Review

Heterogeneity in Bacterial Specialized Metabolism

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Abstract

Specialized metabolites (SMs) like typical antibiotics, signaling molecules or other bioactive compounds of bacterial origin (sometimes also used in human therapy) are often complex natural products that are costly for the cell to make. Several bacterial taxa are known to produce multiple SM classes in parallel and therefore a division of labor within a clonal population of bacteria might be beneficial. In this review, examples of SM of gram-negative and gram-positive bacterial taxa that are produced by different cell types are presented, and the possibility that such a heterogeneity is more widespread in SM biosynthesis is discussed. In addition, tools to study SM production at the single cell level are presented.

Introduction

Natural products of low molecular weight produced by bacteria, fungi or plants are used since ancient times in human society as drugs. Currently, we heavily rely on such molecules especially for anti-infective, anti-cancer and immune-suppressive therapies, since up to 60% of all clinically used drugs are natural products or derivatives thereof [1,2]. In contrast to our strong dependence on these molecules, we know very little about their natural function and the regulatory mechanisms underlying their production has only been extensively investigated in select genera.

Originally, these molecules were termed "secondary metabolites," derived from species-specific pathways, in order to distinguish them from "primary metabolites" common among all organisms [3]. Since they often have no obvious benefit if the producing organism is cultivated alone in rich medium, as was often done in the golden age of natural product research between 1940 and 1970, they were considered "secondary." However, with increasing knowledge regarding organismic interaction, chemical and microbial ecology, it became clear that these molecules are indeed not of secondary importance but have vital functions for the producer, and therefore, the term "specialized metabolites" (SMs) seems to be more appropriate [4].

While essentially all specialized molecules used as clinical drugs have been found by traditional and very successful methods that can be summarized as "grind and find" [5], the hope is that the elucidation of the true ecological function and underlying mechanism leading to their production might allow a more rational approach to find desperately needed new drugs in order to treat emerging diseases, to overcome resistances and to cause fewer side effects.

It is now clear that several prolific SM producers exist in nature including several fungi and bacterial genera. Examination of genome sequences of some families has revealed that they are able to produce multiple SMs, with some strains identified that have the capability to produce >30 different SM types [6,7]. Besides the knowledge gap regarding the structure, function and regulation of these SM, it is also unclear how the producing organisms coordinate the production of these SMs, of which several can be produced in parallel.

This review aims to summarize the role of phenotypic heterogeneity for bacterial SM, and the underlying principles and tools to address them. Although phenotypic heterogeneity has been described for

several aspects of bacterial physiology [8–10], including primary metabolism [11], specialized metabolism has hardly been addressed.

Bacterial SMs

Bacterial SMs in general are small molecules that provide a specific functionality for the producer. These functionalities are broad and may include anything from antibiotic to cell–cell communication. SMs can be found in several bacterial phyla and are of particular interest due to their frequent (but not always) effectiveness as clinical drugs. Actinobacteria currently represent the most important SM producers in the clinic, with antibiotics (erythromycin, tetracycline, vancomycin, kanamycin), anti-fungal (amphotericin, candicidin), anti-cancer (doxorubicin, bleomycin) or immune-suppressive drugs (rapamycin, FK506) all originating from the Actinobacteria [1]. Genome sequencing projects of model Actinobacteria like *Streptomyces coelicolor* or *S. avermitilis* revealed that these bacteria have the ability to produce many more than the already known SM [6], and therefore, they are often regarded as "gifted" or "talented" strains [12]. Similar talents were also found in other bacteria like myxobacteria [13], cyanobacteria [14], *Pseudomonas* [15], *Photorhabdus* and *Xenorhabdus* [16], which have all been a rich source for novel SMs in the last 20 years. These gifted multi-producers of several different SMs in a single strain aside, most bacteria do carry at least one or two BGCs, often involved in the production of iron-binding siderophores. Examples of typical BGCs have even been found in anaerobic bacteria such as clostridia [17,18], as well as in archaea [19].

