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Enhancement of antibiotic production by co-cultivation of two antibiotic producing marine *Vibrionaceae* strains

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Abstract

Deciphering the cues that stimulate microorganisms to produce their full secondary metabolic potential promises to speed up the discovery of novel drugs. Ecology-relevant conditions, including carbon-source(s) and microbial interactions, are important effectors of secondary metabolite production. Vice versa, secondary metabolites are important mediators in microbial interactions, although their exact natural functions are not always completely understood. In this study, we investigated the effects of microbial interactions and in-culture produced antibiotics on the production of secondary metabolites by *Vibrio coralliilyticus* and *Photobacterium galatheae*, two co-occurring marine *Vibrionaceae*. In co-culture, production

of andrimid by *V. coralliilyticus* and holomycin by *P. galatheae*, were, compared to monocultures, increased 4.3 and 2.7 fold respectively. Co-cultures with the antibiotic deficient mutant strains (andrimid$^-$ and holomycin$^-$) did not reveal a significant role for the competitor's antibiotic as stimulator of own secondary metabolite production. Furthermore, we observed that *V. coralliilyticus* detoxifies holomycin by sulphur-methylation. Results presented here indicate that ecological competition in *Vibrionaceae* is mediated by, and a cue for, antibiotic secondary metabolite production.

Introduction

Microbial derived secondary metabolites have enriched humanity with a broad range of medically useful compounds and analysis of microbial whole genome sequences and biosynthetic gene clusters encoding secondary metabolites have demonstrated that many more bioactive compounds can potentially be found than hitherto detected (Machado *et al.* 2015b; Jensen 2016). Therefore, there is a large interest in finding culture conditions that will cause the microorganisms to produce compounds so far not (chemically) detected (Rutledge and Challis 2015; Tomm, Ucciferri and Ross 2019; Hoskisson and Seipke 2020). Increasingly, an ecology-based bioprospecting strategy is pursued using natural carbon sources or co-cultivation systems (Bertrand *et al.* 2014; Giubergia *et al.* 2017; Arora *et al.* 2020). It is often assumed that especially secondary metabolites with antibiotic activity serve as microbial defense molecules in natural microbial communities (Fischbach 2009), however, they may, especially at sub-inhibitory concentrations, also act as cues or signals triggering gene expression and phenotypic changes (Andersson and Hughes 2014). For instance, exposure to sub-inhibitory concentrations of antibiotics induce biofilm formation in *Escherichia coli* and *Pseudomonas aeruginosa* (Hoffman *et al.* 2005; Linares *et al.* 2006) or

production of secondary metabolites in *Streptomyces coelicolor* and *Burkholderia thailandensis* (Craney *et al.* 2012; Seyedsayamdost 2014).

Interactions between living microorganisms can mediate production of antibiotic secondary metabolites (Traxler *et al.* 2013; Abrudan *et al.* 2015; Patin *et al.* 2018; Westhoff *et al.* 2020). For example, antibiotic production by *Streptomyces* soil isolates on solid medium is altered by the presence of a second co-cultured strain as compared to solitary growth (Abrudan *et al.* 2015). Only a few studies have unraveled the underlying mechanisms for induction of antibiotic production in co-cultures. For example, iron competition was identified as induction cue of antibiotic production in a *Myxococcus xanthus* - *Streptomyces coelicolor* co-culture (Lee *et al.* 2020) and in a fungal-bacterial interaction between *Aspergillus nidulans* and *Streptomyces hygroscopicus*, induction of orsellinic acid production by *A. nidulans* is cell contact dependent (Schroeckh *et al.* 2009). However, the exact mechanisms by which bacteria regulate and alter their antibiotic production in response to the presence of other microorganisms is often not known. The hypothesis on so-called competition sensing addresses this (Cornforth and Foster 2013, 2015). This theory suggests that nutrient limitation and cell damage can act as cues for bacteria to sense competitors. Subsequently, this information can be used to regulate complex responses such as production of antibiotics, activation of the type VI secretion system (Russell, Peterson and Mougous 2014) or formation of biofilm (Lories *et al.* 2020).

Studies of antibiotic secondary metabolites have typically focused on soil derived *Streptomyces* due to their importance as source of clinically relevant antibiotics (Bérdy 2012), however, some strains of marine *Vibrionaceae* are producers of potent antibiotics (Mansson, Gram and Larsen 2011) and harbor several biosynthetic gene clusters (BGCs) encoding secondary metabolites (Machado *et al.* 2015b). In *Vibrionaceae*, antibiotic mediated antagonism is prevalent among ecologically and genotypically defined populations,

but individual strains are resistant against attacks from direct ecological neighbors with a similar genetic background (Cordero *et al.* 2012). Antibiotic secondary metabolite production is thus an ecologically important trait for marine *Vibrionaceae,* which makes them excellent candidates to study interaction mediated secondary metabolite production.

We have previously shown that the antibacterial secondary metabolite andrimid, produced by and purified from *Vibrio coralliilyticus* cultures, causes enhanced expression of BGCs encoding secondary metabolites both in a silent BGC reporter fusion and in another marine bacterium, *Photobacterium galatheae* (Buijs *et al.* 2020). *V. coralliilyticus* and *P. galatheae* are members of the *Vibrionaceae* family, inhabit similar ecological niches and produce antibacterial secondary metabolites. Andrimid, produced by *V. coralliilyticus* S2052, is an inhibitor of bacterial fatty acid synthesis (Freiberg *et al.* 2014) and is produced by multiple *Vibrio* strains (Wietz *et al.* 2010; Long *et al.* 2005). *P. galatheae* S2753 produces the antibiotic holomycin, a sulfur containing compound with a zinc chelating mechanism of action (Chan *et al.* 2017). The purpose of the present study was to investigate if stimulation of holomycin production in *P. galatheae* by antibacterial andrimid is an ecological relevant induction mechanism. Towards this, we co-cultured *V. coralliilyticus* and *P. galatheae* and their antibiotic deficient mutants and studied their secondary metabolite mediated interactions.

