Stimulatory Effects of Methyl-β-cyclodextrin on Spiramycin Production and Physical—Chemical Characterization of Nonhost@Guest Complexes

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Abstract: Spiramycin is a macrolide antibiotic and antiparasitic that is used to treat toxoplasmosis and various other infections of soft tissues. In this study, we evaluated the effects of α-cyclodextrin, β-cyclodextrin, or methyl-β-cyclodextrin supplementation to a synthetic culture medium on biomass and spiramycin production by Streptomyces ambofaciens ATCC 23877. We found a high stimulatory effect on spiramycin production when the culture medium was supplemented with 0.5% (w/v) methyl-β-cyclodextrin, whereas α-cyclodextrin or β-cyclodextrin weakly enhanced antibiotic yields. As the stimulation of antibiotic production could be because of spiramycin complexation with cyclodextrins with effects on antibiotic stability and/or efflux, we analyzed the possible formation of complexes by physical—chemical methods. The results of Job plot experiment highlighted the formation of a nonhost@guest complex methyl-β-cyclodextrin@spiramycin I in the stoichiometric ratio of 3:1 while they excluded the formation of complex between spiramycin I and α- or β-cyclodextrin. Fourier-transform infrared spectroscopy measurements were then carried out to characterize the methyl-β-cyclodextrin@spiramycin I complex and individuate the chemical groups involved in the binding mechanism. These findings may help to improve the spiramycin fermentation process, providing at the same time a new device for better delivery of the antibiotic at the site of infection by methyl-β-cyclodextrin complexation, as it has been well-documented for other bioactive molecules.

INTRODUCTION

Spiramycin is a macrolide antibiotic and antiparasitic that is used to treat toxoplasmosis and various other infections of soft tissues. It is produced by the mycelial actinomycete Streptomyces ambofaciens, a prolific producer of bioactive molecules. Besides spiramycin, the reference strain S. ambofaciens ATCC 23877, whose genome sequence has been recently delivered, has been known for the past 60 years to produce congoicidine (netropsin), a pyrrolamide with a broad range of biological activities but no medical applications. More recently, a genome mining-guided approach has revealed the ability of this strain to produce, in addition to these two molecules, the antibiotic kinamycins, the siderophores coelichelin and desferioxamines, the antifungal antimycin and stambomycins, and novel polyketides with antibacterial and antiproliferative activities.

The molecular structure of spiramycin consists of a 16-membered lactone ring that is decorated by two amino sugars (mycaminose and forosamine) and one neutral sugar (mycarose). The 16-membered lactone ring is synthesized by a modular polyketide synthase (PKS) that, apart from the most common polyketide precursors, malonyl-CoA and methylmalonyl-CoA, is predicted to incorporate the less common precursor, ethylmalonyl-CoA, and the functionalized extender unit, methoxymalonyl-CoA. S. ambofaciens ATCC 23877 produces three components forming the "spiramycin complex", namely, spiramycin I, which has a hydroxyl group at the C-3 of aglycone, spiramycin II, in which the hydroxyl group is acetylated, and spiramycin III, in which the same position is propionylated.

Over the past 40 years, a variety of approaches have been described in the literature for improving the industrial production of this natural compound. In addition to the traditional mutate-and-screen method and process improvement, rational selection (2-deoxy-glucose, valine, and soybean oil-resistant mutants, mutants resistant to high concentrations of spiramycin, etc.) genetic modification of metabolic genes, and pathway-specific
Regulators have been reported. Recently, an integrated approach to identify metabolic targets for antibiotics overproduction by systems biology has been described. Regarding process improvement, the effects of glucose, glycerol, inorganic phosphate, ammonium ions, and short-chain fatty acids on the production of spiramycin have been reported. Here, we evaluated the effects of cyclodextrin supplementation to a culture medium on the biomass and spiramycin production by S. ambofaciens ATCC 23877. Cyclodextrins are a family of macrocyclic oligosaccharides linked by α-1,4 glycosidic bonds to form a cone-shaped structure with a hydrophobic cavity. Natural cyclodextrins are α-cyclodextrin, β-cyclodextrin, and γ-cyclodextrin, composed, respectively, of six, seven, and eight units of D-glucopyranose. These cyclic oligosaccharides can form different complexes with compounds: host@guest, which can be inserted into their cavity leading to an increased solubility and consequently to a higher biological activity, and nonhost@guest. In the latter case, the interaction occurs with the external surface and big/small borders of cyclodextrins. Some examples of host@guest complexes involving β-cyclodextrins are those formed with ofloxacin, butylparaben, and triclosan. Among nonhost@guest complexes, those formed between erythromycin and β-cyclodextrin have been well-characterized. Complexation with cyclodextrins has been proposed as a drug delivery system, in addition to other applications. For instance, cyclodextrins can be exploited by imprinted magnetic nanoparticles for the recovery and separation of erythromycin from industrial wastewater. A further industrial application of cyclodextrins is the improvement of secondary metabolites production. For example, in different Mycobacterium strains, hydroxypropyl-β-cyclodextrin increased the bioconversion of phytosterol by a factor of 2.