The ability to produce SMs in general can be easily identified by biosynthetic gene clusters (BGCs) encoded in their genomes since most SMs are derived from only a few basic biochemical pathways. Among these pathways are polyketide synthases (PKSs) [20], non-ribosomal peptide synthetases (NRPSs) [21], terpene synthases and cyclases [22], BGCs involved in the production of ribosomally synthesized and post-translationally modified peptides (RiPPs) [23] and BGCs encoding mixtures of all these pathways. Given the ability to mix pathways, the variety of potential products is immense, with new compounds and their derivatives being regularly discovered.

A number of structurally diverse molecules have been identified to be involved in quorum sensing (QS), which is the intra- or inter-specific cell–cell communication to sense population densities [24]. For this, bacteria produce, secrete and detect low-molecular weight compounds named autoinducers (AIs). Examples for AIs in gram-negative bacteria are *N*-acyl homoserine lactones (AHLs), α-hydroxyketones, quinolone-like compounds or fatty acid derivatives, while from gram-positive bacterial peptides and γ-butyrolactones are known (Fig. 1) [25,26]. Several of the identified AIs are in fact SMs, derived from typical SM pathways as highlighted by photopyrones and dialkylresorcinols from *Photorhabdus* [27,28]. Indeed valdiazen (Fig. 1), a novel class of QS signal, was recently identified from *Burkholderia cenocepacia* H111. It is derived from the NRPS-based biosynthesis pathway of the antifungal compound and metallophore, fragin, that it also regulates [29].

Since the biosynthesis of several SMs involved in virulence or specific growth phases is QS dependent, it is not surprising that QS and SM are tightly connected making the manipulation of QS an attractive target for applied microbial ecology or microbiome manipulation [25,30]. In addition, it is now clear that SMs represent an important part of all microbiomes including the mammalian gut microbiome where SMs contribute to the shaping of the microbiome and the interaction with their host organism [31–34]. There are also several examples where SMs were only produced in mixed cultures or upon the addition of other microbes' SM [35]. These points raise the question of what ecological role these molecules are playing in complex environments.

Specialized Metabolism and Phenotypic Heterogeneity, Division of Labor and Phase Variation in Bacteria

Bacteria, as any organism, must have the ability to respond to changing environments. Examples of such strategies are sporulation/spore germination, biofilm formation/escape from biofilms, motility or change in other traits. Several of the bacteria that are mentioned above and that are talented SM producers show examples of such strategies like heterocyst formation in cyanobacteria, fruiting body formation in myxobacteria, substrate and aerial mycelium formation and sporulation in *Streptomyces* or biofilm formation in *Pseudomonas* or other proteobacteria. SMs are known that are only produced during specific growth phases or in specific cell types with spore pigments in *Streptomyces* being classical examples.

In cyanobacteria heterocysts accumulate granules of cyanophycin (Fig. 2), a non-ribosomally made polymer of arginine and asparagine used for nitrogen storage, while heterocysts do not produce the carotenoids required for photosynthesis. In the myxobacterium *Myxococcus xanthus* unusual ether lipids [36] are produced in myxospores but not in the peripheral rods surrounding the fruiting bodies [37] and cells that are destined to develop into myxospores accumulate lipid bodies composed of different lipid classes not found in other cell types [38]. *M. xanthus* also shows a phase variation between *yellow* cells [39] that produce the pigment DKxanthene as well as the antibiotic myxovirescin and are prolific swarmers, and *tan* cells that do not produce both SM but instead

