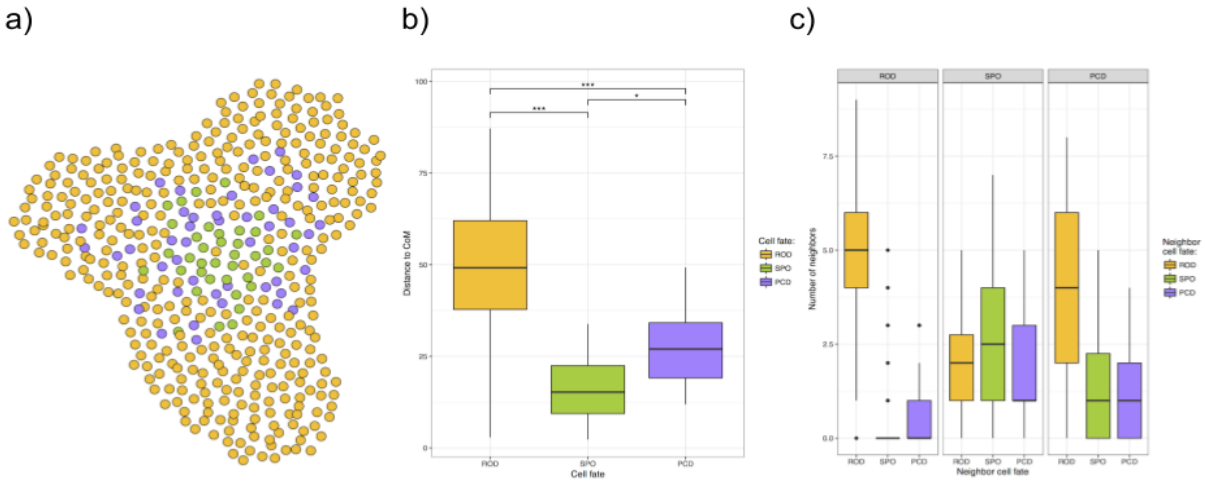

Figure 5. Spatial patterning of cell fates within aggregates. (a) Illustrative figure of the spatial distribution of cell fates in a virtual aggregate. Only aggregates of ≥100 cells were included. Cells are displayed as circles representing the position of the center of mass for each cell. **(b)** Distribution of distance of cell fates from the center of mass (CoM) of each aggregate. ANOVA p-values: (*) $p < 0.05$, (***) $p < 0.001$ **(c)** Distribution of cell fate of the neighbors of each of the cell fates observed for each aggregate.

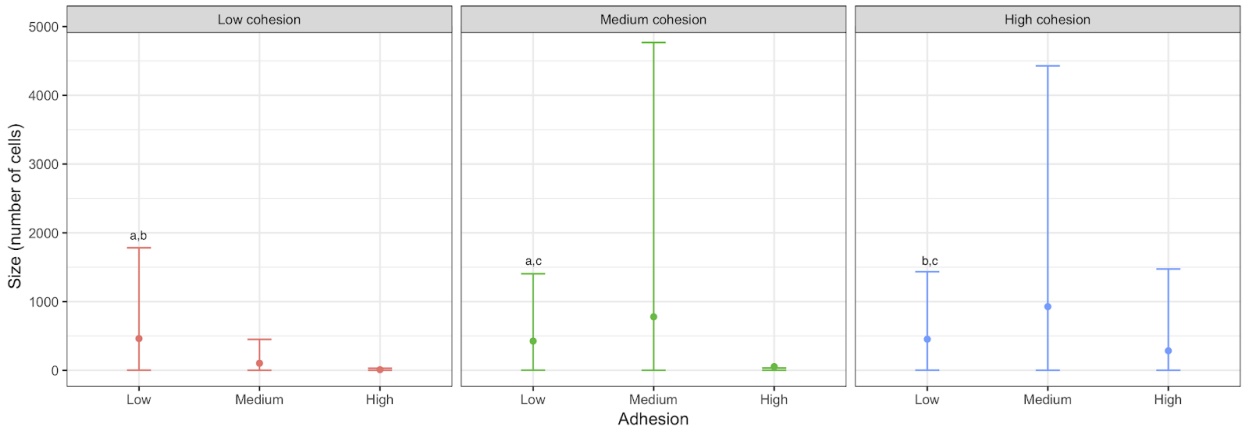

Figure 6. Theoretical effect of the balance between cell-to-medium (adhesion) and cell-to-cell (cohesion) contact energy in aggregate size. For each combination of adhesion and cohesion energy contact, the mean aggregate size (point) and standard deviation (error bars) are shown. Aggregate sizes were measured once the model reached equilibrium. Kolmogrov-Smirnov test to compare distributions; Indexes a, b, and c indicate $p > 0.05$ for pair-wise comparisons. Only non-significantly different pairs of distributions are indicated. *p-values* are adjusted using Holm-Bonferroni method for multiple comparisons.