RESULTS AND DISCUSSION

Effects of Cyclodextrins on Mycelial Growth and Spiramycin Production. The primary goal of the present study was to evaluate the possibility of improving spiramycin production by manipulating the fermentation microenvironment by means of cyclodextrins. The effects of cyclodextrins on the mycelial growth and spiramycin production by S. ambofaciens ATCC 23877 were evaluated in shake-flask batch cultivation using the minimal basal medium (MBM) medium containing 25 mM L-valine and 40 g/L glucose. To this purpose, the bacteria were cultivated for 24 h. Then, the broth cultures were supplemented with 0.5% (w/v) α-cyclodextrin, β-cyclodextrin, methyl-β-cyclodextrin, or potato dextrin. α-Cyclodextrin, β-cyclodextrin, or potato dextrin did not elicit any significant effect on the spiramycin production at 72 h (RG1-T phase), 120 h (RG2 phase), and 168 h (stationary phase) (data not shown). In contrast, methyl-β-cyclodextrin stimulated total spiramycin titers by a factor of 1.96, 2.66, and 3.26 at 72, 120, and 168 h, respectively (Figure 1A,B). The increase was statistically significant at 120 h (p-value < 0.05) and 168 h (p-value < 0.005). Methyl-β-cyclodextrin also produced clear effects on the growth curve. The most noticeable one consisted of a growth slowdown during rapid growth phase 1 (RG1) and rapid growth phase 2 (RG2) and about 25% lower final biomass values. Spiramycin is produced by S. ambofaciens ATCC 23877 as a complex formed by three major components (I, II, and III), which differ in the substituent at position 3 (−H, −CO−CH3, −CO−CH2−H, −CO−CH₂−CO−H).
and −CO−CH₂−CH₃, respectively) of the lactone nucleus. The quantitative analysis of these components by liquid chromatography−mass spectrometry (LC-MS) revealed that in the absence of methyl-β-cyclodextrin, the percentage ratios between spiramycin I, II, and III at 72, 120, and 168 h were, respectively, 65:10:25, 67:9:24, and 70:9:21 (Figure 1C). The addition of methyl-β-cyclodextrin to the broth cultures affected the ratios of these components by increasing the relative amounts of spiramycin I with respect to spiramycin II and III. Under these conditions, the percentage ratios between spiramycin I, II, and III at 72, 120, and 168 h were, respectively, 86:4:10, 83:5:12, and 84:5:11 (Figure 1C). The higher percentage ratios of spiramycin I recovery with respect to spiramycin II and III are of practical interest, as spiramycin I is...
the component of the “spiramycin complex” that is mostly used for human therapy. This finding is consistent with the study by Patzelt and colleagues on polyether antibiotics momensin A in Streptomyces cinnamoneus A3823-S and nigerincin in Streptomyces hygroscopicus V1327 and Streptomyces violaceusniger NRRL B-1865, who demonstrated a clear stimulatory effect of heptakis-2,6-di-O-methyl β-cyclodextrin (DMCD) on the production of these compounds in shaken liquid cultures. Noticeably, they hypothesized a similar stimulatory effect on the biosynthesis of other related antibiotics including macrolides and polyenes.

