Myxococcus xanthus induces actinorhodin overproduction and aerial mycelium formation by Streptomyces coelicolor

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Summary
Interaction of the predatory myxobacterium Myxococcus xanthus with the non-motile, antibiotic producer Streptomyces coelicolor was examined using a variety of experimental approaches. Myxococcus xanthus cells prey on S. coelicolor, forming streams of ordered cells that lyse the S. coelicolor hyphae in the contact area between the two colonies. The interaction increases actinorhodin production by S. coelicolor up to 20-fold and triggers aerial mycelium production. Other bacteria are also able to induce these processes in S. coelicolor though to a lesser extent. These studies offer new clues about the expression of genes that remain silent or are expressed at low level in axenic cultures and open the possibility of overproducing compounds of biotechnological interest by using potent inducers synthesized by other bacteria.

Introduction
Bacterial populations in natural habitats are complex communities containing many species that exhibit competition and/or collaboration in order to survive with limiting nutritional resources. The study of these interactions has attracted much interest (Shank and Kolter, 2009; Straight and Kolter, 2009; Vos and Velicer, 2009). Laboratory co-cultures sometimes trigger the expression of genes that remain silent in pure cultures (Yamanaka et al., 2005). The number of silent or poorly expressed genes under laboratory conditions may be much higher than originally thought (Schneiker et al., 2007). Perhaps in natural communities unknown signals trigger gene expression, sometimes in other organisms (Bassler and Losick, 2006).

In this work we have examined the confrontation between two typical soil inhabitants, immobile Streptomyces coelicolor and the mobile predator Myxococcus xanthus. Both bacteria have genomes in excess of 8 Mb and are endowed with the capacity to produce many secondary metabolites. Streptomyces coelicolor contains 23 gene clusters related to secondary metabolite production (Bentley et al., 2002). However, only four antibiotics have been detected under laboratory conditions, actinorhodin (ACT), undecylprodigiosin (RED) and calcium dependent antibiotic (CDA), synthesized by proteins encoded by the chromosome, and methylenomicin, whose biosynthetic enzymes are encoded by plasmid SCP1. ACT and RED are pigmented and their production is easily visualized. The genome sequence of M. xanthus DK1622 has revealed the presence of at least 18 clusters of polyketide/non-ribosomal peptide genes, most of which are not expressed under laboratory conditions (Wenzel and Muller, 2009). In fact, no antibiotics were identified in M. xanthus cultures until 2005 when the use of high-performance liquid chromatograph mass spectrometry (HPLC-MS) technology provided a sensitive method to identify five antibiotic families (Wenzel and Muller, 2009).

There are several groups of bacterial predators. Myxococcus xanthus has been extensively studied (Berleman and Kirby, 2009; Velicer and Mendes-Soares, 2009a) and moves on solid surfaces by two surface translocation mechanisms, the adventurous (A motility) and social (S motility) motility systems (Mauriello and Zusman, 2007). This myxobacterium preys on a wide variety of microorganisms by secreting lytic enzymes and toxic molecules (Velicer and Mendes-Soares, 2009a). It attacks in groups like a wolf pack by surrounding the prey (Velicer and...
Mendes-Soares, 2009a). Myxococcus is not a specialized predator, and can feed on a single species including *Escherichia coli*, *Corynebacterium glutamicum*, *Micrococcus luteus* and *Saccharomyces cerevisiae* (Hillesland et al., 2007; Berleman and Kirby, 2009). Other predators are specialists and only feed a single species (Velicer and Mendes-Soares, 2009a).

During *M. xanthus* S motility, cells glide in groups. This social behaviour has been more extensively studied during fruiting body development following nutrition depletion. Development culminates when the rod-shaped vegetative cells differentiate into metabolically quiescent, spherical spores that germinate when nutrients are supplied (Dworkin, 1996). Fruiting body development and predation of other organisms induce rippling behaviour during which the cells organize themselves in parallel ridges that move coordinately (Berleman et al., 2006). In addition, the social and multicellular behaviour of this bacterium requires intercellular communication (Kroos, 2007; Velicer and Vos, 2009b).

