2 Metabolomics Approaches in Microbial Research: Current Knowledge and Perspective Toward the Understanding of Microbe Plasticity

Paulo R. Ribeiro, Rhaissa R. Barbosa, and Catherine P. de Almeida

2.1 Introduction

2.1.1 Microbial Metabolomics Research in the Post-genomic Era

The central dogma of molecular biology describes how genetic information stored within genes of a living organism flows into proteins: DNA → RNA → protein (Jafari et al. 2017). The advances conquered during the current post-genomic era have brought to light a paradigm shift in which new genetic research has enabled simultaneous analyses at the level of transcripts, proteins and metabolites (Illig and Illig 2018; Marcone et al. 2018; Singh et al. 2018; Van Der Heul et al. 2018). Enzymes are proteins, except for some catalytic RNA molecules, and their activity depends on several factors, including native protein integrity, conformation, pH, and temperature. Thousands of different enzymes work coordinately to ensure that all required chemical reactions occurs flawlessly within each individual cell (Raveendran et al. 2018; Scrutton 2017). The metabolic signature of an organism can be characterized by identifying the pathways (sets of enzymes) encoded in its genome. However, this metabolic signature is better assessed by applying advanced metabolomics approaches. Metabolomics encompasses the qualitative and quantitative analysis of the complete set of metabolites (metabolome) of an organism (Ribeiro et al. 2018). Therefore, by using a metabolomics approach it is possible to study key molecules of the metabolism of a given organism (Alcalde and Fraser 2016; Barkal et al. 2016; Bean et al. 2016; Beloborodova et al. 2018; D'Sousa Costa et al. 2015; Ribeiro et al. 2015). Metabolomics approaches in microbial research have allowed the identification and characterization of thousands of distinct chemical reactions that occur as the microorganism grow and divide. In general, microbial metabolomics researches

P. R. Ribeiro (✉) · R. R. Barbosa · C. P. de Almeida
Metabolomics Research Group, Departamento de Química Orgânica, Instituto de Química, Universidade Federal da Bahia, Salvador, Brazil
e-mail: pauloribeiro@ufba.br

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apply two different approaches: untargeted and targeted. The untargeted approach encompasses the qualitative or semiquantitative characterization of the entire metabolome without prior knowledge of the metabolites to be analysed, whereas the targeted approach focuses on specific metabolites. These two approaches may focus on intracellular (fingerprinting) or extracellular (footprinting) metabolites (Baptista et al. 2018; Götz et al. 2018; López-Gresa et al. 2017; Sander et al. 2017).

A general workflow of metabolomics in microbial researches encompass four consecutive steps: sampling, extraction, data acquisition, and data processing (Azzollini et al. 2018; Chatzimitakos and Stalikas 2016; Maansson et al. 2016). The sampling step requires the separation, mainly by centrifugation, and quenching of the intra- and extracellular metabolites produced by the microorganism in the biological material. The extraction step includes mechanical cell disruption and the use of buffers or organic solvents to obtain the intra- and extracellular metabolites. The data acquisition step requires the use of advanced chromatographic and spectroscopic tools to determine metabolite content within a sample as well as its molecular structure. In some cases, prior to the data acquisition step it is necessary to perform metabolite derivatization. The last step requires the use of sophisticated softwares and chemometric tools to perform data processing and extraction. At this stage, statistical analysis are applied to identify discriminant group of metabolites responsible for the overall alterations in the studied system (Fig. 2.1).

An important aspect of any metabolomics study is to fully characterize the metabolome of an organism. Therefore, in order to obtain a broad picture of the microbial metabolome, researches usually apply several chromatography and metabolite detection techniques. Gas and liquid chromatography (GC and LC, respectively) are frequently used for metabolite separation, whereas nuclear magnetic resonance (NMR) and mass spectrometry (MS) are the most used techniques for metabolite detection (Fernand et al. 2017; Ortiz-Villanueva et al. 2017; Schelli et al. 2017; Vinci et al. 2018; Wang et al. 2016).

2.1.2 Initial Analysis of the Microbial Metabolomics Studies

In the following section, we will present a detailed systematic literature review of the current knowledge, main findings, and perspective toward the understanding of

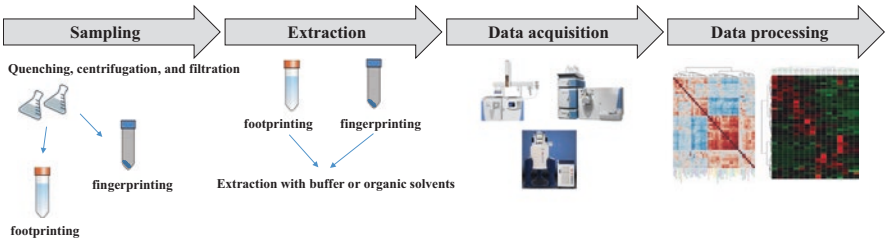


Fig. 2.1 Schematic representation of a general microbial metabolomics workflow

microbe plasticity in microbial metabolomics research. This book chapter covers manuscripts published between January 2014 and August 2018 that were available from scientific databases such as "Google Scholar," "PubMed," "ScienceDirect," "SpringerLink," and "Web of Science – Clarivate Analytics." The search through the scientific databases was performed using the keywords "metabolomics" or "metabolite profiling," along with "microorganism," "microbe," "fungi," "bacteria," "plasticity," and "biofilm." The chemical structures in this paper were drawn using ChemDraw Ultra 12.0.