On a mechanistic point of view, as methyl-β-cyclodextrin does not go through the bacterial cell membrane, there could be two possible and not mutually exclusive mechanisms to interpret its stimulatory effect on spiramycin production. One mechanism could be an indirect effect of methyl-β-cyclodextrin on spiramycin biosynthesis due to perturbation of membrane fatty acid metabolism, as suggested by Patzelt and colleagues for polyether antibiotics. Indeed, they noticed that in Mycobacterium phlei, DMCD was able to stimulate fatty acid biosynthesis catalyzed by fatty acid synthase, possibly because of the ability of cyclodextrins to bind the hydrophobic portion of palmitoyl-CoA. On the basis of the analogy between fatty acid and polyketide biosynthesis, they suggested that DMCD exerted its general effects on the polyether antibiotic production by binding to the partially reduced polyketide chains synthesized by the PKS active in polyether biosynthesis. Moreover, there is evidence that methyl-β-cyclodextrin can perturb the homeostasis of membrane lipids by removing hopanoids in prokaryotic membranes. This perturbation may have effects on spiramycin biosynthesis because membrane lipid metabolism is strictly interconnected with the biosynthesis of spiramycin backbone through common biosynthetic precursors. The other hypothetical mechanism responsible for the enhancement of spiramycin production could be a direct effect of methyl-β-cyclodextrin on the spiramycin stability and/or efflux due to complex formation. This latter hypothesis would also account for the altered percentage ratios between spiramycin I, II, and III and for the selective effect of methyl-β-cyclodextrin on spiramycin I yields. Therefore, we next analyzed the possible interaction between spiramycin I and cyclodextrins by using chemical—physical methods.

Physical—Chemical Characterization of the Cyclodextrin(s)@Spiramycin I Complexes. Cyclodextrins are well-known for their aptitude to provide inclusion complexes where the target molecules could be inserted in the cavity for different applications, such as drug delivery. The Job plot of the continuous variation method is an analytical approach typically employed for the study of the stoichiometry of such complexes and was used in this contribution by means of UV–vis spectroscopy to study the capability of α-, β-, and methyl-β-cyclodextrin to complex spiramycin molecules with particular attention to the stoichiometry of the eventually induced adduct (Figures 2 and 3, and Supporting Information Figure S2). Seven solutions (from A to G, as shown in Table 1) containing different amounts of spiramycin I stock solution and α-cyclodextrin or β-cyclodextrin or methyl-β-cyclodextrin stock solution were prepared to have 100 μmol as the total mole amount in 2 mL of phosphate buffer saline (PBS). Supporting Information Figure S2A reports UV–vis spectra in the 200–500 nm range recorded for the A–G solutions obtained by mixing spiramycin I and α-cyclodextrin. No shifts or new absorption bands were induced by changing the molar ratio between the two species. This result suggests that the formation of a complex α-cyclodextrin/spiramycin I based on strong interactions should be excluded. In fact, the continuous variation method allows the identification of an adduct formed by the species with a chemical affinity enough to consider such an adduct as a new chemical compound with different physical–chemical properties, such as a different molar extinction factor and so a distinct absorption pattern, which can be detected by monitoring the changes in the UV–vis absorption features. The same result was obtained in the case of β-cyclodextrin (Supporting Information Figure S2B). Probably, the chemical affinity between spiramycin I and β-cyclodextrin, also in this case, did not allow the formation of a complex, detectable by observing the changes in the UV–vis spectra of the antibiotic.

Figure 2A depicts the UV–vis spectra obtained for the seven different solutions obtained by mixing a proper molar amount of spiramycin I and methyl-β-cyclodextrin. The presence of a new absorption band located at 279 nm was revealed in the case of solution E (gray line in Figure 2A and in the inset) and, so, when the spiramycin I/methyl-β-cyclodextrin molar ratio was 1:3. The Job plot obtained for the complex formed by spiramycin I and methyl-β-cyclodextrin was obtained by plotting the absorbance variation at 279 nm, calculated by subtracting the recorded absorbances at 279 nm of all solutions by the recorded absorbance at 279 nm of solution A (100 μmol of spiramycin I), versus the molar fraction (χ) of methyl-β-cyclodextrin.