Materials and Methods

Experimental setup. Based on our previous results, we hypothesized that ecologically relevant competition and antibiotic production by competing bacteria are cues for production of secondary metabolites. Environmental co-occurence was inferred by an *in silico* analysis of the abundance of the two species in publicly available microbiome datasets. Then, we measured and compared the antibiotic production of andrimid by *V. coralliilyticus* and

holomycin by *P. galatheae* in (i) monocultures (ii) in co-cultures of the two wild-type strains (*V. coralliilyticus* WT - *P. galatheae* WT) and (iii) in co-cultures of the wild-type strain with an antibiotic deficient mutant competitor (*V. coralliilyticus* WT - *P. galatheae* Δ*hlmE* and *V. coralliilyticus* Δ*adm* - *P. galatheae* WT). The cultures were sampled (1 mL) at 0, 3, 8 and 24 hours after inoculation for DNA extraction and subsequent qPCR analysis to quantify cell abundances of both strains. Samples were taken for chemical extraction and quantification of andrimid and holomycin, as well as analysis of the metabolomes, by high-performance liquid chromatography and mass spectrometry (HPLC-MS).

Abundance analysis of *V. coralliilyticus* and *P. galatheae* of microbiome data. Publicly available 16S rRNA gene amplicon-based microbiome data were searched for co-abundance of *V. coralliilyticus* and *P. galatheae*. The following datasets were retrieved from the NCBI sequence read archive: microbiome of the mussel *Mytilus coruscus* (Li *et al.* 2019a) (SRP196510), microbiome of the mussel *Mytilus galloprovincialis* (Li *et al.* 2019b) (SRP197453), microbiome of the oyster *Crassostrea gigas* (King *et al.* 2019) (SRP139423), *Vibrio*-16S rRNA gene enriched sediment samples from the Chinese marginal seas (Wang *et al.* 2019) (SRP159585) and *Vibrio*-16S rRNA gene enriched samples from and around an oyster shellfish hatchery (Gradoville *et al.* 2018) (SRP118403). Raw paired-end reads were trimmed and merged using fastp (version 0.20.0) default options (Chen *et al.* 2018b). The lengths of the merged sequences are summarized in Table 2. Blast databases were built per microbiome dataset using the merged and trimmed sequences and the 16S rRNA gene sequences of *V. coralliilytiucs* S2052 and *P. galatheae* S2753 were blasted against these databases with the BLAST+ standalone (version 2.9.0), using standard parameters (Camacho *et al.* 2009). Manual inspection of the blast results was done to check if highest identity matches for both bacteria originated from the same sequencing sample.

Bacterial strains, media and culture conditions. *Vibrio coralliilyticus* strain S2052 (from marine sediment) and *Photobacterium galatheae* strain S2753 (from green-shell mussel) were isolated on the Galatheae 3 expedition based on their antibacterial activity against *Vibrio anguillarum* (Gram, Melchiorsen and Bruhn 2010; Machado *et al.* 2015a). Wild-type and gene-knock out mutants (see below) were cultured at 25 °C on Marine Agar (MA, Difco 2216) and in Artificial sea water Peptone Yeast extract (APY) broth consisting of: 19.45 g NaCl, 5.9 g $MgCl_2.6H_2O$, 3.24 g Na_2SO_4, 1.8 g $CaCl_2.2H_2O$, 0.55 g KCl, 3.0 g HEPES, 5.0 g peptone and 3.0 g yeast extract per liter milliQ H_2O, pH = 7.0, as modified from (Lefèvre *et al.* 2010). Liquid cultures of 20 mL in 100 mL shake flasks were aerated by orbital shaking (diameter = 20 mm) at 200 rpm. For cloning, *Escherichia coli* strain GBdir-*pir116* (Wang *et al.* 2016) was cultured on LB-agar (Difco, 244520) and in LB-broth (Difco, 244620). *E. coli* strain WM3064 (Chen *et al.* 2018a) was used for conjugation and cultured in LB media supplemented with 300 µM diaminopimelic acid. Chloramphenicol was used for selection of pDM4 containing cells (see below) at concentrations of 10 µg/mL (liquid culture) and 30 µg/mL (solid agar culture) for both *E. coli*, *V. coralliilyticus* and *P. galatheae* strains when necessary. All strains and genotypes are summarized in Table 1.

Construction of knockout mutant. In-frame, scarless knockout mutants were constructed from parent wild type strains *V. coralliilyticus* S2052 and *P. galatheae* S2753 by suicide plasmid (based on the pDM4 plasmid, using *sacB* - sucrose counter selection) homologous recombination. In brief, a scarless in-frame deletion mutant *P. galatheae ΔhlmE* was constructed as described in (Zhang *et al.*, submitted) and (Buijs *et al.* 2020). *V. coralliilyticus Δadm* was constructed similarly, except that the suicide plasmid was constructed by direct cloning using the RecET system in *E. coli* strain DH5α-pir116. Suicide vector transformation was achieved by intergenic conjugation between *E. coli* strain WM3064 (Chen *et al.* 2018a) and the target strain at an OD600-based ratio of 3:1 (donor:recipient), with a mating time of 4

hours at 37 °C. Single crossover mutants with the suicide vector integrated in the chromosome were selected on APY-Cm30 agar plates. A cell suspension of first crossover mutants was then plated on ½ strength APY plates supplemented with 10% (w/v) sucrose for counter selection, and double crossover mutants were identified by chloramphenicol and sucrose sensitivity tests.