9. Tables

Table 1. Comparison between alternative scenarios of CsgA-mediated intercellular signaling using the two models involving CsgA key properties.

Property	Model 1. Csg-direct	Model 2. Csg-indirect
Description	CsgA acts as an intercellular protein via direct cell-to-cell contact (Løtte-Soggard *et al*., 2001)	CsgA diffuses in the medium and mediates short-range intercellular communication (Muñoz-Dorado 2016; Robeltzki *et al.*, 2008)
Cell fate trajectories	Matches experimental evidence	Matches experimental evidence
Predicted cell fate proportions	SPO < ROD < PCD	SPO < ROD < PCD
Mutant phenotypes	Matches experimental evidence	Matches experimental evidence
Isolated myxospore formation (independent of FB development)	**Not predicted by the model**	**Predicted by the model, in agreement with previous reports (Yang and Higgs, 2014)**

10. Supplementary Figures

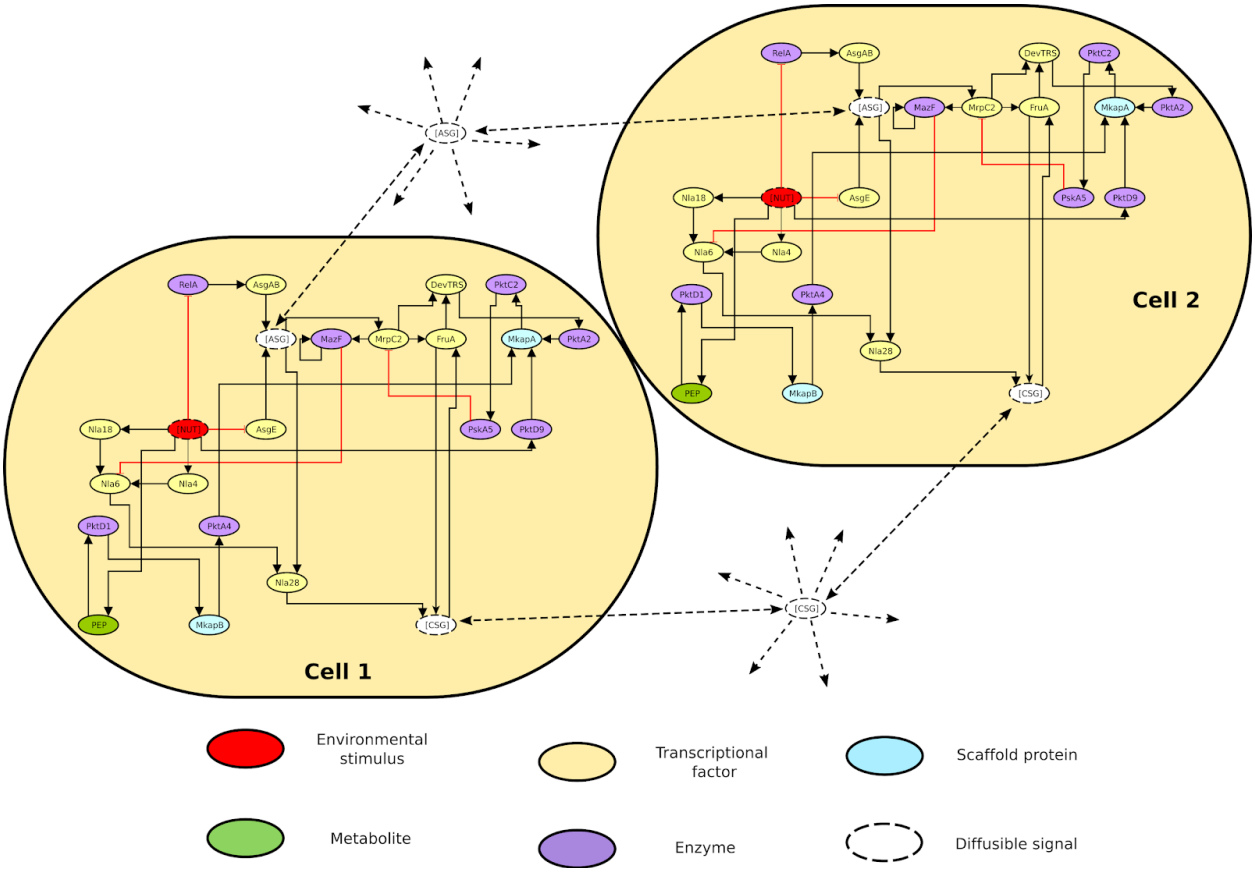

Figure S1. Schematic representation of the internal molecular regulatory network. Two cells are represented (each containing an identical schematic of the molecular regulatory network). Solid lines represent intracellular regulatory interactions. Black and red arrows stand for positive and negative regulatory interactions, respectively. Dotted lines indicates diffusion. Nutrients, A-signal and C-signal are abbreviated as NUT, ASG and CSG, respectively. All other nodes are annotated as usually found in literature.