The molar fraction, χ, was calculated as $\chi = (m_i)/(m_i + m_m)$, where $m_i$ is spiramycin I moles and $m_m$ is methyl-β-cyclodextrin moles.

The Job plot highlighted the formation of a complex methyl-β-cyclodextrin@spiramycin I when the χ of methyl-β-cyclodextrin was 0.75, that is, in the 3:1 stoichiometric ratio (Figure 2B). This result can be rationalized by excluding the formation of a host@guest inclusion complex due to the individuated stoichiometric ratio, steric hindrance, and the dimension of the antibiotic compound, as further confirmed by the in silico modeling of cyclodextrins@spiramycin I complexes study. The

### Table 1. Spiramycin I Solutions Diluted by Specific Amounts of Cyclodextrin Solutions Used for Job Plot Experiments

| spiramycin I stock solution (mL) | α-cyclodextrin or β-cyclodextrin or methyl-β-cyclodextrin stock solution (mL) | PBS (mL) | spiramycin I (μmol) | α-cyclodextrin or β-cyclodextrin or methyl-β-cyclodextrin (μmol) |
|----------------------------------|---------------------------------|----------|---------------------|---------------------------------|
| A 0.20                           | 0                               | 1.8      | 100                 | 0                               |
| B 0.18                           | 0.02                            | 1.8      | 90                  | 10                              |
| C 0.15                           | 0.05                            | 1.8      | 75                  | 25                              |
| D 0.10                           | 0.10                            | 1.8      | 50                  | 50                              |
| E 0.05                           | 0.15                            | 1.8      | 25                  | 75                              |
| F 0.02                           | 0.18                            | 1.8      | 10                  | 90                              |
| G 0                               | 0.20                            | 1.8      | 0                   | 100                             |
Fourier-transform infrared (FT-IR) spectroscopy measurements were carried out to characterize the methyl-β-cyclodextrin@spiramycin I complex and individuate the chemical groups involved in the binding mechanism (Figure 3). Drop-casted films starting from an aqueous solution containing 18 × 10⁻⁸ mol of methyl-β-cyclodextrin and 6 × 10⁻⁸ mol of spiramycin I were obtained directly on the attenuated total reflectance (ATR) plate, and the spectra were recorded after water evaporation, black and light gray spectra in Figure 3A in the 1900–900 cm⁻¹ frequency range. The cyclodextrin spectrum is dominated by C–O, C–C–O, and C–O–C stretching vibration modes of the glucopyranose units in the range of 1200–900 cm⁻¹. The light gray spectrum, corresponding to the antibiotic cast film, is characterized by the IR features typical of the macrocyclic lactone, and in particular by the C==O stretching mode of the cyclic ester absorption located at 1721 cm⁻¹, and of the oligosaccharide moieties, typically C–O stretching modes in the 1200–900 cm⁻¹ interval. A cast film of an aqueous solution containing both cyclodextrin and antibiotic in the proper molar ratio (18 × 10⁻⁸ and 6 × 10⁻⁸ mol, respectively) was further obtained on the ATR plate (gray spectrum, Figure 3A). Because of the molar ratio, the complex spectrum is dominated by cyclodextrin IR features that result almost unchanged after the binding. Nonetheless, some interesting shifts could be observed and allowed to individuate the main groups that are involved in the interaction mechanism, in particular the carboxyl group (COOH) of the antibiotic moiety and the OH groups of both compounds. In fact, the most interesting regions of the complex spectrum are the 1800–1650 cm⁻¹ (Figure 3A) and 1150–800 cm⁻¹ (Figure 3C) ranges. The 1800–1650 cm⁻¹ spectral range is characterized by the C==O stretching mode of the spiramycin COOH group (light gray line, Figure 3B), whose results shifted to a lower frequency (from 1721 to 1715 cm⁻¹) after the complex formation (gray spectrum, Figure 3B), suggesting a key role of the C==O group in the interaction, probably thanks to hydrogen bonds formation. Besides, the 1150–800 cm⁻¹ range of the complex spectrum (gray spectrum, Figure 3C) is characterized by the C–O stretching modes, as already reported, mainly ascribable to cyclodextrin, and by a broad band at about 910 cm⁻¹ due to O–H out-of-plane bending mode (squared in Figure 3C). In particular, such a vibration mode is located at about 915 cm⁻¹ in the case of cyclodextrin (black line Figure 3C) and at about 905 cm⁻¹ in the case of spiramycin (light gray line, Figure 3C). Therefore, hydroxyl groups were also demonstrated to play an important role in the binding mechanism. In fact, the O–H out-of-plane bending vibrations in the complex spectrum provided a broad band different from both OH out-of-plane modes of antibiotic and cyclodextrin (squared in Figure 3C) underlining the OH involvement in the complex formation, probably through hydrogen bonds.