Bacteria was, by far, the most well-studied microorganism with 44% of the published manuscripts, followed by fungi (30%), and lichen (7%) (Fig. 2.2a). Intracellular metabolome (fingerprinting) was assessed by 62% of the published manuscripts, whereas extracellular metabolome (footprinting) was assessed by 38% (Fig. 2.2b). Liquid chromatography–mass spectrometry (LC-MS) was used by 45% of the published manuscripts, followed by gas chromatography–mass spectrometry (GC-MS) (36%) and nuclear magnetic resonance (NMR) (19%) (Fig. 2.2c).

2.1.3 Metabolomics for Microbial Bioactive Metabolites

The worrisome diffusion of antibiotic-resistant microorganisms has encouraged new and advanced research to development new and more efficient antimicrobial metabolites (Bosso et al. 2018; Santos et al. 2018; Tracanna et al. 2017). Microorganisms constitute an important source of new bioactive metabolites that aid the development of new drugs and chemicals used for industrial and agricultural purposes (Honoré et al. 2016; Kildgaard et al. 2014; Romoli et al. 2014; Yogabaanu et al. 2017). Potentially new bioactive metabolites can be obtained by associating the discovery of new natural products with semisynthetic remodeling (Mgbeahuruike et al. 2017; Pintilie et al. 2018; Yang et al. 2018). Metabolomics is an important ally in drug discovery since it provides new strategies and methods to perform reliable and fast identification of new bioactive metabolites from different organisms

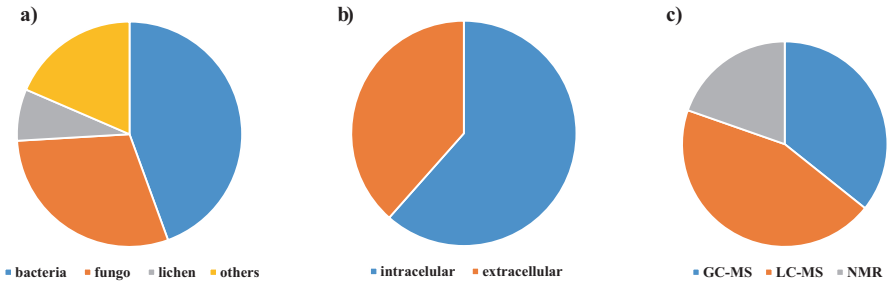


Fig. 2.2 (**a**) Type of microorganisms, (**b**) portion of the metabolome, and (**c**) techniques used for data acquisition of microbial metabolomics studies

(Bittencourt et al. 2015; Hakeem Said et al. 2017; Koistinen et al. 2018; Liao et al. 2018; Maansson et al. 2016; Santos et al. 2018).

An innovative approach using accurate dereplication by ultra-high performance liquid chromatography (UHPLC) and a high-resolution mass spectroscopy (HRMS) was used to find new bioactive metabolites from species of Aspergillus, Penicillium, and Emericellopsis from marine origin (Kildgaard et al. 2014). Dereplication techniques are key components of natural product screening and discovery since they allows rapidly and efficiently discrimination between previously known compounds and potential new bioactive metabolites within a crude extract (Hubert et al. 2017). Several metabolites were identified including small polyketides, non-ribosomal peptides, terpenes, and meroterpenoids. Four new metabolites related to asperphenamate were identified from *Penicillium bialowiezense* (Fig. 2.3). Asperphenamate is a natural anticancer phenylalanine dipeptide analog derivative with an *N*, *N*′-substituted phenylalanine–phenylalaninol ester framework (Liu et al. 2016b). Asperphenamate was initially isolated from *Aspergillus flavus* and later on from raw malt, which was used to treat hyperplasia of mammary glands (Clark et al. 1977), and it exhibits antitumor activity toward a number of cell lines (Li et al. 2012; Yuan et al. 2012). Therefore, dereplication by UHPLC-HRMS allowed the identification of potentially new bioactive metabolites.