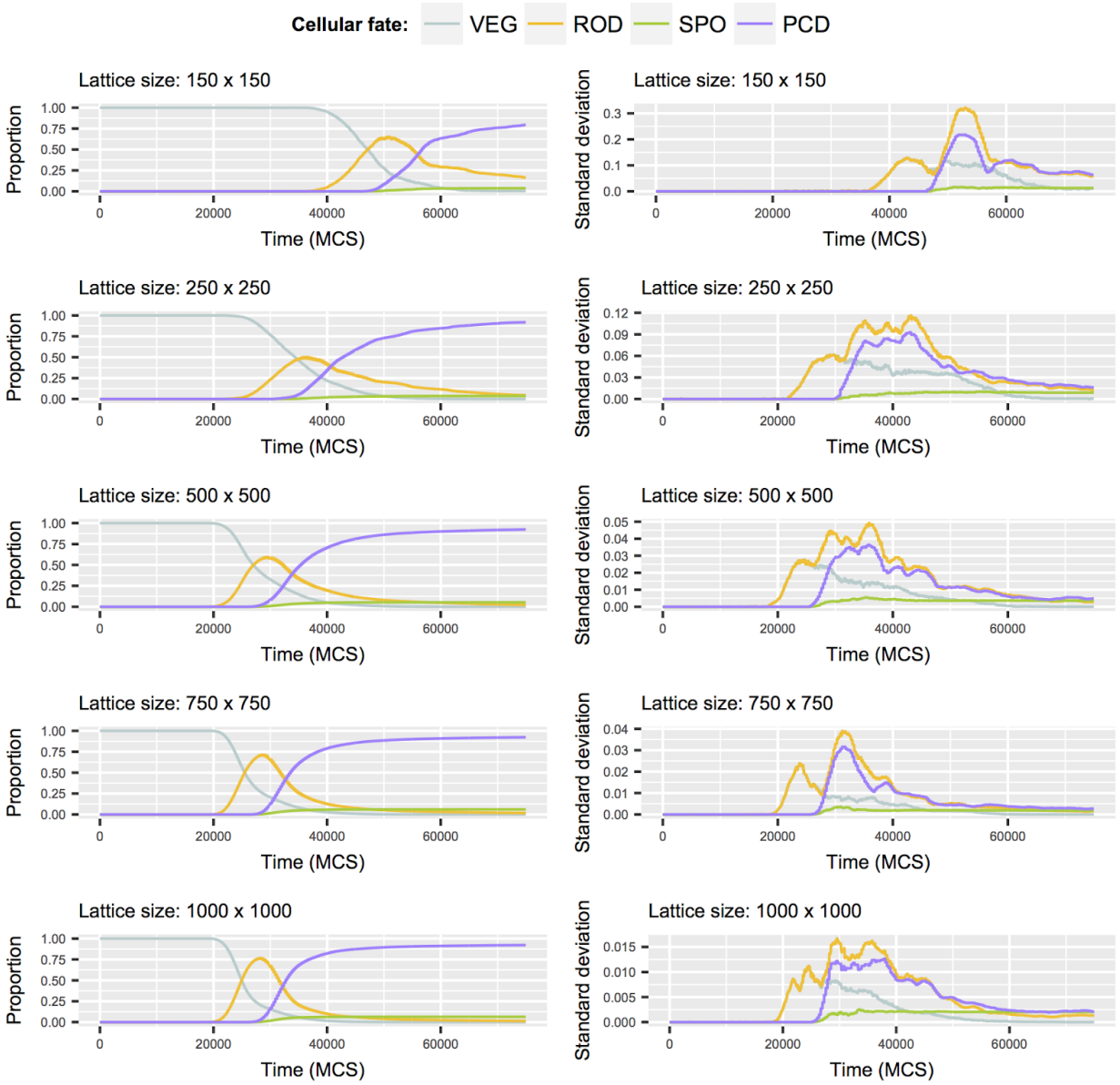

Figure S2. Robustness of system dynamics for large lattice sizes. Each row represents the results obtained for the cell fate trajectory over time when different lattices sizes are used. The left column shows the cell fate trajectories over time (line shows mean of N = 30 simulations). The right column shows the standard deviation for cell fate trajectories obtained from N = 30 simulations. Note that for small lattice sizes, trajectories are delayed and noisier (larger standard deviation) compared to larger lattices sizes.