Altogether, these findings indicate that antibiotic compound size and geometry did not allow being included in the cyclodextrin basket, as already reported for erythromycin and cyclodextrin complexes. In particular, the Job plot experiments highlighted the formation of a methyl-β-cyclodextrin@spiramycin complex in the 3:1 stoichiometric ratio and no complex formation for the other two kinds of cyclodextrin. Such a result could be rationalized, considering that the presence of methyl substituents could induce geometric rearrangement favoring the chemical communication among the two species. FT-IR spectroscopy investigations allowed characterizing the obtained nonhost@guest complex from a chemical point of view. Such an approach highlighted that the binding mechanism is ruled by noncovalent chemical interactions, presumably hydrogen bonds, involving the alcoholic functionalities of both constituents and carboxylic functions of spiramycin I. Such interactions, although non-specific, takes place only in the case of methyl-β-cyclodextrin because of the steric hindrance of the methyl goup.

**In Silico Modeling of Methyl-β-cyclodextrin@Spiramycin I Complex.** The interaction between spiramycin and cyclodextrins was then modeled by using in silico docking tools. Models of spiramycin I, α-cyclodextrin, β-cyclodextrin, and methyl-β-cyclodextrin were generated as detailed in the Methods section and used as inputs for in silico docking by using AutoDock Vina. As a single molecule of spiramycin I was expected to accommodate up to three molecules of each α-cyclodextrin, β-cyclodextrin, or methyl-β-cyclodextrin, three or more consecutive docking cycles were carried out starting from different configurations (docking 1–5 in the Supporting Information Dataset S1) and selecting the best result from each cycle (Supporting Information Figures S3 and S4). According to the computational data, β-cyclodextrin is a better complexing agent for spiramycin I than α-cyclodextrin, probably because of the slightly larger space of the internal
cavity. The comparison of complexes formed by spiramycin I with β-cyclodextrin or methyl-β-cyclodextrin indicated slightly lower values of free energy of the complexes with β-cyclodextrin. This finding seems to be in contrast with the Job plot data, indicating that only methyl-β-cyclodextrin formed a complex with spiramycin I (Supporting Information Figure S1 and Figure 2). However, the docking cycles illustrated in Supporting Information Figure S5 might explain this incongruence: β-cyclodextrin seems to have a higher tendency to aggregate in the solution than methyl-β-cyclodextrin that may be more available for physical complexation with the antibiotic.

The arrangement of methyl-β-cyclodextrin@spiramycin I complex during fermentation may account for the stimulation of antibiotic production. Indeed, spiramycin I complexation with methyl-β-cyclodextrin may affect the antibiotic stability and/or antibiotic efflux through the cell membrane that has been associated with the activity of SrmB coding for the ATP-binding component of an ABC transporter.\textsuperscript{45} Methyl-β-cyclodextrin may facilitate the efflux of spiramycin through the cell membrane, thereby decreasing the intracellular concentration of the antibiotic and stimulating its biosynthesis. A similar mechanism has been proposed to be involved in a high stimulatory effect of β-cyclodextrin on the production of lankacin C by \textit{Streptomyces rochei} because of the formation of an inclusion complex with β-cyclodextrin in the fermentation broth and abrogation of feedback repression.\textsuperscript{46} Our finding, however, does not rule out the possibility that additional mechanisms may be involved in the stimulatory effects of cyclodextrin on spiramycin production. As mentioned above, methyl-β-cyclodextrin can perturb the homeostasis of membrane lipids by removing hopanoids in prokaryotic membranes.\textsuperscript{47} This metabolic perturbation may indirectly affect the spiramycin biosynthesis. In fact, the membrane lipid metabolism is strictly interconnected with the biosynthesis of spiramycin, whose macrolactone ring is built up by a PKS that, in addition to one unit of each methylmalonyl-CoA, ethylmalonyl-CoA, and methoxymalonyl-CoA, incorporates as many as four units of malonyl-CoA, a key precursor of the membrane fatty acid biosynthesis.\textsuperscript{19} Thereby, in addition, to be valuable for increasing antibiotic yields in batch fermentations and for the isolation of new metabolites that are otherwise produced in only very small amounts,\textsuperscript{48} cyclodextrins can be further exploited to investigate the complex interconnections between polyketide biosynthesis and membrane lipid metabolism.