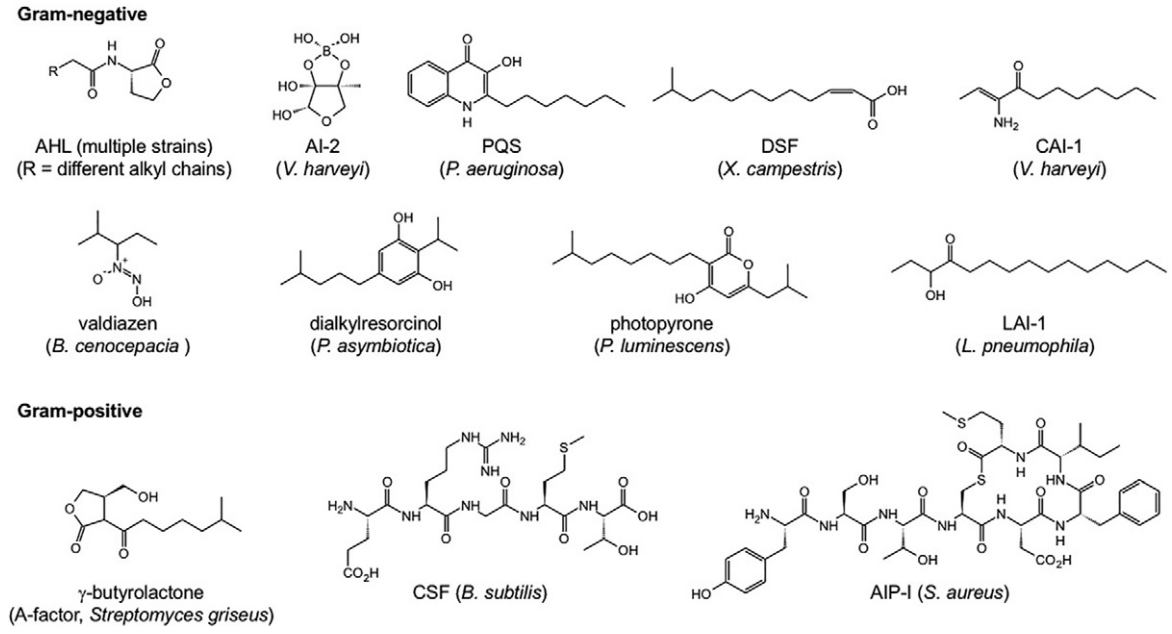

Fig. 1. Selected SMs acting as QS signals in gram-negative and gram-positive bacteria.

produce the siderophore myxochelin and are only mediocre swarmers [40]. Interestingly, the *tan* cells predominantly end up as myxospores [40] but require the DKxanthene produced by the fruiting body surrounding peripheral rods for the generation of fully functional spores [41].

In biofilms of *Bacillus subtilis* ultimately leading to endospore formation [42], a subset of cells produce surfactin [43], an NRPS-derived peptide acting as an antimicrobial SM and signal, triggering production of the biofilm matrix. Meanwhile other cells (not producing surfacting) produce this matrix and the structural protein, TasA, that assembles into amyloid fibers that attach to the cell walls [44]. In contrast to typical signaling in bacteria where the same cells usually produce and respond to the produced signal (see below), surfactin acts as a paracrine signal that induces a phenotype only in other cells [43].

Biofilms of *Pseudomonas aeruginosa* PAO1 show a typical mushroom-like architecture with a stalk composed of non-motile cells and a cap with motile cells [44]. These sub-populations interact also at the level of SMs in that the stalk cells produce the siderophore pyoverdine and the QS signal PQS as common goods, both used by the cap cells [45].

In *Pseudomonas putida* IsoF, it has been shown that the NRPS-derived peptide putisolvin is required for motility and escape from the biofilm [46]. Its production is regulated only in a small fraction of cells within the biofilm by an AHL-based QS mechanism. This AHL production is stochastically regulated and here the AHL acts as an internal signal and not in neighboring cells. Therefore, only the AHL-producing cells also produce putisolvin, which is not a common good, but is used for biofilm escape of the individual AHL- and putisolvin-producing cell, in contrast to the usually assumed AHL-mediated group behaviour. The reason for this unexpected observation might be due to the very low number of AHL molecules (1–2 per cell) required for expression of putisolvin-producing genes. Recently, "self-sensing" has also been observed in *B. subtilis* indicating that such mechanisms are more widespread than initially thought [47].