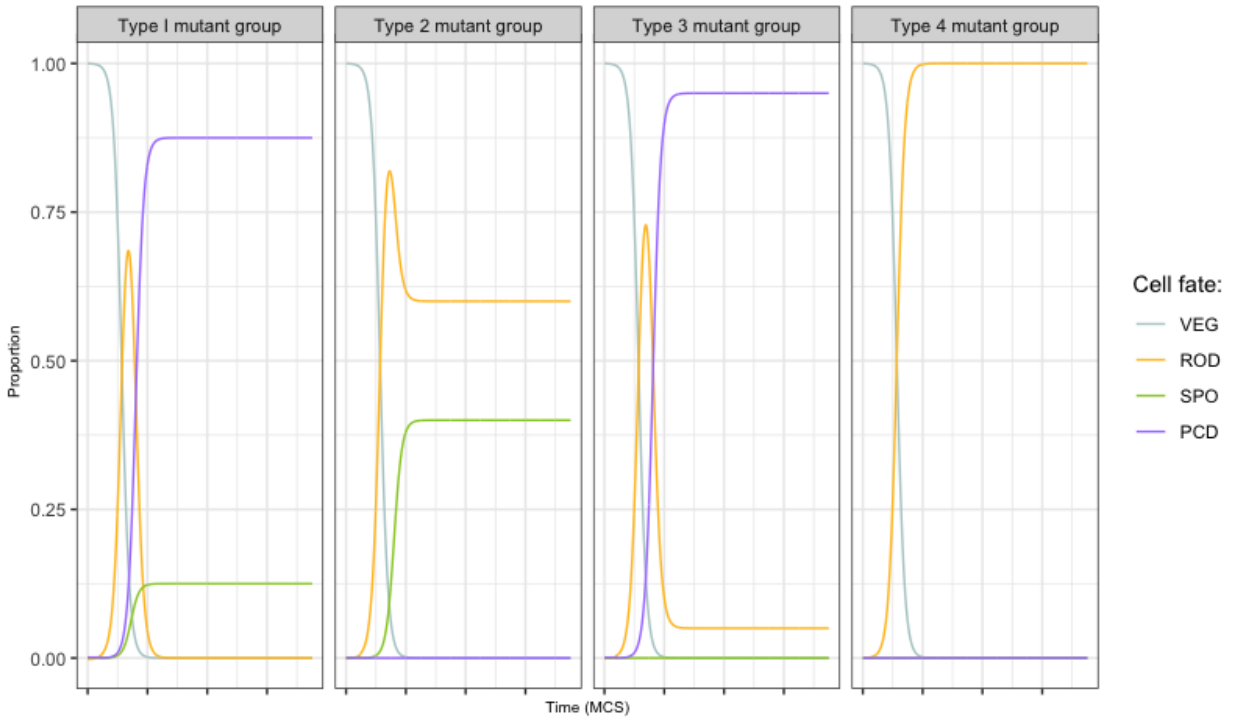

Figure S3. Effect of *in silico* knock-out mutants on cell fate trajectories. In each panel, the change of cell fate proportion over time is shown for a different mutant group. A mutant group comprises variables in the model (gene or proteins) that exhibit similar behaviour. No effect (Type 1), depletion of PCD (Type 2), depletion of SPO (Type 3), depletion of both PCD and SPO (Type 4). For variables contained in each mutant group see **Table S3**.