Another very interesting application fields of cyclodextrins in clinical pharmacology of antibiotic therapy concerns drug delivery. Cyclodextrins are biocompatible and do not elicit an immune response in humans.\textsuperscript{49} Thus, they are used as potential drug carriers to enhance therapeutic drug efficiency and consequently to reduce toxicity by the direct transportation to specific targets.\textsuperscript{47,48} Recent accumulating evidence indicates β-cyclodextrin complexation as an effective drug delivery system [cyclodextrin inclusion complexes (ISCOMs)] for different classes of antibiotics to circumvent the limitations of conventional formulations and to treat drug resistance.\textsuperscript{45,50} With reference to macrolide antibiotics, complexes between β-cyclodextrin and erythromycin have been proposed as a drug delivery device for orthopedic application.\textsuperscript{51} Very promising applications of ISCOMs include treatment of biofilm-resident microbes that are also refractory to conventional antibiotic therapy.\textsuperscript{51} Spiramycin I is used to treat toxoplasmosis and certain bacterial infections of soft tissues. In these tissues, the antibiotic treatment lacks efficiency in spite of the documented susceptibility of the causative pathogen to the administered antibiotic.\textsuperscript{52} Delivery of spiramycin with methyl-β-cyclodextrin might augment the level of the bioactive drug at the site of infection while reducing the dosage and the dosing frequency.

### CONCLUSIONS

In this study we propose a new approach to improve the spiramycin production process by manipulating the micro-environment of the fermentation medium with methyl-β-cyclodextrin. By using physical–chemical methods, we characterized the methyl-β-cyclodextrin@spiramycin I non-host@guest complex and modeled the interactions between methyl-β-cyclodextrin and spiramycin I by in silico docking. Our findings will be useful not only to understand the molecular mechanism(s) underlying the stimulatory effect of methyl-β-cyclodextrin on spiramycin I production but also to develop a new device for better delivery of the antibiotic at the site of infection by methyl-β-cyclodextrin complexation.

### METHODS

**Bacterial Strains, Media, and Growth Conditions.** \textit{S. ambofaciens} ATCC 23877 was obtained from the American Type Culture Collection. The strain was stored in 1 mL cryotubes at −80 °C as a frozen mycelium in a yeast–starch (YS) medium containing 15% glycerol at a biomass concentration of approximately 0.25 g dry cell weight/mL or at −20 °C as spores in 20% glycerol at a titer of approximately 5 × 10\textsuperscript{8}/mL. The composition (per liter) of the media used in this study for the propagation and growth of \textit{S. ambofaciens} growth are reported here. 2XYT (medium for spore resuspension): 16 g of tryptone, 10 g of yeast extract, and 5 g of NaCl; YS broth (or agar): 2 g of yeast extract, 10 g of soluble starch, and 18 g of agar in YS agar (pH 7.0); and MBM (15): 1 g of MgSO\textsubscript{4}, 15 mg of ZnSO\textsubscript{4}, 2 g of KH\textsubscript{2}PO\textsubscript{4}, 5 g of CaCO\textsubscript{3}, 20 g of NaCl, and 0.3 mg of CoCl\textsubscript{2}. Glucose (40 g/L) and 25 mM L-valine were added as the carbon and nitrogen sources, respectively. When indicated, the bacterial broth cultures in MBM containing glucose and L-valine were supplemented with 0.5% (w/v) α-cyclodextrin, β-cyclodextrin, methyl-β-cyclodextrin, or potato dextrin during the growth.