Additionally, helvolic acid was identified in the culture of *Emericellopsis* sp. strain (IBT 28361). Helvolic acid is a nortriterpenoid first isolated from an endophyte fungal, *Xylaria* sp. (Fig. 2.4) (Ratnaweera et al. 2014). Helvolic acid showed antibacterial activity against *Bacillus subtilis*, *Enterococus faecalis,* methicillin-resistant *Staphylococcus aureus*, *Mycobacterium tuberculosis*, *Pseudomonas aeruginosa, Ralstonia solanacearum, Streptococcus pneumonia,*

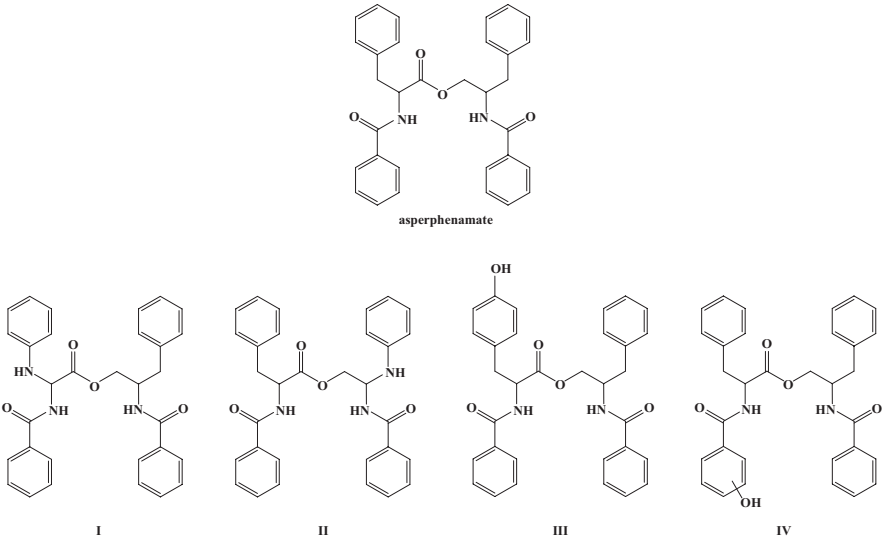


Fig. 2.3 Chemical structure of asperphenamate and its four new related metabolites (I–IV)

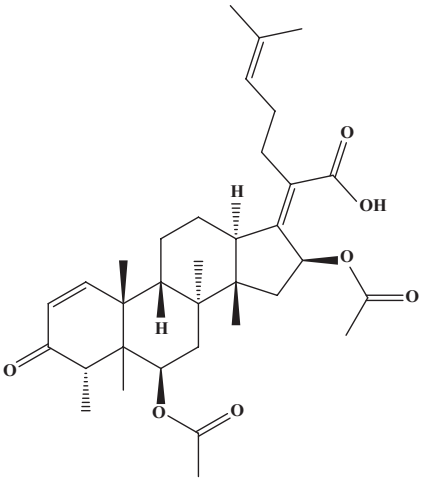


Fig. 2.4 Chemical structure of the antibacterial metabolite helvolic acid

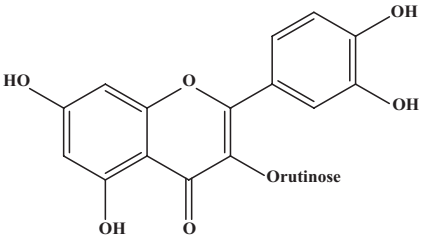


Fig. 2.5 Chemical structure of rutin

and *Xanthomonas campestris* (Luo et al. 2017; Ratnaweera et al. 2014; Sanmanoch et al. 2016; Yang et al. 2017).

Aspergillus flavus was isolated as an endophytic fungus from the Indian medicinal plant *Aegle marmelos* (Patil et al. 2015). Extracts produced from this fungus culture showed great antibacterial activity against *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella abony*, *S. typhi*, *Bacillus subtilis*, and *Staphylococcus aureus*. Additionally, extracts showed DPPH scavenging activity, and membrane-stabilizing activity. Targeted high performance liquid chromatography (HPLC) identified rutin (Fig. 2.5) was the main compound produced in the extracts and most likely responsible for the observed activities. The chemical structure of rutin encompass the flavonol quercetin attached to a disaccharide rutinose moiety. This flavonoid is mainly found in plants, with rare occurrence in fungi (Patil et al. 2015). Therefore, these results shows that endophytic fungi are potential sources of bioactive metabolites and the combination with advanced metabolomics analysis allows their exploitation for medicinal, agricultural, and industrial uses.