Sequencing of *V. coralliilyticus* strains and confirmation of loss of 224 kb replicon. For the *V. coralliilyticus Δadm* mutant, no target region could be amplified by PCR, prompting whole genome sequencing of the mutant strain on an Illumina MiSeq system at the DTU Center for Biosustainability (Kgs. Lyngby, Denmark). Sequence reads were aligned to the genome of *V. coralliilyticus* S2052 (parent strain) using bowtie2 (Langmead and Salzberg 2012) v2.3.5, which showed the loss of a 224 kb contiguous region (including the andrimid BGC) in the genome of the mutant. The genome of *V. coralliilyticus* S2052 WT was sequenced with the minION from Oxford Nanopore Technologies (Oxford, UK) using the SQK-RAD004 sequencing kit and protocol version RSE_9046_v1_revM_14Aug2019. Long sequencing reads from nanopore sequencing and short illumina sequencing reads (Machado *et al.* 2015b) were used as input to close the genome with Unicycler (Wick *et al.* 2017). The genome sequence is available under GenBank accession numbers CP063051-CP063053.

Growth curves of monocultures. Monocultures of *V. coralliilyticus* WT, *V. coralliilyticus Δadm*, *P. galatheae* WT and *P. galatheae ΔhlmE* were inoculated from overnight liquid cultures (~10^9 CFU/mL) at a starting cell density of approximately 1 x 10^3 CFU/mL. Growth curves were measured by colony counts, using MA plates containing a total concentration of 2.5% agar, which reduced swarming of *P. galatheae* colonies. Maximum specific growth rates μ (h^{-1}) were calculated using the datapoints at T = 2 and T = 6 hours.

Liquid co-culture experiments. Wild type and deletion mutant strains of *P. galatheae* and *V. coralliilyticus* were inoculated from overnight cultures in precultures at an OD600 of 0.01 (*V. coralliilyticus*) and 0.02 (*P. galatheae*) and incubated for 2.5 hours. Exponentially growing cultures (OD600 between 0.1 and 0.5) were used as inoculum in co-cultures in 20 mL APY media; *V. coralliilyticus* was always inoculated at an OD600 of 0.001 and *P. galatheae* was inoculated at 0.0005 for a 2:1 ratio, 0.00025 for a 4:1 ratio or 0.00017 for a 6:1 ratio. In parallel, monocultures were inoculated at an OD600 of 0.001. Samples of 1 mL were taken at T = 0h, 3h, 8h and 24h for DNA extraction, and at T=24h a 10 mL culture aliquot was taken for chemical extraction. All co-culture experiments were done with four biological replicates.

Genomic DNA extraction. Genomic DNA from the mono- and co-cultures was extracted using the NucleoSpin Tissue kit (740952.250 Machery-Nagel) with the following modifications. Aliquots of 1000 µL (T = 0h), 100 µL (T = 3h) and 10 µL (T = 8 and 24h) were centrifuged for 5 min at 8,000 g, the cell pellet was resuspended in 180 µL T1 buffer + 25 µL proteinase K solution and stored at -20 °C for maximum 3 days before further processing. After thawing, samples were pre-lysed (according to manufacturer's protocol) for 2 hours.

Quantitative PCR. Strain abundances in the co- and monocultures were determined by quantitative PCR (qPCR) on a Mx300P (Agilent Technologies) using Luna Universal qPCR Master Mix (New England Biolabs) with a reaction volume of 20 µL in Optical Tube 8x Strip (Agilent Technologies), using 1 µL of extracted gDNA sample as template and a primer concentration of 0.4 µM. Two sets of primers were used in the qPCR for the specific quantification of *P. galatheae* and *V. coralliilyticus* (Table S1). Primers were designed against sequences in strain specific biosynthetic gene clusters. qPCR reactions were carried out with an annealing temperature of 60 °C. Water controls as well as gDNA extracted from

the monoculture of the different strain (i.e., *P. galatheae* gDNA with *V. coralliilyticus* primers and vice versa) were included as negative controls. Cycle-threshold (C_T) values were converted to CFU/mL by means of a standard curve. To obtain the standard curve, cell suspensions of approximately $1*10^8$ CFU/mL of each strain were 10-fold serial diluted and quantified for colony forming units. In parallel, 500 µL aliquots of both cell suspensions' dilution series were mixed and subjected to gDNA extraction and qPCR quantification. The standard curve was based on biological triplicates.

Metabolome analysis, holomycin and andrimid detection. Chemical extractions, metabolome analysis and detection of the compounds holomycin and andrimid was performed as described previously (Buijs *et al.* 2020). In short, 10 mL culture aliquots were extracted with an equal volume of ethyl acetate, which was evaporated by a flow of nitrogen gas and re-dissolved in 100 µL methanol. High-performance liquid chromatography coupled to diode array detection and quadrupole time-of-flight mass spectrometry (HPLC-DAD-QTOFMS) analysis was performed on an Agilent Infinity 1290 UHPLC equipped with a diode array detector and connected to an Agilent 6545 QTOF MS, using the method described in Isbrandt et al 2020 (Isbrandt *et al.* 2020), however using an Agilent Poroshell 120 phenyl-hexyl column (2.1 x 100 mm, 1.9 µm) and a gradient of only 10 minutes. Relative quantification of holomycin, andrimid, hydroxyandrimid and chloroandrimid was based on peak areas of extracted ion chromatograms (EICs) of the following compound masses (holomycin: $[M+H]^+ = 214.9943$, andrimid: $[M+H]^+ = 480.2493$, hydroxyandrimid: $[M+H]^+ = 496.2442$, chloroandrimid: $[M+H]^+ = 514.2103$) and UV chromatograms extracted at 380±5 nm for holomycin, and 300±5 nm for andrimid, hydroxyandrimid and chloroandrimid. All area values were normalized based on cell density.

Holomycin addition to *V. coralliilyticus* cultures. A *V. coralliilyticus* S2052 preculture was inoculated from an overnight culture and grown for 2.5 hours until OD600 = 0.2-0.5. This

preculture was used to inoculate four new cultures of 20 mL APY supplemented with 5, 3 and 1 μM holomycin and methanol (solvent, control) respectively, at an OD600 of 0.001. Cultures were incubated at 25 °C, 200 rpm for 24 hours, after which OD600 was measured and 10 mL culture aliquots were extracted with ethyl acetate for quantification of andrimid production. Pure holomycin was purchased from Toronto Research Chemicals (Toronto, Canada).