Several other examples are known where the expression of AI synthase genes or QS-dependent genes is heterogeneous at a single cell level, even in supposed homogeneous cultures within isogenic populations [8], suggesting that the AI-dependent production of SM might also be heterogeneous. However, in contrast to all examples mentioned above that include heterogeneity due to different cell types spatially separated in biofilms, fruiting bodies or in filaments, so far the only example for SM heterogeneity in a homogenous culture is the production of anthraquinones (AQ) in *P. luminescens* (Fig. 2) [48]. The orange AQ pigments give *P. luminescens* its characteristic color, and they are produced by a type II PKS encoded by the genes *antABCDEFGHI* [49]. Downstream from the *ant* BGC lies *antJ,* encoding a transcription factor with a DNA-binding domain and a shortened WYL-domain that may act as a ligand binding domain. AntJ is a positive regulator of AQ production that directly binds to the *antA* promoter P_{antA}. Surprisingly, the P_{antA} activity is heterogeneous at the single-cell level with increasing amounts of AntJ leading to an increasing P_{antA} activity. Ultimately, this leads to all cells expressing the *ant* genes and

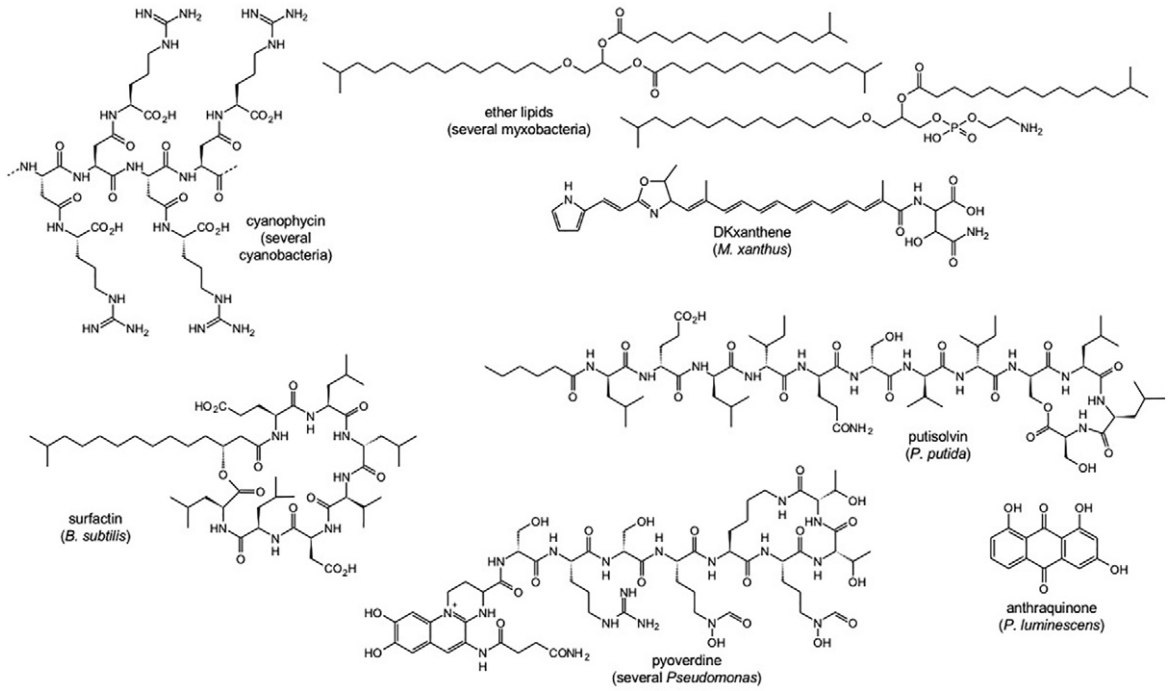

Fig. 2. Examples of heterogeneously produced SMs in different bacteria.