For fermentation experiments in shake-flasks, spores in frozen aliquots were collected by centrifugation, resuspended in 2XYT medium, and readily separated by vortexing. The spore suspension (0.1 mL) (with and OD\textsubscript{600nm} of 1.22) was inoculated into 50 mL YS broth in a 300 mL Erlenmeyer flask. The cultures were incubated for about 24 h at 28 °C on an orbital shaker at 250 rpm until OD\textsubscript{600nm} reached 1.8. Then, 1 mL of YS broth culture was used to inoculate 50 mL MBM containing glucose and L-valine. After 24 h of growth, when indicated, the MBM broth cultures were supplemented with 0.5% (w/v) α-cyclodextrin, β-cyclodextrin, or methyl-β-cyclodextrin or potato dextrin. At different time intervals, the biomass (OD\textsubscript{600nm}) and spiramycin concentration were determined.

**Spiramycin Production Assay.** Spiramycin production by \textit{S. ambofaciens} broth cultures was assessed by high-performance liquid chromatography–electrospray ionization-MS (HPLC–ESI-MS). At different time intervals, supernatants were filtered through a Phenex-RC membrane (0.45 mm; Phenomenex). Five microliters of a 1 ppm solution of erythromycin (Sigma-
Aldrich) in acetonitrile and water (30:70, v/v) and 210 μL of acetonitrile were added to 1 mL. Eppendorf tubes containing 500 μL of filtrated samples; the tubes were vortexed and centrifuged at 4 °C for 5 min, and then 2 μL of the supernatant was injected for spiramycin determination.

The HPLC–ESI-MS apparatus was constituted by a Thermo Surveyor MS quaternary pump coupled to a Finnigan LCQ Deca XP Plus mass detector equipped with an ESI source and an ion trap quadrupole. The mass spectrometer was operated in the positive-ion mode, with a capillary temperature of 325 °C and capillary voltage of 1.5 kV. 25 The mass spectra were recorded between 250 and 1000 m/z. Spiramycin I, II, and III were separated on a reverse phase C18 analytical column (Bio Basic C-18, 150 × 2.1 mm, particle size 5 μm; Thermo Scientific) at a flow rate of 200 μL min⁻¹ using 5 mM ammonium formate adjusted to pH 7.0 (solvent A) and LC-MS-grade acetonitrile (solvent B) applying the following gradients of B: 0 min, 15%; 1 min, 15%; 15 min, 70%; 20 min, 70%; 21 min, 15%; and 25 min, 15%. Purified spiramycin complex (consisting of about 80% spiramycin I, 5% spiramycin II, and 10% spiramycin III) (Sigma-Aldrich) or purified spiramycin I (Sanofi) freshly dissolved in 5 mM ammonium formate (pH 7) was used as the standard. To avoid the interference of cyclodextrins in the mass spectrometric determination and their deposition on the source, the flow was directed to the source only during erythromycin and spiramycin elution by using the divert valve on the MS instrument. Analyte quantification was performed with the internal standard (IS) method: spiramycin responses were normalized to the erythromycin IS area. The recoveries of the method, evaluated by spiking the samples with low (200 ng) and high (2 μg) levels of analyte, were 97 and 93%, respectively. The limit of detection and the limit of quantification determined at S/N ratios of 3 and 10 for spiramycin were, respectively, 20 and 67 ng/mL.

**Job Plot Experiments and FT-IR Characterization.** α-Cyclodextrin, β-cyclodextrin, and methyl-β-cyclodextrin were purchased from Sigma-Aldrich and used as received without further purifications. Purified spiramycin I was provided by Sanofi. For Job plot experiments, spiramycin I, α-cyclodextrin, β-cyclodextrin, and methyl-β-cyclodextrin stock solutions containing 250 μmol of each compound dissolved in 0.5 L of PBS 1× at pH 7.2 were prepared. Then, seven dilutions (from A to G) of spiramycin I stock solution with different amounts of cyclodextrin stock solutions were prepared as indicated in the Table 1 to carry out three different experiments. A total volume of 2 mL was chosen.