Holomycin methylation and MIC assay. For conversion of holomycin into dimethyl-holomycin, holomycin (500 μg) was first dissolved in methanol (MeOH) (500 μL). Excess of tris(2-carboxyethyl)phosphine hydrochloride (TCEP) (2.5 mg) was added to reduce the disulfide bond, followed by addition of 30 μL methyl iodide (MeI). The reaction mixture was stirred for 15 min and dried under a flow of nitrogen to remove solvent and residual MeI. Excess TCEP was removed by redissolving the reaction mixture in MeOH and subjecting the samples to strong anion exchange using a Biotage Isolute SAX column, and collecting dimethyl-holomycin as the non-retained fraction, followed by drying. Finally, non-retained impurities (e.g. mono-, di-, and trimethylated TCEP) from the SAX was removed using a Strata C18 SPE column, obtaining pure dimethyl-holomycin by dissolving the sample in 5% methanol in water and eluting with the same solvent, to yield 200 μg pure dimethyl-holomycin. A minimum inhibitory concentration (MIC) assay was performed to test toxicities of holomycin and dimethyl-holomycin towards *V. coralliilyticus*. Holomycin and dimethyl-holomycin were two-fold diluted in a 96 well plate (Thermo Scientific 262162) starting from 320 μM down to 0.63 μM, in a volume of 100 μL APY medium. Overnight cultures of *V. coralliilyticus* were diluted to an OD600 of 0.02, and 5 μL of this cell suspension was used to inoculate the well cultures. The 96 well plate was incubated for 24 hours at 25 °C at a plate shaker (Fisher Scientific, cat. no. 88861023) with orbital shaking at 300 rpm. The lowest concentration without growth, as observed by visual inspection and measured by a plate

reader (SpectraMax i3, Molecular Devices), was determined to be the MIC value. Biological triplicate measurements did yield the same MIC value.

Statistical methods. Statistical analyses were performed in R (version 3.6). For statistical analysis of cell abundances, CFU/mL values were log-transformed before calculating means and standard deviations and performing statistical tests. The effect of the co-cultivation strain on cell abundance over time was tested by a repeated-measures mixed model ANOVA using the lmer() function from the lme4 package to set up the model using replicates as random effects. The lsmeans() function from the emmeans package was then used for post-hoc pairwise Tukey's test of the mixed model. Differences in cell-normalized antibiotic production between the monoculture, co-culture with the WT competitor, and co-culture with the antibiotic deficient mutant was tested for statistical significance using one-way ANOVA followed by post-hoc Tukey's test.

Results

***Vibrio coralliilyticus* and *P. galatheae* coexist in nature.** An analysis of five publicly available 16S-rRNA amplicon sequencing datasets (see Materials and Methods) was carried out to investigate the possible coexistence of *V. coralliilyticus* and *P. galatheae* in the marine environment. The consensus rRNA genes of *V. coralliilyticus* S2052 and *P. galatheae* S2753 were used as queries in nucleotide BLAST searches against each dataset. Amplicon sequences with high identity (>97%) to both strains were found in three datasets, and in two cases the highly identical hits co-occur in the same sample (Table 2).

Production of antibiotic secondary metabolites confers an advantage in co-cultures. The impact of antibiotic production on growth performances of the two bacteria was measured. Maximum growth rates of the WT strains in monocultures were 2.03 h^{-1} and 2.10 h^{-1} for *V. coralliilyticus* and *P. galatheae* respectively (Fig. S1). Despite the similar growth rates of

both strains, *P. galatheae* outcompeted *V. coralliilyticus* in co-cultures with an inoculation ratio of 1:1. This prompted co-cultivations with decreased inocula of *P. galatheae* to study the interactions between the bacteria when co-occurring and the effects of co-culture on their antibiotic secondary metabolites. The effect of holomycin antibiotic production by *P. galatheae* on the growth performance of *V. coralliilyticus* was tested by comparing co-cultures of *V. coralliilyticus* with *P. galatheae* WT and *P. galatheae ΔhlmE*, inoculated at a ratio of 2:1 (*V. coralliilyticus*:*P.galatheae*) (Fig. 1A & 1B). A repeated-measures ANOVA demonstrated a significant effect of time and co-cultivation strain (*P. galatheae* WT vs *ΔhlmE*, $p < 0.0001$, type II Wald F test) as well as a significant interaction between co-cultivated strain and time ($p < 0.0001$) on abundance of *V. coralliilyticus,* suggesting that the effect of co-cultivation strain was dependent on time. Indeed, abundance of *V. coralliilyticus* cells was similar at time points 0h and 3h, but was significantly higher in co-culture with *ΔhlmE* compared to the co-culture with *P. galatheae* WT at t = 8h (3.4-fold, $p < 0.0001$, Tukey's test) and t = 24h (15-fold, $p < 0.0001$, Tukey's test). Furthermore, *V. coralliilyticus* abundance decreased between 8 and 24 hours in both co-cultures. These data indicate that, under these conditions, the ability to produce holomycin facilitates *P. galatheae* killing of *V. coralliilyticus*, although there is also holomycin-independent killing.