resulting in AQ overproduction upon overexpression of *antJ* [48] as was shown by stable integration of reporter gene constructs into the *P. luminescens* genome [50]. *P. luminescens* also demonstrates a phase variation between so-called primary and secondary cells that differ in multiple traits, including AQ-based pigmentation [51]. Analysis of both cell types revealed that they show a comparable transcript and protein level of AntJ, suggesting that the difference in P_{antA} activity might be mediated via a ligand with fluctuating concentrations similar to the putisolvin production mentioned above [46].

Besides the already mentioned low amount of signaling molecules (AI or transcription factors) leading to stochastic gene expression that are a consequence of the burst-like nature of transcription, other mechanisms might be the cause of the phenotypic heterogeneity. This includes periodic oscillations, the cellular age of cells that divide asymmetrically, or response to cues from other cells (as in QS) [9]. Recently, stochastic gene expression has been shown in exponentially growing *B. subtilis* cultures that divide into distinct interacting metabolic sub-populations that produce and release distinct metabolic products including toxic byproducts [11]. If such a division of labor regarding the primary metabolism is frequent in clonal populations, wouldn't it be even more likely for the energy consuming specialized SM? Especially since it was recently shown that growth rate of cross-feeding populations that trade certain cellular building blocks can be significantly increased [52].

The Need for Phenotypic Heterogeneity for SM Production?

The contribution of SMs to microbial ecology [53,54] as well as to human health [55] is still an under-explored field, but accepted in principle. It suggests that the microbiome or any microbial community including the SMs produced and exchanged among their individual members might be regarded as a functional unit that as a whole performs the tasks required for the respective situation in time and space.

What has not been studied at all is the role of genetically identical or clonal cells of a single species with respect to SM production. From analyses of multiple strains from different genera [56–58] using mass spectrometry-based methods and computational analysis [59], it has become obvious that, especially in talented strains, multiple SMs are produced in parallel even under artificial lab conditions.

The genomic content required for the production of all SMs in a given strain can reach up to 10%. Single BGCs involved in the production of one SM class can easily be >80 kbp resulting from the giant PKS or NRPS they encode. The megasynthases are among the largest enzymes known and in *Photorhabdus luminescens* the 1.81-MDa NRPS Kol is currently the largest bacterial NRPS and the corresponding 49.1 kbp *kol* gene represents 1% of the *P. luminescens* genome. The Kol NRPS is composed of 15 modules for the formation of a linear 15-mer peptide with alternating D- and L-configuration and has overall 45

catalytically active domains as part of a single protein [60]. Assuming the speed of *Escherichia coli* for transcription (80 nt/s) and translation (20 aa/s) (http://book.bionumbers.org/), the production of Kol might take longer than the actual growth rate. Also taking into account the energy required for the generation of the required building blocks including NTPs for transcription, charged tRNAs for translation and activated amino acids required as building blocks for the actual peptide synthesis, it is clear that the parallel production of several such SMs needed for various interactions in complex environments is very costly. Theoretical modeling of QS [61], assuming growth impairment resulting from AI production [62] as was experimentally confirmed [63,64], showed that ecological and population dynamics are coupled, leading to phenotypic heterogeneity in microbial populations.

Even when resistance mechanisms are encoded as part of SM producing BGCs, SM production can impair the growth of the producing cells when the SM is produced inside the cell as it is usually the case. However, cells not producing the SM might be protected against the SM, since it cannot enter the target (e.g., ribosome, RNA polymerase) inside these cells easily due to the structure of the cell wall, which it cannot penetrate once secreted by the producer cell. This is especially true for gram-negative bacteria and is probably also the reason why it is easier to find antibiotics against gram-positive bacteria.