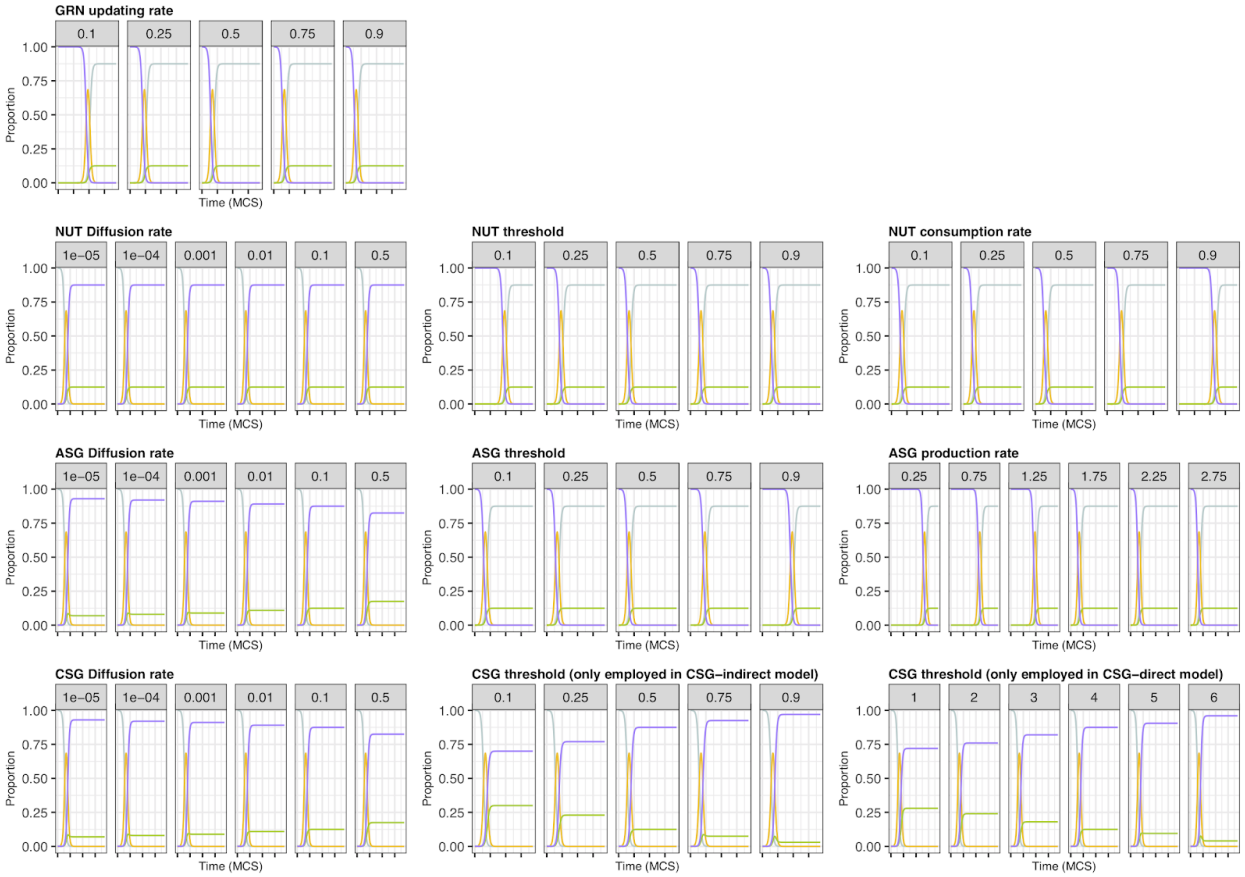

Figure S4. Sensitivity analysis for cell fate trajectories to parameter variation over time. Each panel shows cell fate trajectories over time upon variation in a single parameter. The modified parameter is annotated at the top of each panel (bold) and the specific value for each set of realization are shown in each sub-panel (gray boxes). Colored lines represent the proportion of individuals with each cell fates over time. Cell fates are VEG (cyan), ROD (yellow), SPO (green) and PCD (purple).

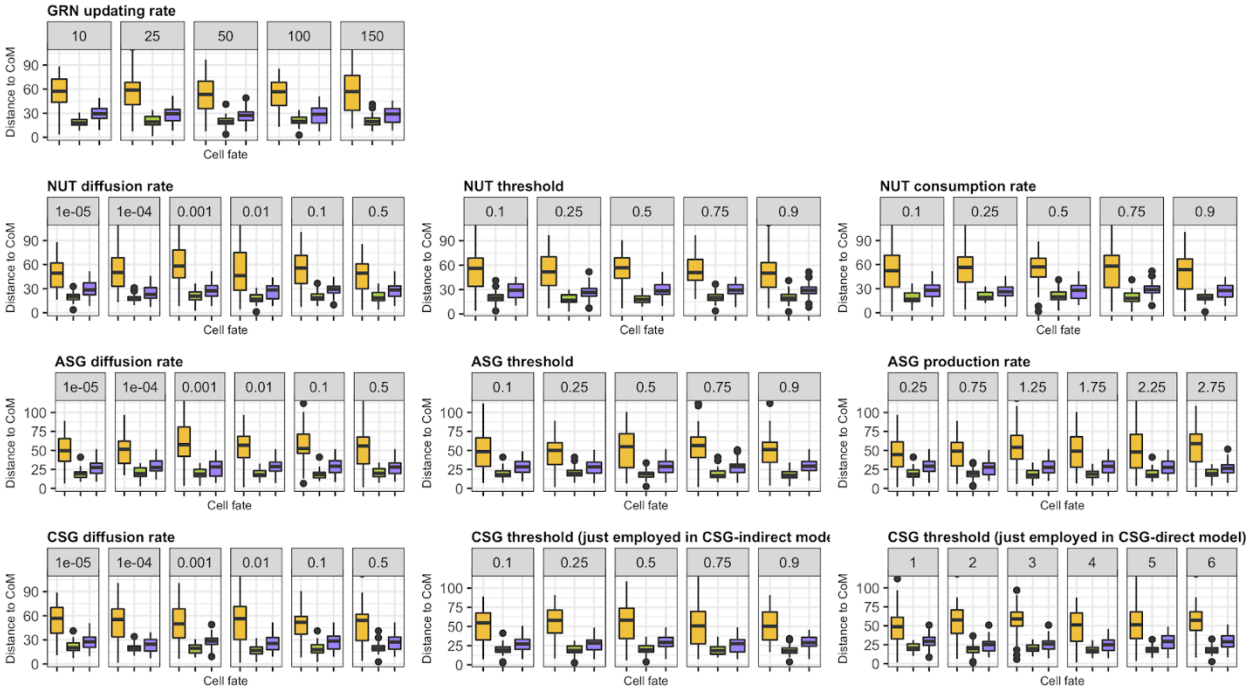

Figure S5. Sensitivity analysis of cell fate patterning to parameter variation. Each panel shows the distribution of distance relative to center of mass (CoM) for each cell fate upon variation in a single parameter. The modified parameter is annotated at the top of each panel (bold) and the specific value for each set of realizations are shown in each sub-panel (gray boxes). Boxplots represent the distribution of distance relative to center of mass for individual cell fates. Cell fates are ROD (yellow), SPO (green) and PCD (purple).