Analogously, the effect of andrimid production on cell abundances of both bacteria in co-culture was investigated. To this end, an andrimid deficient mutant of *V. coralliilyticus* was constructed. This mutant has lost a 234 kb extrachromosomal replicon that contains the BGC for andrimid production, as determined by whole genome sequencing (Fig. S2). No other BGCs were identified to reside on the lost extrachromosomal replicon. Under the conditions used in this study (APY liquid medium, 25 °C, 200 rpm shaking), this *Δadm* mutant grew with the same growth rate and reached the same maximum cell density as the *V. coralliilyticus* WT strain in monocultures (Fig. S1A). Repeated-measures ANOVA of cell

abundances in co-cultures of *P. galatheae* WT with *V. coralliilyticus* WT and *Δadm*, inoculated at a ratio of 6:1, demonstrated no significant effects of co-cultivation strain on growth performance of *P. galatheae* ($p = 0.71$, type II Wald F test). Comparison of *V. coralliilyticus* WT and *Δadm* growth performances in co-culture with *P. galatheae* demonstrated that the ability to produce andrimid had, on average across all time points, no significant effect on cell abundance of *V. coralliilyticus* ($p = 0.16$, type II Wald F test). However, there was a significant effect for the interaction between time and strain ($p = 0.003$, type II Wald F test), and a post-hoc analysis demonstrated a significantly higher *V. coralliilyticus* cell abundance at t = 24h for WT compared to *Δadm* ($p = 0.002$, Tukey's test), corresponding to a 22-fold higher cell density (Fig. 1C and 1D). This result suggests that the ability of *V. coralliilyticus* to produce andrimid is a benefit under these co-culture conditions.

Antibiotic production by *V. coralliilyticus* and *P. galatheae* is increased in co-cultures. *V. coralliilyticus* S2052 and *P. galatheae* S2753 were co-cultured in liquid APY medium as we have demonstrated that pure andrimid from *V. coralliilyticus* increases holomycin production in *P. galatheae* monoculture (Buijs *et al*. 2020). Growth rate and maximum cell density of the two strains in co-cultures were very sensitive to inoculation ratios. Inoculation ratios lower than 4:1 (*V. coralliilyticus*:*P. galatheae*, based on OD600) resulted in decreasing cell densities of *V. coralliilyticus* towards the end of the 24 hour culture period (as measured by qPCR). In contrast, inoculation ratios higher than 8:1 led to co-cultures in which *P. galatheae* was not able to grow above cell densities of 5×10^7 CFU/mL. Inoculated at a 4:1 ratio, *P. galatheae* produced 2.7-fold more holomycin per cell in co-culture as compared to monoculture, although this difference was not statistically significant at an $\alpha = 0.05$ (Tukeys test: $p = 0.067$) (Fig. 2A). Surprisingly, we also observed an increase in andrimid production by *V. coralliilyticus* (Fig. 2B); in co-culture with *P. galatheae*, *V. coralliilyticus* produced 4.3-fold more andrimid per cell compared to monocultures (Tukeys test: $p < 0.001$). Thus,

while the increase in holomycin production by *P. galatheae* was not statistically significant, both strains increased their antibiotic secondary metabolite production when co-cultured. In addition to andrimid, LC-MS/MS analyses revealed two novel andrimid analogues to also be present in the co-cultures (Fig. S3). The two analogues were tentatively identified as an oxygenated analogue, hydroxyandrimid ([M+H]+ = 496.2451, C27H33N3O6, calc. m/z = 496.2442) and a chlorinated analogue, chloroandrimid ([M+H]+ = 514.2109, C27H32N3O5Cl, calc. m/z = 514.2103) (Fig. S3). Based on MS/MS experiments, tentative structures for both analogues could be proposed, since the three andrimid analogues show highly similar fragmentation patterns. Notably, a key fragment (m/z 193.0972), only observed for hydroxy- and chloroandrimid supports the tentatively suggested structures, only allowing placing the hydroxyl and chlorine groups on the methyl group of the five membered ring (Fig. S4).

Effect of andrimid on holomycin production in co-culture. Holomycin production by *P. galatheae* was measured in co-cultures with *V. coralliilyticus* WT and the *Δadm* mutant strain respectively (Fig. 2A). *P. galatheae* produced 2.4-fold more holomycin in co-culture with *V. coralliilyticus* WT compared to co-culture with *Δadm*, but the difference was not statistically significant ($p = 0.091$, Tukeys test). To investigate if holomycin played a role in the increase in andrimid production by *V. coralliilyticus* in co-culture, andrimid production was measured in co-cultures with *P. galatheae* WT and *ΔhlmE.* Andrimid production in co-cultures of *V. coralliilyticus* with *P. galatheae* WT and the holomycin deficient mutant *ΔhlmE* were similar (Fig. 2B). This result was corroborated by addition of pure holomycin to monocultures of *V. coralliilyticus* and subsequent measurement of andrimid concentration; none of the tested holomycin concentrations (1, 3 and 5 μM) had a stimulatory effect on andrimid production in monoculture of *V. coralliilyticus* (Fig. S5).

***V. coralliilyticus* detoxifies holomycin by methylation.** The LC-MS data of extracts from monocultures and co-cultures were compared and a compound that was only present in co-culture extracts was observed (Fig. 3). Using HRMS analysis, the compound was determined to have the molecular formula $C_9H_{12}N_2O_2S_2$, based on an observed $[M+H]^+$ = 214.0414 (calc. m/z = 214.0413), with the isotopic pattern confirming the presence of the two sulphur atoms. Furthermore, the compound had similar fragment ions compared to holomycin, when analyzed by MS/MS. Li *et al.* reported a peak with the same m/z value and assigned this to a dimethylated form of holomycin (Li *et al.* 2012) (calculated m/z = 245.0413). The role of *V. coralliilyticus* in the production of this compound was explored by adding pure holomycin to monocultures of *V. coralliilyticus*. The same peak corresponding to dimethyl-holomycin was observed in these culture extracts, demonstrating that *V. coralliilyticus* methylates holomycin. To test the (relative) toxicity of dimethyl-holomycin towards *V. coralliilyticus*, dimethyl-holomycin was synthesized from holomycin, and the MIC values of both holomycin and dimethyl-holomycin were measured. Holomycin was a potent antibiotic against *V. coralliilyticus* with a MIC value of 20 µM (4.3 µg/mL), whereas dimethyl-holomycin did not show growth inhibition at the concentrations tested (MIC > 320 µM).

Discussion

Co-cultivation of microorganisms is a widely used method to study microbial interactions (Nai and Meyer 2018) and has also been employed to induce production of secondary metabolites (Bertrand *et al.* 2014; Arora *et al.* 2020). In this work, we studied the competition and antibiotic mediated interactions between two marine *Vibrionaceae* that inhabit similar niches, with a focus on their antibiotic secondary metabolite production.