Streptomyces also has a complex developmental cycle that begins with the germination of a spore to form multigenomic substrate mycelia. Some mycelia erect aerial mycelia that generate unigenomic spores by transverse division of the tips (Chater, 1993; Flardh and Buttner, 2009). All this summarized process needs the action of a wide number of genes and signals among which the surfactant SapB and eight chaplins play an important role in the development of aerial mycelium (Capstick et al., 2007).

In nature, actinomycetes are very abundant and they contribute to the fertility of soil degrading organic material and interacting with other organisms that live free or form part of the rhizosphere (Mazzola, 2007; Tamilarasi et al., 2008; Chater et al., 2010). These interactions may trigger the induction of otherwise silent secondary metabolite pathways and they are starting to be described (Straight et al., 2007; Kurosawa et al., 2008; Schroechk et al., 2009).

In this article we show that *M. xanthus* induces antibiotic production and differentiation by *S. coelicolor*. These results reinforce the idea that examination of interactions between microorganisms can increase the production of secondary metabolites and/or lead to the discovery of new metabolic compounds.

**Results**

**Predation and competition between Myxococcus and Streptomyces**

The interaction between *Myxococcus* and *Streptomyces* was examined in co-culture on an agar surface. A lawn of *M. xanthus* DK1622 cells was inoculated on a CTT agar plate (3 x 10^7 cells per plate). One line of *S. coelicolor* M145 and another of *Streptomyces lividans* 1326 were streaked across the *M. xanthus* cells. The plates were incubated at 30°C for 5 days. Production of the blue antibiotic ACT was observed around the *S. lividans* (Sc) cells (Fig. 1A). However, ACT production by *S. coelicolor* M145 was very low in a control plate on which *S. coelicolor* M145 and *S. lividans* 1326 were inoculated alone (Fig. 1B).

Interactions between *M. xanthus* and *S. coelicolor* were examined in more detail by inoculating drops of each microorganism next to each other. Two drops of *Streptomyces* at the same distance were used as a control. Three *M. xanthus* strains were examined, the fully motile wild-type (wt) strain DK1622, the one with reduced S motility DZF1 strain and the non-motile mutant DK6204. Only the two motile strains moved towards *Streptomyces* (Fig. 1C). The mgl mutant (DK6204) was unable to migrate towards the *Streptomyces* but it induces coloured antibiotic production and aerial mycelium formation suggesting produc-
tion of a bioactive compound or lytic enzyme. More detailed visualization of predation can be observed in the movie attached as supplementary information.

M. xanthus induces formation of abnormal S. coelicolor hyphae

As shown in the movie, M. xanthus DK1622 cells moved towards S. coelicolor spores lysing them even from some distance. However, Streptomyces recovers coincident with spore germination suggesting that growing Streptomyces cells are partially resistant (see below). As the M. xanthus cells enter the S. coelicolor colony intense lysis of Streptomyces hyphae occurs. The use of the LIVE/DEAD BacLight kit demonstrates that control cultures of S. coelicolor without Myxococcus contained mainly living cells (Fig. 2A). However, the co-cultures were predominantly red indicating a high proportion of dead mycelia (Fig. 2A). Scanning electron microscopy showed that the cells at the distal edges of the Streptomyces and Myxococcus drops that did not have contact with each other looked healthy (Fig. 2B). Myxococcus cells in the interaction zone also appeared healthy having the normal bacillar shape whereas Streptomyces hyphae exhibited aberrant morphologies (Fig. 2B). The cell density for both bacterial species in this zone was lower than at the distal edges due to competition and predation.