11. Supplementary Information 1

Specifications for the hybrid Boolean/ODEs GRN model.

S1a … $d[NUT]_i/dt = -NUT[NUT]_i + DNUT\nabla[NUT]_i$

S1b … $d[ASG]_i/dt = rASG[ASG_i](1 - ASG_i) + DASG\,\nabla[ASG]_i$, if *ASGAB(t) ∧ ASGE(t)*

$= -rASG[ASG_i]^{\wedge 2} + DASG\,\nabla[ASG]_i$, otherwise

S1c … $RELA_i(t + \tau) = [NUT]_i < \theta_{NUT}$

S1d … $ASGAB_i(t + \tau) = RELA_i(t)$

S1e … $ASGE_i(t + \tau) = \neg\, RELA_i(t) \wedge ASGAB_i(t)$

S1f … $NLA4_i(t + \tau) = RELA_i(t)$

S1g … $NLA18_i(t + \tau) = RELA_i(t)$

S1h … $NLA6_i(t + \tau) = \neg\, MAZF_i(t) \wedge (NLA4_i(t) \vee NLA18_i(t))$

S1i … $NLA28_i(t + \tau) = NLA6_i(t) \wedge ([ASG]_i < \theta_{ASG})$

S1j … $CSGA_i(t + \tau) = NLA28_i(t)$

S1k … $PKTD9_i(t + \tau) = [NUT]_i < \theta_{NUT}$

S1l … $PEP_i(t + \tau) = [NUT]_i < \theta_{NUT}$

S1m … $PKTD1_i(t + \tau) = PEP_i(t)$

S1n … $MKAPB_i(t + \tau) = PKTD1(t)$

S1o … $PKTA4_i(t + \tau) = MKAPB_i(t)$

S1p … $MKAPA_i(t + \tau) = PKTD9_i(t) \vee PKTA2_i(t) \vee PKTA4_i(t)$

S1q … $PKTA2_i(t + \tau) = DEVTRS_i(t)$

S1r … $PKTC2_i(t + \tau) = MKAPA_i(t)$

S1s … $PSKA5_i(t + \tau) = PKTC2_i(t)$

S1t … $MRPC2_i(t + \tau) = ([ASG]_i < \theta_{ASG}) \wedge \neg\, PSKA5_i(t) \wedge \neg\, MAZF_i(t)$

S1u … $FRUA_i(t + \tau) = MRPC2_i(t) \wedge (\theta_i \geq \theta_{CSG})$

S1v … $DEVTRS_i(t + \tau) = MRPC2_i(t) \vee FRUA_i(t)$

S1w … $MAZF_i(t + \tau) = ([NUT]_i < \theta_{NUT}) \wedge \neg DEVTRS_i(t) \wedge (MRPC2_i(t) \vee MAZF_i(t))$

In equation S1u, θ_i is the number of neighbors to cell *i* with CSGAj(t) = 1.

12. Supplementary Tables

Table S1. Model parameters. All parameters are measured in arbitrary units unless stated otherwise.

Symbol	Description	Nominal value	Values tested	Eq.
Potts models - *Cellular level*				
J(0,1)	Cell-to-medium adhesion energy	12.0	-	1
J(1,1)	Cell-to-cell adhesion energy	7.0	-	1
$V_0(\sigma)$	Target volume	25.0	-	1
ƛ	Spring constant	2.0	-	1
T	Temperature	10.0	-	2
Boolean/ODEs GRN model - *Sub-cellular level*				
τ	GRN updating rate	50 MCS	5, 10, 25, 50, 100	S1a-u
r_{ASG}	A-signal production rate	1.75	0.1, 0.25, 0.5, 1.0,	S1b
γ_{NUT}	Nutrient consumption rate	0.5	0.1, 0.25, 0.75, 0.9	S1a
θ_{NUT}	Nutrient threshold for starvation	0.1	0.2, 0.25, 0.5, 0.75	S1c,k,l
θ_{ASG}	A-signal activation threshold	0.5	0.1, 0.25, 0.75, 0.9	S1i,t
θ_{CSG}	Active neighbors threshold	5 cells	1, 2, ..., 6	S1u

Partial differential equations - *Chemical field level*				
D_{ASG}	A-signal diffusion rate	0.5	10^{-5}, 10^{-4}, …, 10^{-2}, 0.1	S1b
D_{NUT}	Nutrient diffusion rate	0.1	10^{-5}, 10^{-4}, …, 10^{-2}, 0.5	S1a
ρ	Nutrients released by dead cells	10.0	0, 1, 5, 20, 100	3

Table S2. Summary of key parameters and their effects on properties in the model: 'Yes' means that the given property is sensitive to the parameter variation and 'No' that it is not.