Co-occurrence of *V. coralliilyticus* and *P. galatheae* in the marine environment was demonstrated by homology searches through publicly available microbiome sequence data. Homology searches of the 16S rRNA gene of *V. coralliilyticus* and *P. galatheae* showed that especially *V. coralliilyticus* strains (≥99% sequence identity) are abundant in e.g. sediment and bivalve microbiomes. *P. galatheae* (or closely related) strains were less abundant than *V. coralliilyticus*, although sequences with a 98% identity to the 16S rRNA of *P. galatheae* were identified in e.g. sediments. The presence of sequences with high identity to *P. galatheae* in sediments (the isolation environment of *V. coralliilyticus* S2052), and sequences with high identity to *V. coralliilyticus* in mussels (the isolation environment of *P. galatheae* S2753) further demonstrates the likeliness of co-occurrence of these two species. The co-occurrence analysis was based on the 16S rRNA gene, which is not the preferred phylogenetic marker for species identification in *Vibrionaceae* due to low sequence divergence (Sawabe *et al.* 2013; Machado and Gram 2015) and the data should thus be interpreted carefully. Despite these shortcomings, 16S rRNA gene sequence databases are still the most widely available sequence repositories that can serve the purpose of our study.

Vibrionaceae often show antagonistic activity (Gram, Melchiorsen and Bruhn 2010; Long *et al.* 2005) which seems to be an important trait for their ecological population structure (Cordero *et al.* 2012). Thus, co-cultivation of *V. coralliilyticus* and *P. galatheae* represents an interesting model to study antibiotic mediated interactions that bear ecological relevance. It is however noted that co-abundance of two phylogenetically close species is not proof of ecological interactions *per se* (Blanchet, Cazelles and Gravel 2020).

Establishing co-cultures of *V. coralliilyticus* and *P. galatheae* in which the abundance of both strains was stable was very sensitive to inoculation ratios. *P. galatheae* outcompeted *V. coralliilyticus* when inoculated at OD600 ratios of 2:1 and lower (1:1, 1:2, etc., *V. coralliilyticus*:*P. galatheae*), despite similar maximum growth rates (Fig. S1). Such

observations in liquid co-cultivations are common but are often, at least partly, the result of exploitative competition (Hibbing *et al.* 2010) in which one of the strains grows significantly faster than the other (Chignell *et al.* 2018; Khan *et al.* 2018; Sathe and Kuemmerli 2020), and that was not the case in this study. Sampling at multiple time points showed decreasing abundance of *V. coralliilyticus* cells in the stationary phase, indicating interference competition. Holomycin production by *P. galatheae* was a component of the observed late stage interference competition, albeit not the sole mechanism as the *ΔhlmE* mutant was also able to decrease *V. coralliilyticus* abundance. Likewise, the andrimid deficient mutant reached a lower cell density in the co-cultivations compared to *V. coralliilyticus* WT. These results add to other examples showing the importance of antibiotic secondary metabolites in microbial interactions and competition (Moons *et al.* 2006; Chandler *et al.* 2012; Dragos *et al.* 2020).

Beyond the impact of antibiotic secondary metabolites on growth dynamics and competition, we focused on interaction mediated induction of secondary metabolite production. First, it was observed that both bacteria increased their antibiotic production in co-cultures compared to monocultures. These findings support the idea that competitive interactions play an important role in the production of antibiotics (Traxler *et al.* 2013; Abrudan *et al.* 2015; Netzker *et al.* 2018). In *V. coralliilyticus* and *P. galatheae*, expression of BGCs and production of antibiotics is also influenced by carbon source as growth on chitin increased production of andrimid in *V. coralliilyticus* and holomycin in *P. galatheae* (Giubergia *et al.* 2017). Chitin is an important source of carbon and nitrogen in the marine environment (Gooday 1990) and since both chitin and the antibiotic production of a fellow Vibrionaceae enhances antibiotic production, bacterial competition for access to chitin could be an important ecological cue for antibiotic secondary metabolite production in these *Vibrionaceae*.

We recently demonstrated that andrimid, purified from *V. coralliilyticus* cultures and added in sub inhibitory concentrations, induced transcriptional activity of biosynthetic gene clusters and production of holomycin in *P. galatheae* (Buijs *et al.* 2020). It was therefore expected that andrimid would play an important role in the increase of holomycin production by *P. galatheae* in the co-culture experiment. Compared to the system with both wild type antibiotic producers, we measured a decrease in holomycin production by *P. galatheae* in co-culture with *V. coralliilyticus Δadm*. However, because the observed difference was not statistically significant, we cannot conclude that andrimid production by *V. coralliilyticus* is the key driver for increase in holomycin production by *P. galatheae* in our co-culture system. Possibly, andrimid is a component of the co-culture dependent induction of holomycin production, next to additional unknown factors. Our measurements are not sufficiently sensitive to test this hypothesis. Co-cultivation of *V. coralliilyticus* WT and the *P. galatheae ΔhlmE* showed that holomycin was not important for andrimid induction in co-culture.

How do *V. coralliilyticus* and *P. galatheae* sense each other and respond with an increase of antibiotic production? The competition sensing hypothesis predicts that nutrient limitation and cell damage are important cues (Cornforth and Foster 2013). Presumably, nutrient limitation will occur equally in mono and co-cultivations and holomycin induced cell damage of *V. coralliilyticus* did not increase production of andrimid. Although speculative, interference competition by alternative mechanisms such as type VI secretion mediated attack (Guillemette *et al.* 2020) may have been a relevant cue to sense competition. In contrast, the antibiotic hygromycin A of *Streptomyces hygroscopius* induced violacein production by *Chromobacterium violaceum* in a co-culture (Lozano *et al.* 2020). Other examples of induction of secondary metabolism in co-cultivations demonstrate specific roles for iron competition and cell-cell contact (Schroeckh *et al.* 2009; Lee *et al.* 2020). Taken together,

microbial interaction dependent induction of secondary metabolism is regulated by a variety of cues and mechanisms.