Some bioactive metabolites presents biopreservation properties, especially of food related products. Honoré et al. (2016) used reversed-phase liquid chromatography-mass spectrometry (LC-MS) in an untargeted footprinting approach to assess the metabolome of *Lactobacillus paracasei*. Bioassay-guided

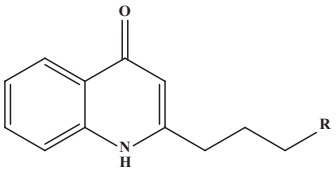
fractionation and comprehensive screening was applied to identify potential antifungal metabolites. The antifungal property was measured by the capacity to inhibit the relative growth of the two *Penicillium* strains. The untargeted footprinting approach allowed the identification of glucose, amino acids such as leucine, isoleucine, phenylalanine, methionine, tryptophane, proline, and tyrosine, along with adenosine, and adenine. Additionally, a series of 2-hydroxy acids were identified: lactic acid, 2-hydroxy-4-methylpentanoic acid, 2-hydroxy-3-phenylpropanoic acid, 2-hydroxy-3-(4-hydroxyphenyl) propanoic acid, 2-hydroxy-3-phenylpropanoic acid, 2-hydroxy-(4-hydroxyphenyl) propanoic acid, and 2-hydroxy-4-methylpropanoic acid. These metabolites showed minimal inhibitory concentration for 50% inhibition (MIC_{50}) varying from 5 to 10 $mg.mL^{-1}$ against two *Penicillium* strains. Three undescribed antifungal metabolites, along with three known were detected from *Lb. paracasei* (Honoré et al. 2016).

Several studies supports that microorganisms isolated from marine samples are a promising source of new bioactive metabolites. Kim et al. (2016b) used the intestine of the golden sea squirt to isolate the wild-type bacterial strain. The strain was identified as Pseudoalteromonas sp. by 16S rDNA sequence analysis. LC-MS analysis of the ethyl acetate extract revealed that presence of nine metabolites belonging to the 4-hydroxy-2-alkylquinoline class and were identified as pseudane III, IV, V, VI, VII, VIII, IX, X, and XI (Fig. 2.6).

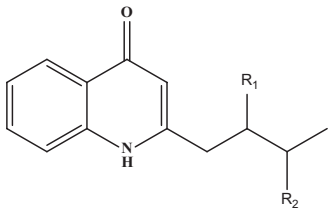
Additionally, two new metabolites from marine bacteria were identified: 2-isopentylqunoline-4-one and 2-(2,3-dimetylbutyl)qunoline-4-(1H)-one (Fig. 2.6). Pseudane VI and VII possess anti-melanogenic and antiinflamatory activities (Kim et al. 2016b, 2017), whereas pseudane IX showed strong anti-Hepatitis C virus activities (Wahyuni et al. 2014).

Betancur et al. (2017) isolated actinobacteria strains from sediment, invertebrate and algae samples collected from coral reefs in the Colombian Caribbean Sea. Species belonging to the genera *Streptomyces*, *Micromonospora*, and *Gordonia* were identified within the isolated bacteria by 16S rRNA gene sequencing. They used LC-MS analysis to identify new antimicrobial and quorum quenching metabolites against pathogens from the isolated actinobacteria strains. Six out of the 24 isolates showed promising results regarding the antimicrobial activities. Dereplication was applied to identify new bioactive metabolites by excluding well-known active metabolites or inactive natural products.

Twenty-eight entities did not present any hits and may represent new compounds. Dereplication indicates the presence of possible antibacterial and anthelmintic activity pyridine derivatives from *Streptomyces tendae* and *S. piericidicus*, δ-lactones inducer of anthracycline production from *S. viridochromogenes*, antibacterial fatty acid derivatives from *S. globisporus*, antifungal and antibacterial anthraquinone derivatives from Streptomyces sp., antitumor and antifungal alkaloids from *S. thioluteus*, and antibacterial macrolides from *S. griseus* (Betancur et al. 2017). Some of the well-known active metabolites included streptomycin-D, youlenmycin, inostamycin-b, pterulamide III, bistheonellic acid B, and mechercharmycin A (Fig. 2.7) (Betancur et al. 2017). Streptomycin is an antibiotic used to treat several types of infection (Schatz et al. 1944) and it shows quorum sensing inhibitory



pseudane III, (R = H)
pseudane IV, (R = CH_3)
pseudane V, (R = C_2H_5)
pseudane VI, (R = C_3H_7)
pseudane VII, (R = C_4H_9)
pseudane VIII, (R = C_5H_{11})
pseudane IX, (R = C_6H_{13})
pseudane X, (R = C_7H_{15})
pseudane XI, (R = C_8H_{17})



2-isopentylqunoline-4-one, (R_1 = H; R_2 = CH_3)
2-(2,3-dimetylbutyl)qunoline-4-(1H)-one, (R_1 = CH_3; R_2 = CH_3)

Fig. 2.6 Chemical structure of bioactive metabolites produced by a wild-type bacterial strain isolated from the intestine of the golden sea squirt (*Halocynthia aurantium*)

activity in *Acinetobacter baumannii* (Saroj and Rather, 2013). Inostamycin-b showed antimicrobial activities against *Staphylococcus aureus*, *Micrococcus luteus*, *Bacillus anthracis*, *B. subtilis*, *Corynebacterium bovis*, and *Mycobacterium smegmatis* (Odai et al. 1994). Pterulamide III is a cytotoxic linear peptide isolated from Pterula species (Lang et al. 2006), whereas mechercharmycin A is a antitumor cyclic peptide-like isolated from *Thermoactinomyces* sp. obtained from marine source (Kanoh et al. 2005).