Parameter	Property		
	Temporality	**Cell fate proportion**	**Cell fate patterning**
GRN updating rate	No	Yes	No
ASG diffusion rate	No	Yes	No
NUT diffusion rate	No	Yes	No
CSG diffusion rate	No	Yes	No
CSG threshold (direct model)	No	Yes	No
CSG threshold (indirect model)	No	Yes	No
ASG production rate	Yes	No	No
NUT consumption rate	Yes	No	No

NUT threshold	Yes	No	No
ASG threshold	Yes	No	No

Table S3. Classification of nodes included in the gene regulatory network by their 'knock-out' phenotypic effects.

Mutant group	Phenotype	'Knock-out' nodes
Type 1	Wild-type	PktA2, PktA4, PktD1, PskA5, PktC2, MkapA, MkapB
Type 2	No PCD	MazF
Type 3	No SPO	DevTRS, FruA, Nla4, Nla18, Nla6, Nla28, CsgA
Type 4	Neither PCD nor SPO	AsgAB, AsgE, MrpĆ2

Discusión general

El linaje de las myxobacterias ha emergido como un sistema modelo para el estudio del desarrollo multicelular y su evolución (Arias Del Angel et al., 2017). En contraste con las plantas y los animales, que se desarrollan mediante un mecanismo de división clonal, las myxobacteria lo hacen mediante la agregación de células individuales en respuesta a la falta de nutrientes. Las implicaciones de este mecanismo en la evolución del desarrollo continúan por ser definidas, pero se ha sugerido que la agregación limita la evolución de la complejidad en este tipo de organismos (medida en número de tipo celulares y diversidad de tipos celulares; Grosberg & Strathmann, 2007). En este contexto, el estudio de diferentes linajes de organismos multicelulares y modos de desarrollo son relevantes para profundizar nuestro entendimiento sobre la evolución de la diversidad y complejidad en sistemas biológicos.

El presente trabajo busca aportar al entendimiento del desarrollo multicelular de myxobacteria. Aquí, mediante una estrategia de modelos dinámicos y su implementación computacional, se integra la evidencia experimental respecto a los componentes, y la interacciones entre estos, asociados al desarrollo multicelular de *Myxococcus xanthus*, la especie más extensamente estudiada de este linaje. Las estrategias de modelado dinámico han sido enriquecedoras en el estudio de otros sistemas biológicos, reales o hipotéticos, donde han ayudado a elucidar los mecanismos que subyacen la emergencia de propiedades no lineales a partir de la interacción entre los componentes individuales (Turing 1952; Furusawa & Kaneko, 2002; Benítez et al., 2008; Mora Van Cauwelaert et al., 2015; Varahaan et al., 2019). La estrategia de modelado dinámico tomada aquí provee un enfoque complementario respecto a los estudios bioinformáticos previamente realizados en myxobacteria y enfocados en el estudio de secuencias moleculares y de expresión génica en la escala genómico (Müller et al., 2010; Huntley et al., 2011, 2014; Muñoz-Dorado et al., 2019).

Motivado por el marco de la biología evolutiva del desarrollo presentado en el Capítulo 1 (Arias Del Angel et al., 2017), esta tesis presenta un estudio teórico para el estudio del desarrollo de *M. xanthus*. En el Capítulo 2 se presenta un estudio de como los componentes identificados para estar asociados al desarrollo integran una red multiestable que específica los diferentes destinos celulares observados durante el desarrollo de *M. xanthus* (Arias Del Angel et al., 2018). El modelo presentado en el capítulo 3 apunta a los mecanismos que subyacen la

capacidad de una población de células inicial homogéneamente para diferenciarse en los diferentes destinos celulares, así como permitir su coexistencia temporal dentro de los agregados multicelulares (Arias Del Angel et al., *en preparación*). En este modelo, el acoplamiento de la dinámica interna de las células individuales es crucial para esta diferenciación espacial pero también para la especificación de transiciones específicas entre los diferentes destinos celulares. Aunque una primera impresión pareciera indicar que estos modelos carecen de un componente evolutivo, se plantea que un entendimiento del desarrollo como proceso dinámico es crucial como punto de partida para la resolución de preguntas de interés evolutivo.