SMs are often made of initial and basic structure intermediates that are subsequently decorated with sugars or are modified by additional enzymes. In addition, unusual building blocks (e.g., amino acids or carboxylic acids, sugars) are required that need to be synthesized first. These intermediates or building blocks can often be exchanged between cells, which is an outcome of several precursor directed biosynthesis or mutasynthesis experiments that were used to generate non-natural SM derivatives [21,65]. Therefore, division of labor of different cell types for the production of one SM might also be possible as it was shown for the primary metabolism in *B. subtilis* recently [11].

In complex environments such as biofilms or as a part of a microbiome, one can see that heterogeneity in terms of division of labor would be useful for bacteria, with close proximity to neighboring cells facilitating easy resource sharing. Depending on the function of the SM, there are other possible reasons for heterogeneity in populations. These include differences in nutrient availability (or a change in nutrient source as demonstrated in *Lactococcus* [66]), the age of bacterial cells or even the occurrence of spontaneous mutants that may be "supported" by the population. The energy cost of (some) SMs would be prohibitive for an entire population as discussed above and although division of labor has been demonstrated in biofilms, it has yet been shown to be beneficial with respect to SM production under ecologically "real" conditions. Therefore, it is imperative that we develop new approaches to understanding the specific reason(s) and regulatory mechanisms that lead to these phenomena in microbial communities.

Experimental Approaches to Study SM Heterogeneity

In order to study any aspect of phenotypic heterogeneity, single cells must be studied. Often, reporter gene assays based on the production of fluorescent proteins are used that should be integrated into the genome to ensure stability and to avoid plasmid heterogeneity as observed previously [50]. These reporter constructs can be integrated at a neutral site in the genome (if one is indeed available) or at the genomic locus of the original promoter. However, low numbers of transcription factors or their ligands might result in stochastic gene expression as outlined above. Furthermore, the introduction of a second promoter might change the heterogeneity outcome. Alternatively, when the original gene is replaced for a reporter gene, this problem can be avoided, but no SM can be produced anymore due to the loss of the biosynthesis enzyme. Therefore, the only possibility is to use translational fusions between genes encoding a biosynthesis enzyme involved in SM production and reporter genes. Although this approach must ensure that the fusion enzyme is fully functional, the multidomain character of several SM enzymes belonging to the NRPS or PKS family [20,21] might suggest that an additional "reporter domain" would not impair the enzyme function, since these enzymes can also be engineered using domain exchanges [67]. Such an approach was pioneered to localize the PKS machinery responsible for the production of bacillaene in *B. subtilis* [68]. However, in order to maintain the required protein–protein interactions often mediated by specific docking domains [69] between the different PKS and/or NRPS enzymes that form the functional multienzyme complex, the best fusion point would be the at the C-terminal end of the last megaenzyme of the biosynthesis pathway of interest. This should result in fully functional enzymes containing C-terminal reporters allowing the correlation between protein production (and reporter activity) and SM production.

When this approach was applied to different NRPS from *Photorhabdus* or *Xenorhabdus*, fully functional NRPSs that showed no difference in either the timing or amount of SM production were indeed obtained (Yvonne Engel, Samine Atri, Christoph Spahn, Mike Heilemann, H.B. Bode, unpublished results). As an example, the fluorescence of a Ypet fusion at the C-terminal end of the ririwpeptide (Fig. 3a) producing NRPS Plu3123 (Fig. 3b) is shown that is induced