UV–vis absorption spectra of A–G solutions in the three experiments were recorded by means of an Agilent Cary 5000 instrument. ATR-FTIR measurements were taken with a PerkinElmer Spectrum One instrument. In particular, a cast film of spiramycin I, a cast film of methyl-β-cyclodextrin, and a cast film of the complex methyl-β-cyclodextrin/spiramycin I in the stoichiometric 3:1 ratio were obtained directly on the ATR plate starting from the aqueous solution. Sixty four scans were acquired in each case.

**In Silico Modeling.** Models of spiramycin I, α-cyclodextrin, and β-cyclodextrin were downloaded by using the RCSB database, and hydrogen was added by Avogadro specific function. Models of methyl-β-cyclodextrin, starting from the model of β-cyclodextrin, were edited by using Avogadro software to add methylation in different positions and to optimize the molecular geometry. These models were used as inputs for in silico docking by using AutoDock Vina,33 using spiramycin I as a receptor and cyclodextrins as ligands. As a single molecule of spiramycin I was expected to accommodate up to three molecules of each α-cyclodextrin, β-cyclodextrin, or methyl-β-cyclodextrin, three or more consecutive docking cycles were carried out starting from the different docking configuration (docking 1–5 in the Supporting Information Dataset S1) and selecting the best result from each cycle. The first cycle aimed to define the relative position between spiramycin I and cyclodextrin, and the second cycle aimed to define the position of second and third cyclodextrins using a receptor from the first selected result (spiramycin/cyclodextrin 1:1 complex). Finally, in the last docking, we used spiramycin as a ligand and cyclodextrins as receptors to obtain a better configuration of the complex and to compute the total energy of affinity (kcal/mol). The best predictions were used to generate the final images with Chimera.44 For methyl-β-cyclodextrin, which contains randomly 2-O, 3-O, or 6-O-methylated glucose moieties, we modified β-cyclodextrin in the predicted complex by sequentially adding methyl groups in different positions (Supporting Information Figure S1). Different combinations were tested and the best docking outputs were used to generate the final images with Chimera. Finally, we performed a docking cycle to clarify the self-aggregation of cyclodextrins in the solution and the mechanism of complex formation: the ligand and receptor molecular model is the same (cyclodextrins methylated or not). More detailed information is reported in the Supporting Information Dataset S2.

**ASSOCIATED CONTENT**

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.7b01766.

**Modeling data; UV–vis spectra; and docking analysis images and dataset (PDF)**

Results of 3D docking model α-cyclodextrins and β-cyclodextrins versus spiramycin (3:1), screening of best docking model of randomly methylated β-cyclodextrins versus spiramycin (1:1), docking model of randomly methylated β-cyclodextrins versus spiramycin (3:1), docking model of cyclodextrins versus cyclodextrins aggregations (XLSX)

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M.C. and S.B. contributed equally to this work. P.A., L.S., G.E.D.B. and L.V. conceived the experimental design and planned the experiments. M.C., M.D.S., S.M.T. and A.T. optimized the growth conditions and carried out the fermentation experiments. F.D., D.F., and G.E.D.B. performed HPLC–ESI-MS determinations. S.B., R.P., and L.V. carried out Job plot experiments and FT-IR characterization. M.C. performed in silico modeling. P.A., G.E.D.B., S.B., and L.V.
wrote the draft of the work. All authors critically revised and finally approved the manuscript.

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**ABBREVIATIONS**

ATCC, American Type Culture Collection; ATR-FT-IR, attenuated total reflection Fourier-transform infrared spectroscopy; DCW, dry cell weight; ESI, electrospray ionization; FT-IR, Fourier-transform infrared spectroscopy; HPLC, high-performance liquid chromatography; HPLC–ESI-MS, high-performance liquid chromatography–electrospray ionization–mass spectrometry; IS, internal standard; LCMS, liquid chromatography mass spectrometry; LOD, limit of detection; LOQ, limit of quantification; MBM, minimal basal medium; MS, mass spectrometry; PBS, phosphate saline buffer; PKS, polyketide synthase; RG1, rapid growth phase 1; RG2, rapid growth phase 2; YS, yeast starch YS

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