A pesar de lo anterior, los modelos desarrollados como parte de este proyecto arrojan ideas de interés dentro del marco de la biología evolutiva del desarrollo. Durante el estudio de los procesos de determinación de los destinos celulares presentados en el Capítulo 2, el modelo predice la capacidad del sistema para especificar un destino celular, los esferoplastos, que de acuerdo con la evidencia ocurre de manera independiente al desarrollo multicelular (Arias Del Angel et al., 2018). Experimentalmente, este tipo celular emerge en respuesta a otras condiciones ambientales tales como alta presión osmótica o la presencia de antibióticos (Müller et al., 2010). Este resultado sugiere que el conjunto de elementos regulatorios incluido en la red de regulación podría haber constituido un mecanismo mediando la respuesta a condiciones estresantes y haber sido cooptada durante la transición a la multicelularidad en el linaje de myxobacteria. Esto podría reforzar la hipótesis de que durante la transición hacia la multicelularidad no fue necesaria la aparición de nuevas familias génicas sino la reutilización de módulos regulatorias potencialmente presentes en el ancestro unicelular (True & Carroll, 2002) Posteriormente, en el trabajo presentado en el Capítulo 3, el modelo sugiere que el destino celular de células periféricas (ROD) emerge como una consecuencia del acoplamiento de la dinámica interna de las células individuales sin la necesidad de variación genética enotros (Arias Del Angel, *en revisión*). Esto es similar a lo observado en otros estudios, Turing, 1952; Furusawa & Kaneko, 2002; Benítez et al., 2008; Varahan et al., 2019). Esta observación contrasta con la idea de que el genoma de las células individuales contiene la capacidad intrínseca de especificar el espectro completo de tipos celulares observados en los organismos multicelulares, así como con estrategias empleando modelado matemático que buscan entender esta capacidad en el contexto de una sola célula (Kauffman, 1969; Espinosa-Soto et al., 2004; Azpeitia et al., 2013).

A pesar de la aparente utilidad de los modelos para estudiar el desarrollo de *M. xanthus*, su aplicación en preguntas concernientes al desarrollo y la evolución en otras especies de myxobacteria es debatible. Lo anterior debido al fenómeno de deriva de los sistemas de desarrollo abordado en el Capítulo 1, el cual se sugiere que subyace el patrón de conservación de los genes asociados al desarrollo en este linaje (Arias Del Angel et al., 2017). Considerando la baja conservación génica de los genes involucrados en el desarrollo, ¿cuál es la utilidad de un modelo planteado para *M. xanthus* en el estudio del resto de las especies? En la propuesta de la deriva de los sistemas de desarrollo, este fenómeno es facilitado por la robustez topológica y dinámica del sistema de regulación que permite su realambrado. Además, se sugiere las propiedades dinámicas asociadas a la determinación de los tipos celulares son conservadas incluso cuando la identidad de los componentes es modificada (True & Haag, 2001). En este sentido, la implementación de modelos dinámicos ha dado indicios de los mecanismos mediante los cuales ciertos comportamientos emergen de manera inherente debido a la conectividad entre los componentes y de manera relativamente independiente a la identidad específica de esto (Alon, 2007; Guzmán et al., 2019). Por ejemplo, las asas de retroalimentación positiva generan biestabilidad e histéresis. Por otro lado, las asas de retroalimentación negativa están involucradas en reducción de la variabilidad, en procesos de filtrado del ruido y generación de comportamientos oscilatorios. En resumen, la implementación de modelos dinámicos podría ayudar a identificar motivos, u otros procesos genéricos (ver Hernández-Hernández et al., 2012) asociados a ciertos comportamientos que son conservados a pesar del efecto de la deriva de los sistemas de desarrollo y que subyacen comportamientos similares a través de las especies incluso en ausencia de señales de homología reconocibles a nivel molecular.

Un enfoque basado en el análisis de procesos dinámicos (incluyendo el de motivos en redes), en lugar de entidades (genes), provee un marco para realizar estudios comparativos entre y dentro linajes que exhiban poca conservación a nivel de genes, tales como el de las myxobacteria, y que permita revelar aspectos generales y específicos que caractericen a los diferentes linajes de organismos multicelulares (Hernández-Hernández et al., 2012). Estos enfoques requieren replantear una visión dominante en la que este tipo de estudios se realizan de manera casi exclusiva a través del análisis de genes. Del mismo modo, estos análisis llaman a un trabajo altamente interdisciplinario que incluya los campos de biología, física, matemáticas y computación. A partir de este tipo de enfoques, y apoyado en herramientas de modelado matemático, las myxobacteria y otros microorganismos pueden proveer claves para el

entendimiento de los mecanismos que subyacen la evolución de la complejidad en organismos vivos y de los factores que facilitan la emergencia recurrente de paralelismos respecto a las morfologías dentro y entre linajes de organismos multicelulares agregativos. Finalmente, desde un punto de vista técnico, los modelos dinámicos representan una herramienta crucial para la continua integración de nueva evidencia empírica, así como en la generación de hipótesis que puedan dirigir de manera informada el trabajo experimental, generando de esta manera una retroalimentación entre este y el trabajo teórico.

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