M. xanthus induces antibiotic production and differentiation in S. coelicolor

In S. coelicolor colonies growing next to M. xanthus more ACT is produced in the interaction zone after 48–72 h of incubation than in distal regions of the spots or with the S. coelicolor only controls (Fig. 3). ACT production is upregulated by each of the three different M. xanthus strains used in this experiment suggesting that motility is not necessary (Fig. 1C). In addition, aerial mycelia containing grey spores are formed near the interaction zone (Figs 1C and 3). Aerial mycelia were induced by 48 h with each of the three M. xanthus strains suggesting that production is not dependent on movement or direct contact between organisms, as illustrated by non-motile mutant DK6204
(Fig. 1C). If anything, the non-motile mutant seems to stimulate more sporulation than wt M. xanthus cells (Fig. 1C).

Production of ACT and aerial mycelia is also stimulated by co-culture with other microorganisms (Table 1). Induction of ACT was observed in co-cultures with Bacillus megaterium, B. subtilis, B. thuringensis and Serratia, although to much lower extent than with M. xanthus. Some of these bacteria were also able to induce the aerial mycelium formation (Table 1). From organisms such as Klebsiella pneumoniae, which induces aerial mycelia but not ACT and B. megaterium, which induces ACT but not aerial mycelia it would appear that the two processes are not strictly coupled.

**ACT overproduction is induced in liquid co-culture of S. coelicolor and M. xanthus**

Stimulation of antibiotic production was also studied in CTT liquid cultures. ACT was clearly overproduced by S. coelicolor in co-culture with Myxococcus strains DK1622 or DZF1 (Fig. 4A). Colorimetric quantification of ACT indicated that the presence of either M. xanthus strain increased Streptomyces ACT production profusely. However, very low and similar levels of CDA were detected under the conditions assayed in the control or the co-cultures (data not shown).

Ultra high pressure liquid chromatography (UHPLC) allowed quantification of the extracted compounds produced in the control cultures, S. coelicolor (Fig. 4B) or M. xanthus DK1622 (Fig. 4F), and in the co-culture with both strains (Fig. 4D). Several compounds eluting in the region between 4.5 and 7 min in the Streptomyces control culture (Fig. 4B) were overproduced in the co-cultures (Fig. 4D), and absent in the M. xanthus DK1622 control (Fig. 4F). These peaks shared the same absorption spectrum, which corresponded to that of the ACT family. When optimized to 500 nm, a wavelength suited to detect ACT, the types of ACT molecules were similar between the S. coelicolor control and the co-culture, but the amounts were roughly 20-fold higher in the co-culture (Fig. 4C and E; notice the difference in the scale). These compounds

### Table 1. Induction of ACT and aerial mycelia by S. coelicolor in co-culture with different bacteria species.

| Bacteria                  | ACT  | AM  |
|---------------------------|------|-----|
| Myxococcus xanthus        | ++   | ++  |
| Bacillus laterosporus     | –    | –   |
| Bacillus licheniformis    | –    | –   |
| Bacillus megaterium       | ++   | +   |
| Bacillus subtilis         | +    | ++  |
| Bacillus thuringensis     | +    | ++  |
| Micrococcus sp.           | –    | –   |
| Mycobacterium phlei       | –    | –   |
| Mycobacterium smegmatis   | –    | –   |
| Staphylococcus aureus     | –    | –   |
| Escherichia coli          | –    | –   |
| Klebsiella pneumoniae     | –    | ++  |
| Proteus sp.               | –    | –   |
| Salmonella sp.            | –    | –   |
| Serratia sp.              | +    | ++  |

**a.** ACT indicates actinorhodin production.

**b.** AM indicates the development of aerial mycelia.

++ and + indicate actinorhodin production or aerial mycelia development after 48 and 72 h, respectively, and – indicates that actinorhodin or aerial mycelia were observed at the same time as the control which contains only S. coelicolor cells.
were not detected in the *M. xanthus* control culture (Fig. 4G). HPLC-MS analysis of the same peaks gave m/z[H⁺] values ranging from 631 to 666, as expected for ACT family members (data not shown). RED was not detected in the control culture or in the co-culture. These experiments confirm that *M. xanthus* increases production of ACT by *S. coelicolor*.