Gnavi et al. (2016) also worked with marine-derived microorganism, but instead of bacteria they isolated sterile mycelia from *Flabellia petiolata* collected in the Mediterranean Sea. Species belonging to the genera *Biatriospora*, *Beauveria*, *Massarina*, *Microascacea*, *Roussoellacea*, and *Knufia* were identified within the isolated bacteria by sequencing the nrDNA internal transcribed spacer (ITS) and large ribosomal subunit (LSU) partial regions. Antibacterial activity was assessed against the multidrug-resistant (MDR) bacteria *Burkholderia metallica*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Staphylococcus aureus*. These bacteria are involved in cystic fibrosis and nosocomial infections. LC-MS analysis was applied to identify intra- and extracellular metabolites produced from each fungal strain and revealed the presence of 2-aminodocosa-6,17-dien-1,3-diol, 2-aminooctadecan-1,3,4-triol, 2-aminooctadecan-1,3-diol, phytoceramide C2, aphidicolin, scopularide A, bis(2-ethylhexyl) hexanedioic acid, fusoxysporone, and ergostane derivatives ergosta-5,7,22-trien-3-β-ol and ergosta-3,5,7,9(11),22-pentaene (Fig. 2.8). These metabolites might be responsible for the antibacterial activity of the extracts (Gnavi et al. 2016).

The actinomycete *Streptomyces sparsus* VSM-30 was isolated from deep sea sediments of Bay of Bengal. LC- and GC-MS analyses of the extracellular metabolites from the ethyl acetate extract revealed that presence of tryptophan dehydrobutyrine diketopiperazine, maculosin, 7-o-demethyl albocycline,

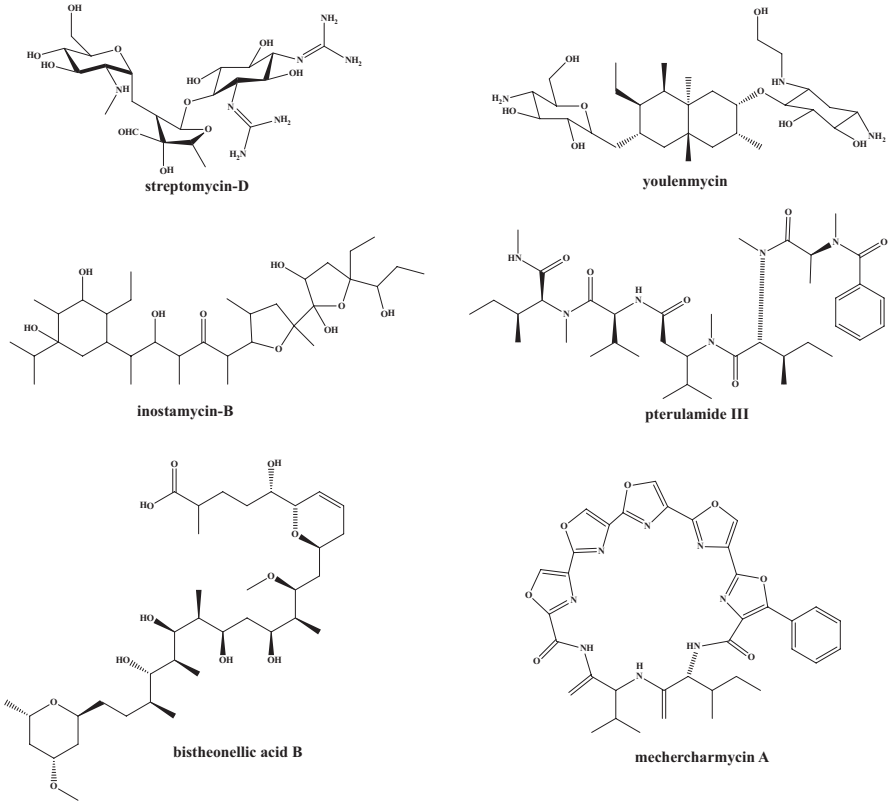


Fig. 2.7 Chemical structure of antimicrobial produced by actinobacteria strains from sediment, invertebrate and algae samples collected from coral reefs in the Colombian Caribbean Sea