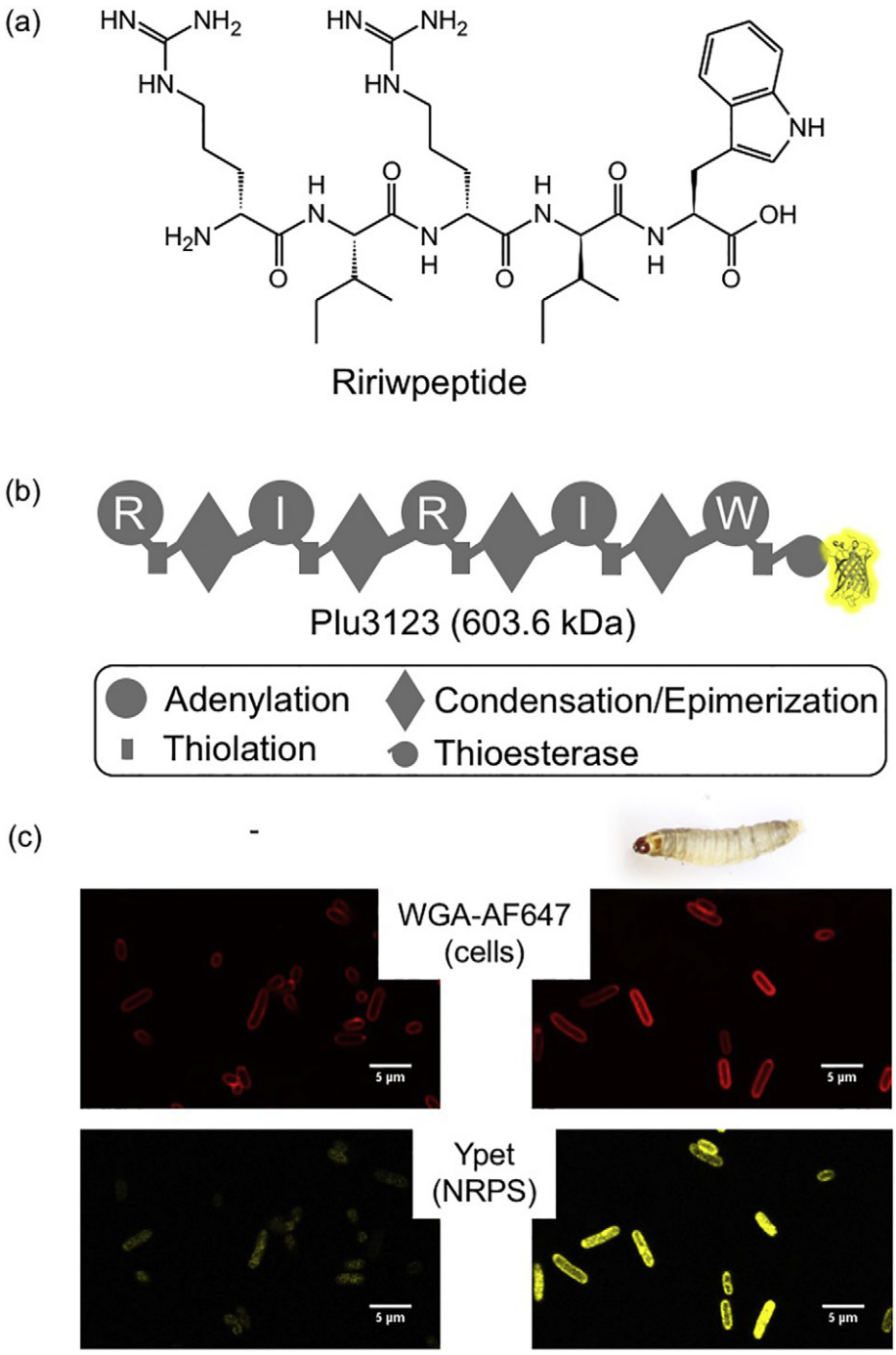

Fig. 3. Fusions between biosynthetic enzymes and reporter proteins as tools to analyze SM heterogeneity. Structure of ririwpeptide (a), the domain structure of the ririwpeptide-producing NRPS Plu3123 (b), and *plu3123-ypet* expression in medium with and without insect lysate using a genomic *plu3123-ypet* fusion in *P. luminescens* (c). Domain organization and amino acid specificity (in standard one letter code) of adenylation domains are shown.

upon cultivation of *P. luminescens* in medium containing insect lysate (Fig. 3c). When the same approach with a Ypet and a mCherry fusion was applied to two NRPS systems in *Xenorhabdus nematophila* producing rhabopeptides and xenortides, respectively, heterogeneous production of both NRPS systems could also be detected (Fig. 4).