**S. coelicolor ACT biosynthesis mutants repel *M. xanthus* less effectively**

To determine whether ACT production provides an advantage to *S. coelicolor* strains against *M. xanthus* predation, mutants impaired in the production of RED (M510), ACT (M511) or both antibiotics (M512) were used. ACT was produced by strains M145 (wild type) and M510 (Fig. 5).

RED was detected in M145 and M511 (Fig. 5). Migration of *M. xanthus* DK1622 cells towards all the *Streptomyces* strains was observed. However, this migration was more evident with the *Streptomyces* strains that did not produce ACT (M511 and M512 strains). *Myxococcus xanthus* seemed to more aggressively attack strains lacking ACT (see the blue arrows in Fig. 5 taken after 200 h co-culture). This observation predicted that strain M512 would be more sensitive to *M. xanthus* attack. However, the general appearance of the *Streptomyces* colonies suggested that the three mutants were as resistant to *M. xanthus* predation as the wt strain.

To obtain a clearer understanding predation was quantified by dilution plating. *Myxococcus xanthus* DK1622 was co-cultured in liquid CTT medium with the *S. coelicolor* strains M145 or M512. After incubation for 3 days,
cells were diluted and inoculated onto R2YE plates (Myxococcus does not grow in this medium). The number of S. coelicolor colonies that survive co-culture with M. xanthus was similar for both strains, approximately one-fifth of the colonies obtained when the Streptomyces strains were grown in the absence of M. xanthus. This result demonstrates that the double antibiotic mutant has a similar level of resistance to Myxococcus predation.

Discussion

Genome sequencing efforts have revealed that, under laboratory conditions, microorganisms have a wide number of genes that remains silent. Recently co-culture of two microorganisms has been considered as a strategy to partially mimic natural communities that exchange chemical signals. This new approach has permitted the discovery of new capabilities that remained silent in axenic cultures. For example, previous work on Streptomycyes interactions has demonstrated alteration of its developmental program due to availability of new carbon sources during co-culture of S. lividans with yeasts (Santamaria et al., 2002). Bacillus subtilis production of a surfactant inhibits chaplin and SapB production, which are required for aerial mycelium formation and sporulation in S. coelicolor (Straight et al., 2006). Description of the interaction of Streptomycyes olivaceoviridis with Aspergillus proliferans via a protein targeting chitin on the fungus cell wall has been done (Siemieniewicz and Schrempf, 2007). Direct physical interaction between Streptomycyes hygroscopicus and the fungus Aspergillus nidulans is necessary for induction of polyketide synthesis. In this interaction the bacterium also triggers the production of lecanoric acid by the fungus, a metabolite that inhibits ATP synthesis and may be used in self-defence by the fungus (Schroeckh et al., 2009).

Myxobacteria and actinomycetes are normal inhabitants of soil where they act as scavengers to recycle cellular debris using hydrolytic enzymes. Both types of organisms produce molecules with antibiotic activity that may act in defence or as communication signals. Myxococcus xanthus, the model myxobacterium, behaves as an active predator, able to consume other microorganisms and even worms. To do so, myxobacterial cells detect potential preys and surround them to facilitate predation. Non-motile Streptomycyes, the most abundant actinomycete in soil, produces dense colonies of mycelia in which the position of cells in the colony determine the pattern of gene expression. Although Streptomycyes is not a motile predator, it lyses other organisms by secreting antibiotics and hydrolytic enzymes. In the soil, both Streptomycyes and Myxococcus coexist and there is evidence for horizontal gene transfer between these bacteria in the case of an endoglucanase (CelA) gene transferred from...
Streptomyces to Myxococcus ancestors (Quillet et al., 1995). Horizontal transfer between Gram-positive and Gram-negative bacteria is widely represented in other genomes and could originate with predation and lead to the incorporation of prey DNA in the genome of the predator.

Interspecies signalling has the potential to induce silent metabolic pathways or to obtain new hybrid compounds. Genome sequencing projects have revealed the capacity for horizontal transfer between Gram-positive and Gram-negative bacteria like Myxococcus because it is not able to enter in the cell bind DNA. However, S. coelicolor strains that produce ACT are not surrounded by *M. xanthus* in the same manner as those that do not synthesize this antibiotic. It remains possible that *S. coelicolor* uses ACT as a repellent signal for *Myxococcus*. In addition, antibiotic production in *S. coelicolor* by a signal produced by other bacteria might have important biotechnological applications to improve the yield of clinically relevant antibiotics.