albocycline M-2, 7-o-demethoxy-7-oxo albocycline, dotriacontane, 11-decyl-tetracosane, diheptyl phthalate, 1-hexadecanesulfonyl chloride, L-alanyl-L-tryptophan, phthalic acid ethyl pentyl ester, 4-trifluoroacetoxyhexadecane, and 1H-imidazole 4,5-dihydro-2,4-dimethyl. These metabolites contribute for the biological activities of the extract (Managamuri et al. 2017). Yogabaanu et al. (2017) used high performance liquid chromatography (HPLC) to identify variations in the extracellular metabolome of soil fungi in response to temperature variation and to screen for antimicrobial metabolites. These fungi are found at the Arctic and Antarctic regions and were obtained from the National Antarctic Research Centre Fungal Collection, from the University of Malaya, Kuala Lumpur. The antimicrobial activity of these fungi was assessed against *B. subtilis*, *B. cereus*, *E. coli*, *E. faecalis*, and *P. aeruginosa* by disk diffusion assay through the inhibition zone produced. They showed that culture temperature influenced the metabolome of the fungal strains. However, they failed to identify the metabolites detected in the crude extracts, limiting their results to the presentation of the retention times of the metabolites (Yogabaanu et al. 2017). Romoli et al. (2014) also worked with

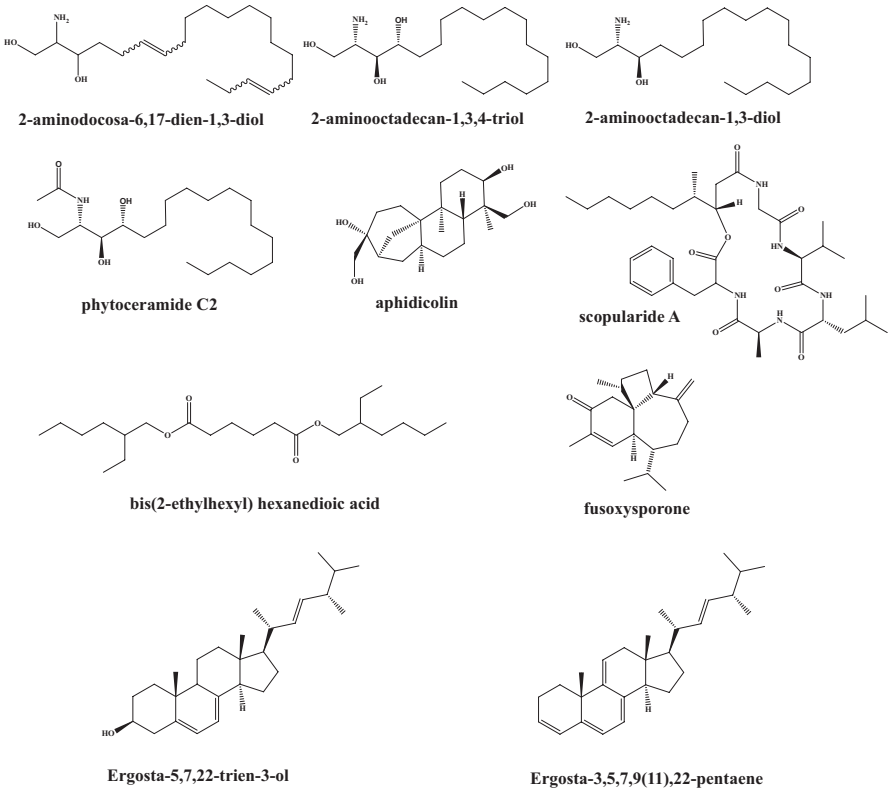


Fig. 2.8 Chemical structure of the metabolites produced by *Flabellia petiolata* sterile mycelia

microorganisms that inhabits the Antarctic region, more specifically the *Pseudoalteromonas* TB41 bacteria strain. These bacteria produced a wide range of volatile compounds (VOCs) that inhibit the growth of *Burkholderia cepacia* complex (Bcc) strains. The Bcc strains are opportunistic pathogens of cystic fibrosis patients (Sfeir 2018; Van Dalem et al. 2018). Solid phase micro extraction (SPME) gas chromatography–mass spectrometry (GC-MS) analysis allowed the identification of 30 VOCs, including some alcohols, along with some nitrogen- and sulfur-rich compounds (Romoli et al. 2014). Microbes produce a diverse range of volatile compounds that may function as key components in the cross-microbial relationships within a microbiota. However, the authors did to correlate the concentration of the identified VOCs with the possible inhibitory effects on the growth of Bcc strains.