Besides the co-culture interactions leading to the increased production of antibiotic secondary metabolites, we also observed dimethyl-holomycin in co-cultivations and found that *V. coralliilyticus* was responsible for production of dimethyl-holomycin. *S. clavuligerus*, a native producer of holomycin, produces dimethyl-holomycin when lacking the *hlmI* gene, which is responsible for oxidizing the toxic intermediate dihydroholomycin into the closed bicyclic disulfide form (Li and Walsh 2011; Li *et al.* 2012). Li *et al.* also determined that dimethylated holomycin is less toxic to *S. clavuligerus* strains (Li *et al.* 2012). Likewise, our experiments show that *V. coralliilyticus* is not sensitive to dimethyl-holomycin, but is inhibited by holomycin. We speculate that *V. coralliilyticus* methylates holomycin as a detoxification strategy to protect itself from holomycin produced by *P. galatheae*. It should be noted that S-methylation also occurs in the production of the secondary metabolite tropodithietic acid by *Phaeobacter inhibens*, and that the addition of the methyl group makes the compound less toxic (Phippen *et al.* 2019). Detoxification of antagonistic secondary metabolites has been reported before (Völkl *et al.* 2004; Thierbach *et al.* 2017; Venkatesh and Keller 2019), but documented cases with production and detoxification in one (co-culture) system are scarce. One example is the conversion of phenazine, produced by *P. aeruginosa*, by the fungus *Aspergillus fumigatus* during a solid co-culture setup (Moree *et al.* 2012). However, besides detoxification by sulfonation, conversion of phenazine by *A. fumigatus* also produced compounds with a higher toxicity. To the best of our knowledge, this is the first example of an antibiotic production-detoxification interaction between two *Vibrionaceae*.

Overall, our results demonstrate the importance of antibiotic as mediators of competition between *V. coralliilyticus* and *P. galatheae*. Furthermore, the competitive

interaction in co-culture resulted in increased antibiotic production by both marine *Vibrionaceae*.

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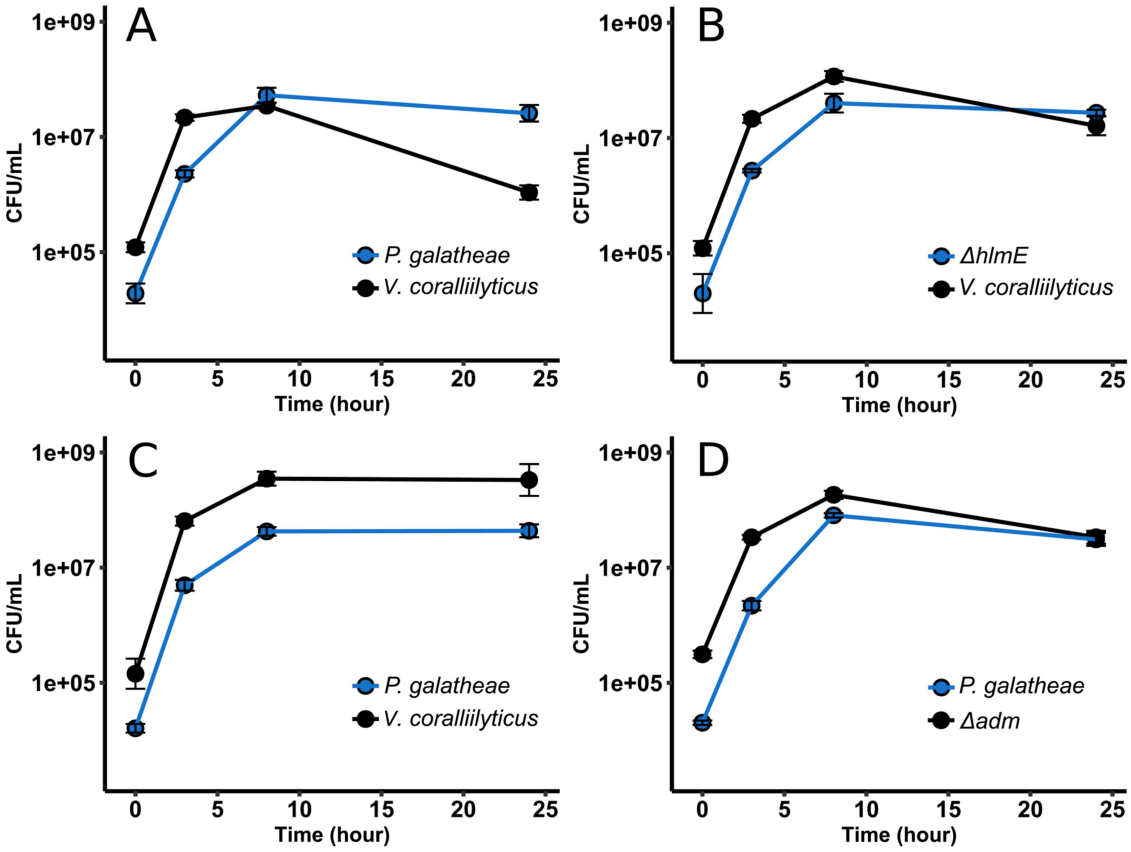

Figure 1. Growth of *V. coralliilyticus* and *P. galatheae* during co-cultivation inoculated at an OD600 ratio of 2:1 (A), *V. coralliilyticus* and *P. galatheae ΔhlmE* inoculated at a ratio of 2:1 (B), *V. coralliilyticus* and *P. galatheae* inoculated at a ratio of 6:1 (C) and *V. coralliilyticus Δadm* and *P. galatheae* inoculated at a ratio of 6:1 (D). Data points are means of four biological replicates and error bars represent the standard deviations.