What is currently missing are simple approaches that also allow SM detection at the single cell level in order to correlate enzyme and SM production.

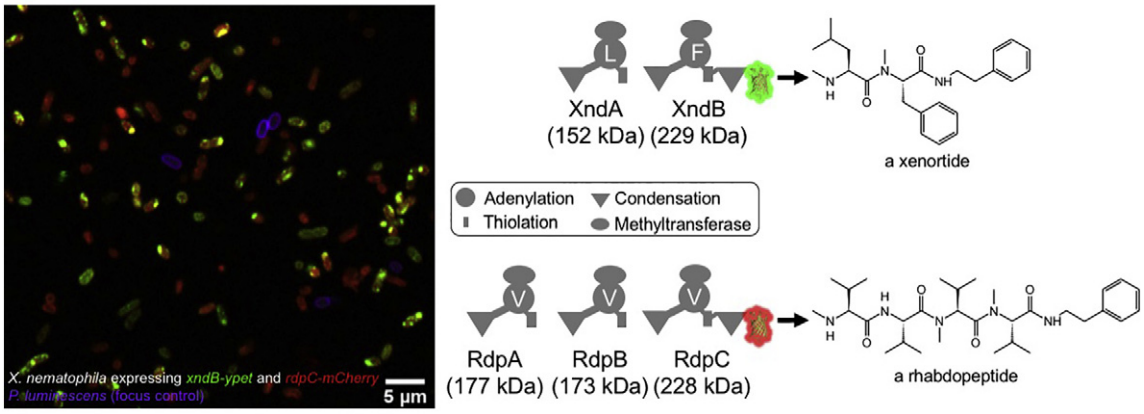

Fig. 4. Heterogeneity of SM production in *X. nematophila* grown in LB medium in shaking flasks. Parallel production of NRPSs producing xenortide and rhabdopeptide using dual *xndAB-ypet* (yellow) and *rdpABC-mCherry* (red) fusions in *X. nematophila*. Blue cells are *P. luminescens* cells labeled with WGA-AF647 that were needed for focusing. Domain organizations and selected SM derivatives produced by both NRPS systems are shown.

In cases where the respective SMs are not secreted into the environment but bind to the producing cell this might be feasible if the SM can be specifically labeled with stable isotopes or using specific building blocks. This would allow SM detection using Raman microscopy or nanoscale secondary ion mass spectrometry (nanoSIMS) that both have a resolution more suited to these types of studies than other MS-based imaging methods [70,71]. As an example, Raman microscopy-based detection of rhabduscin in *X. nematophila* and a heterologous *E. coli* system producing this SM has been achieved, since rhabduscin contains a rare isonitrile moiety that shows a characteristic Raman resonance [72].

Conclusion

Phenotypic heterogeneity with respect to SM production might be a widespread phenomenon in bacteria. Not only for bacteria with different cell types that can be relatively easily differentiated from each other as in biofilm-forming species or filamentous cyanobacteria, but also for bacteria showing homogenous growth. Here the goal must be (i) to develop and apply tools for SM detection at the single cell level and not only to detect the biosynthesis enzymes, (ii) to elucidate the mechanisms that lead to this heterogeneity in order to understand its biological function and (iii) to manipulate this heterogeneity in order to, for example, increase the biotechnological production of desired SMs.

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Abbreviations used:
SM, specialized metabolite; BGC, biosynthetic gene cluster; PKS, polyketide synthases; NRPS, non-ribosomal peptide synthetases; QS, quorum sensing; AI, autoinducer; AHL, *N*-acyl homoserine lactone; AQ, anthraquinone.

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