Co-culture also stimulates aerial mycelium formation by *S. coelicolor* suggesting a chemical induction pathway. It could be argued that nutrient depletion by *M. xanthus* may lead to more rapid development of *S. coelicolor*. However, co-cultures of *M. xanthus* and *S. lividans* do not induce aerial mycelia in this bacteria (data not shown). In addition, aerial mycelia are not observed when *S. coelicolor* colonies are juxtaposed or confronted with other bacterial strains. These results suggest that a signal produced by *Myxococcus* is recognized by receptor encoded by *S. coelicolor* but not *S. lividans*. It will be of interest to determine whether the *M. xanthus* molecule that induces ACT production in *S. coelicolor* is the same as the one that stimulates differentiation.

**Experimental procedures**

**Bacterial strains and media**

*Streptomyces coelicolor* M145 and *S. lividans* 66 (Kieser et al., 2000) were used as prey along with mutant derivatives of *S. coelicolor* M145: Δ(redD), M511 (ΔactII-ORF4) and M512 (ΔredD-ΔactII-ORF4) (Floriano and Bibb, 1996) that do not produce the antibiotics RED, ACT or both respectively. The wt *M. xanthus* DK1622 (Kaiser, 1979), the pilQ1 mutant DZF1, leaky in S motility (Morrison and Zusman, 1979), and the non-motile *mgl* mutant DK6204 (Hartzell and Kaiser, 1991) were used as predators. CTT solid (1.5% Bactoagar) and liquid media were used to grow *M. xanthus* (Hodgkin and Kaiser, 1977). R2YE was used for *Streptomyces* cultures (Kieser et al., 2000). Several other bacteria have been used to examine *S. coelicolor* antibiotic production and aerial mycelium formation. All of them were grown in Luria–Bertani medium (Sambrook and Russell, 2001). These bacteria were obtained from the ‘Colección del Departamento de Microbiología’ (Universidad de Granada, Spain) (*Bacillus litoralisporus*, *B. licheniformis*, *B. megaterium*, *B. subtilis*, *B. thuringiensis*, *Mycobacterium smegmatis*, *K. pneumoniae*, *Salmonella* sp. and *Serratia* sp.), and from the ‘Colección Española de Cultivos Tipo’ (CECT) (*Micrococcus* sp. (CECT 241), *Mycobacterium phlei* (CECT 3009), *Staphylococcus aureus* (CECT 240), *E. coli* (CECT 101) and *Proteus* sp. (CECT 484)).

**Predation experiments**

*Myxococcus xanthus* strains were grown in CTT for 24 h, washed twice with sterile TM buffer (10 mM Tris-HCl, pH 7.6, 1 mM MgSO4) and concentrated to a final cell density of 4.5 × 10⁵ cells ml⁻¹. Drops of 5 or 10 µl were deposited on the surface of CTT agar plates and allowed to dry. Next, drops of 5 or 10 µl of *Streptomyces* spores (2 × 10⁷ spores ml⁻¹) were spotted close to the *Myxococcus* spot to leave a separation of no more than 1 mm. Plates were incubated at 30°C and images were taken directly with a digital camera or under a Zeiss Stemi SV11 or Wild-Heerbrugg disecting microscope. Each experiment was repeated at least four times. The same approach was used when *S. coelicolor* was plated next to other bacterial and yeast species.