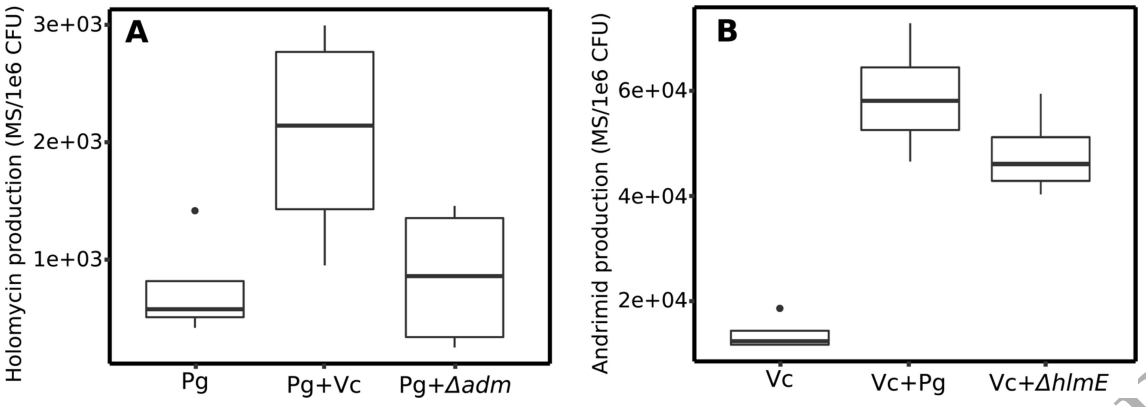

Figure 2. Holomycin production by *P. galatheae* (A) and andrimid production by *V. coralliilyticus* (B) are increased in co-cultures. Data displayed in A are from co-cultures inoculated at an OD600 ratio of 4:1 (Vc:Pg), co-cultures corresponding to data in B were inoculated at a ratio of 6:1. Vc = *V. coralliilyticus* WT, Pg = *P. galatheae* WT. Data are from four biological replicates.

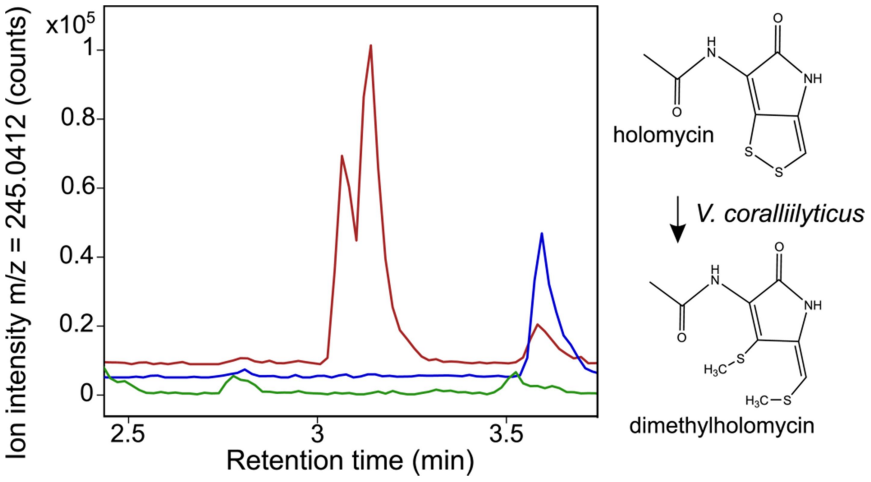

Figure 3. *V. coralliilyticus* dimethylates holomycin from *P. galatheae*. Representative overlaid MS chromatograms of the dimethyl-holomycin ion of culture extracts from co-culture (red trace), *V. coralliilyticus* monoculture (blue trace) and *P. galatheae* monoculture (green trace). Overlaid traces are shifted vertically for improved visualization.

Strain	Description	Source
P. galatheae S2753	Wild type, isolated from a mussel.	(Gram, Melchiorsen and Bruhn 2010; Machado *et al.* 2015)
V. coralliilyticus S2052	Wild type, isolated from marine sediment.	(Gram, Melchiorsen and Bruhn 2010)
P. galatheae ΔhlmE	Holomycin deficient mutant of *P. galatheae* S2753. *ΔhlmE.*	Zhang *et al*.
V. coralliilyticus Δadm	Andrimid negative mutant of *V. coralliilyticus* S2052. Loss of 224 kb extrachromosomal replicon.	This study
E. coli GBdir-*pir116*	DH10B, *fhuA*::IS2, Δ*ybcC,* Δ*recET*, *pir116*	(Wang *et al.* 2016)
E. coli WM3064	*thrB1004 pro thi rpsL hsdS lacZΔM15* RP4-*1360 Δ(araBAD)567 ΔdapA1341::[erm pir]*	Strain constructed by William Metcalf at UIUC

Table 1. List of strains used in this study.

Table 2. Abundance and similarity percentages of 16S-rRNA sequences of *V. coralliilyticus* S2052 and *P. galatheae* S2753 in publicly available microbiome databases.

Microbiome dataset	Highest identity match % (n[1])		Same sample(s)?	Ref	SRA accession	Amplicon lengths
	P. galatheae	*V. coralliilyticus*				
Mussel *Mytilus coruscus*	96% (2)	99% (3)	Yes	(Li *et al.* 2019a)	SRP196510	460 - 470 bp
Mussel *Mytilus galloprovincialis*	96% (1)	99% (3)	Yes	(Li *et al.* 2019b)	SRP197453	460 - 470 bp
Shellfish hatchery	98% (>499)	100% (>499)	Yes	(Gradoville *et al.* 2018)	SRP118403	490 - 491 bp
Sediments Chinese marginal seas	98% (2)	99% (>499)	Yes	(Wang *et al.* 2019)	SRP159585	545 - 550 bp
Oyster *Crassostrea gigas*	97% (18)	100% (13)	No	(King *et al.* 2019)	SRP139423	500 - 501 bp

[1]: n = number of reads with this identity percentage