2.1.4 Metabolomics for Microbial Biofilms

Microbial biofilms consist of a multicellular microbial conglomerate embedded in complex extracellular matrix adhered on a solid surface. In contrast, planktonic

cells are single-cell organisms that may drift or disperse in a liquid medium (Azeredo et al. 2017). Microbial biofilms are ubiquitous in nature and may constitute a microorganism survival strategy to unfavorable environmental conditions (Landini et al. 2010). The formation of a microbial biofilm formation involves microbial adhesion, and accumulation of an extracellular matrix. This extracellular matrix is composed of macromolecules such as proteins, polysaccharides, humic substances, and extracellular DNA. Microbial biofilm formation is tightly regulated by a combination of environmental and physiological cues, such as nutrient availability, and cellular stress (Assaidi et al. 2018; Favre et al. 2018; Jin et al. 2018; Landini et al. 2010). They can be beneficial or have a negative impact providing tolerance to antibiotic treatment, and enhancing virulence of many pathogenic bacteria. This is especially concerning when biofilms are formed on industrial settings or on medical devices (Azeredo et al. 2017; Jin et al. 2018; Landini et al. 2010). Due to its versatility, metabolomics has been applied to biofilm research in order to identify the biochemical changes between the planktonic and biofilm phenotypes, to assess the chemical composition of biofilms, and to monitor in vivo biofilm formation and development.

Microbe cells within biofilms and their planktonic equals are morphologically and physiologically distinct from each other. There is a strong correlation between pathogenic biofilms and diseases, since microbe biofilms are more resistant to unfavorable environmental conditions. Therefore, it is crucial to develop strategies to assess in vivo biofilm formation to understand the mechanism underlying the dynamic biochemical changes occurring during the transition between the planktonic and biofilm phenotypes. Bacterial membranes encompass a diverse panel of amphiphilic phospholipids, such as phosphatidylglycerol, phosphatidylethanolamine, cardiolipin, phosphatidylcholine, and phosphatidylinositol, as well as ornithine lipids, glycolipids, and sphingolipids (Sohlenkamp and Geiger 2015). *Escherichia coli* has been used for a long time as the perfect organism to study membrane lipids. However, bacterial membrane lipid composition may vary among different bacteria species and environmental growth conditions (Sohlenkamp and Geiger 2015). Benamara et al. (2014) assessed the dynamics of the phospholipid composition of the *P. aeruginosa* membranes (fingerprint) throughout biofilm development on glass wool. They applied gas chromatography-mass spectrometry (GC-MS) to assess the lipidome dynamics in response to the biofilm age (i.e., from 1-, 2-, to 6-day-old biofilm). Phosphatidylethanolamines (PE 30:1, 31:0, 38:1, 38:2, 39:1, and 39:2) and phosphatidylglycerols (PG 31:0, 38:0, 38:1, 38:2, 39:1, and 39:2) were the predominant lipids on *P. aeruginosa* inner and outer membrane. Lipidome changes was more significant for the biofilm phenotype than for the planktonic counterpart. Heavier and branched-chains phospholipids decreased in the outer membrane, whereas cyclopropylated phospholipids increased in both membranes with the biofilm age. Curiously, the lipidome of the oldest biofilms were more similar to the metabolome of the planktonic phenotype (Benamara et al. 2014). Accumulation of phosphatidylethanolamine derivatives were observed in the biofilm phenotype of *Pseudoalteromonas lipolytica*, whereas ornithine lipids were preferably produced by the planktonic phenotype (Favre et al. 2018). Analysis

of the intracellular metabolome of *Desulfovibrio vulgaris* planktonic and biofilm phenotypes by GC- and LC-MS showed that metabolites related to fatty acid biosynthesis such as lauric, mysistic, palmitoleic, and stearic acids were up-regulated in the biofilm as compared to the planktonic phenotype (Zhang et al. 2016). These results support the hypothesis that membrane related metabolites are important for the formation, maintenance and function of microbial biofilms as well as their differentiation from the planktonic phenotype. Membrane composition re-modelling supported *Streptococcus intermedius* growth and adaptation to anaerobic conditions. *S. intermedius* plasticity under oxygen depletion allows it to coexist in biofilms, both as a commensal and a pathogen (Fei et al. 2016).