**Liquid co-cultures and antibiotic quantification**

Co-cultures of *S. coelicolor* with *M. xanthus* were carried out in liquid CTT. A total of 10⁴ *S. coelicolor* spores were inoculated into 100 ml baffled flasks containing 10 ml of CTT and incubated at 28°C for 24 h. In parallel a culture of *M. xanthus* DK1622 was incubated under identical conditions. Different amounts of *M. xanthus* DK1622 (from 3 × 10⁷ to 3 × 10⁸ cells ml⁻¹) were added to the *S. coelicolor* cultures, except to the control where only *Streptomyces* was grown. Incubation was continued for 3–5 days. Production of the coloured antibiotics was quantified by colorimetric assays (Kieser et al., 2000) and production of CDA was determined by bioassay using *B. subtilis* as the sensitive organism.

**Chromatographic analysis**

Culture supernatants from 10 ml of liquid cultures were extracted twice with an equal volume of ethyl acetate containing 1% formic acid. The solvent was evaporated and the residue redissolved in 100 µl of dimethyl sulfoxide : methanol (50:50). These samples were fractionated by reversed phase in an Acquity UPLC with a BEH C18 column (1.7 μm, 2.1 × 100 mm, Waters) using acetonitrile and 0.1% trifluoroacetic acid in water. Samples were eluted with 10% acetoni-trile for 1 min, followed by a linear gradient from 10% to 100% over 15 min at a flow rate of 0.5 ml min⁻¹ and a column temperature of 30°C. For HPLC-MS analysis, an Alliance chromatographic system coupled to a ZQ4000 mass spectrometer and a Symmetry C18 (2.1 × 150 mm, Waters) was...
used. Solvents were the same as above and elution was performed with an initial isocratic hold with 10% acetonitrile for 4 min followed by a linear gradient from 10% to 88% acetonitrile over 26 min, at 0.25 ml min\(^{-1}\). MS analysis were performed by electrospray ionization in the positive mode, with a capillary voltage of 3 kV and a cone voltage of 20 V. Detection and spectral characterization of peaks was performed in both cases by photodiode array detection and Empower software (Waters).

**Fluorescence microscopy**

Cellular viability was detected by using the LIVE/DEAD BacLight Bacterial Viability kit L-13152 (Molecular Probes), which includes the dyes SYTO 9 (Green) and propidium iodide (red). SYTO 9 stains living cells green while propidium iodide stains damaged cells red (Haugland, 2002). The kit was used as indicated by the manufacturer to stain control cultures of *Streptomyces* or co-cultures of both organisms. Samples were observed in a Leica DMRXA microscope equipped for bright-field and epifluorescence and photographed with an Orca-ER C4742-80 camera (Hamamatsu, Bridgewater, NJ).

**Scanning electron microscopy**

Approximately 72 h co-cultures of *S. coelicolor* and *M. xanthus* on CTT agar plates were fixed with 3% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4 for 24 h at 4°C. Then, samples were washed three times (5 min each) with the same buffer. Dehydration was accomplished by a graded series of ethanol. Samples were then critical-point dried and sputter coated with carbon. Photographs were taken in a LEO 1530 scanning electron microscope.

**Videomicroscopy**

Cell spots of a co-culture of *S. coelicolor* and *M. xanthus* were filmed with a Wild Heerbrugg M7 S dissecting microscope at room temperature. Photographs were taken every 5 min as jpg files with a Spot Insight 2 camera using SPOT software v4.5 (Diagnostic Instruments). The movies were compiled from the images using Quicktime Pro (Apple) at six frames per second. The field of view is 3.8 mm across. The movie was compressed using the H.264 video codec in QuickTime Pro 7.

**Acknowledgements**

This work has been supported by grants from Ministerio de Ciencia e Innovación (EU2008-03631 to R.I.S.; BFU2009-07565/BMC, 70% funded by FEDER, and the programme CONSOLIDER-INGENIO 2010, CSD2009-00006, both to J.M.-D.), Spain. This material is based on work supported in part by the National Science Foundation under Grant 0742976 to L.J.S. Thanks are due to Dr. M. Bibb for *S. coelicolor* M510, M511 and M512 strains and to M.J. Jiménez Rufo for her excellent technical work.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Movie S1. Interaction between M. xanthus (left) and S. coelicolor (right) on solid CTT medium. The pictures were taken every 5 minutes and compiled with Quicktime Pro (Apple).

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