Microbial biofilm development on biotic and abiotic surfaces act as a continual source of contamination. Microbial biofilm formation by Salmonella spp. has profound consequences in many industries, since they are notoriously difficult to eradicate (Corcoran et al. 2013; Keelara et al. 2016; Patel et al. 2013). Wong et al. (2015) applied GC-MS to detect biochemical change between intracellular and extracellular metabolites produced by the biofilm and planktonic phenotypes of Salmonella spp. cells and Salmonella biofilms of different ages. Alanine, glutamic acid, glycine, and ornithine showed the major contribution to discriminate between the extracellular metabolome of planktonic and biofilm phenotypes, whereas succinic acid, putrescine, pyroglutamic acid, and N-acetylglutamic acid acted as major contributors to discriminate between the intracellular metabolome of planktonic and biofilm phenotypes. Similarly, amino acids were responsible for the main discrimination among the samples of different days of biofilm growth. However, the intracellular showed no significant differences in response to age (Wong et al. 2015). Undoubtedly, central carbon and nitrogen metabolism plays a crucial role on the differentiation of the intracellular and extracellular metabolome of planktonic and biofilm phenotypes. For example, Stipetic et al. (2016) reported that *Staphylococcus aureus* planktonic and biofilm phenotypes showed differences in in arginine biosynthesis. Ząbek et al. (2017) applied quantitative NMR to assess the metabolome of the planktonic and biofilm phenotypes (1 and 2-day-old biofilm) of *Aspergillus pallidofulvus* and reported that the levels of the extracellular leucine, arginine, choline, betaine, *N*-acetylglucosamine, and phenylalanine were upregulated after 24 h of growth. Additionally, organic acids such as threoic, aspartic, docosanoic, malonic, hydrobenzoic and keto-gluconic, as well as the carbohydrates fructose, mannose, cellobiose, and maltose are important intracellular metabolites produced by the planktonic and biofilm phenotypes of *Vibrio fischeri* ETJB1H (Chavez-Dozal et al. 2015).

Borgos et al. (2015) used high-resolution liquid chromatography-mass spectrometry fingerprinting as a rapid, sensitive and noninvasive technique to assess the formation and development of *P. aeruginosa* biofilm between 0 and 196 h after inoculation. Despite the fact that these authors applied an untargeted approach, they identified, in the positive ESI mode, a compound with m/z 211.0867 ($M+H^+$ ion) that changed in response to both strain and sampling time. The unknown compound was unambiguously annotated as pyocyanine (Borgos et al. 2015). Pyocyanin is a virulence factor produced by *P. aeruginosa,* which shows antimicrobial activity

against Gram-positive bacteria (Gharieb et al. 2013). The authors, however, failed to present a time-course analysis of the pyocyanin content, limiting their discussion to the ANOVA results. We can only infer that as an antimicrobial compound, pyocyanin is produce in order to ensure proper formation of the microbe conglomerate preventing the incorporation of unwanted microorganisms.

Ammons et al. (2014) applied quantitative NMR to assess dynamic biochemical changes between the planktonic and biofilm phenotypes of methicillin-resistant and methicillin-susceptible *Staphylococcus aureus*. *S. aureus* is considered a wound bioburden since it forms a sort of colonizing biofilm as major contributor to nonhealing wounds (DeWitt et al. 2018; Kim et al. 2018). Principal component analysis based on both intracellular and extracellular metabolites differentiated the phenotypes. Amino acid uptake, lipid catabolism, and butanediol fermentation are key features distinguishing the phenotypes (Ammons et al. 2014). Additionally, they claimed that a shift in metabolism from energy production to assembly of cell-wall components and matrix deposition may also play a role in distinguishing between the planktonic and biofilm phenotypes. This is a farfetched hypothesis since they did not identified any cell-wall metabolite components, with the exception of some pyrimidine nucleotides that may serve as precursors for synthesis of teichoic acids and peptidoglycan in *S. aureus*. Schelli et al. (2017) used HPLC-MS/MS to assess the metabolome of two *S. aureus* strains in response to methicillin exposure. As expected, methicillin exposure disturbed the metabolome of the methicillin susceptible *S. aureus* in a greater extent than of the methicillin resistant strain (Schelli et al. 2017).

2.1.5 Metabolomics for Microbial Biomarkers

2.1.5.1 Abiotic Stresses: Light and Oxygen Availability and Salinity

Microbial biomarkers can be classified in three types: exposure, effect and susceptibility. The identification of reliable biomarkers is important for a wide number of purposes, and may provide information related to microbial metabolism concerning exposure, growth and adaptation under biotic and abiotic stress conditions. Abiotic stress conditions encompass light, oxygen, and water availability along with salinity and low and high temperature.

Ultra performance liquid chromatography was used to compare the extracellular metabolome of *Aspergillus nidulans* during fungal development in the dark or light (Bayram et al. 2016). Light not only accelerated asexual development of *A. nidulans*, but also induced the production of the antitumoral metabolites terrequinone A and emericellamide (Fig. 2.9). Terrequinone A is a bisindolylquinone derivative with tumor growth inhibitory activity (He et al. 2004), whereas emericellamide is an antibiotic compound of mixed origins with polyketide and amino acid building blocks (Chiang et al. 2008; Newman 2016). Dark conditions, however, led to the preference of the sexual development and the accumulation of the polyketide mycotoxin sterigmatocystin, along with the antraquinones asperthecin, and emodin (Fig. 2.9) (Bayram et al. 2016). These metabolites could be used as potential

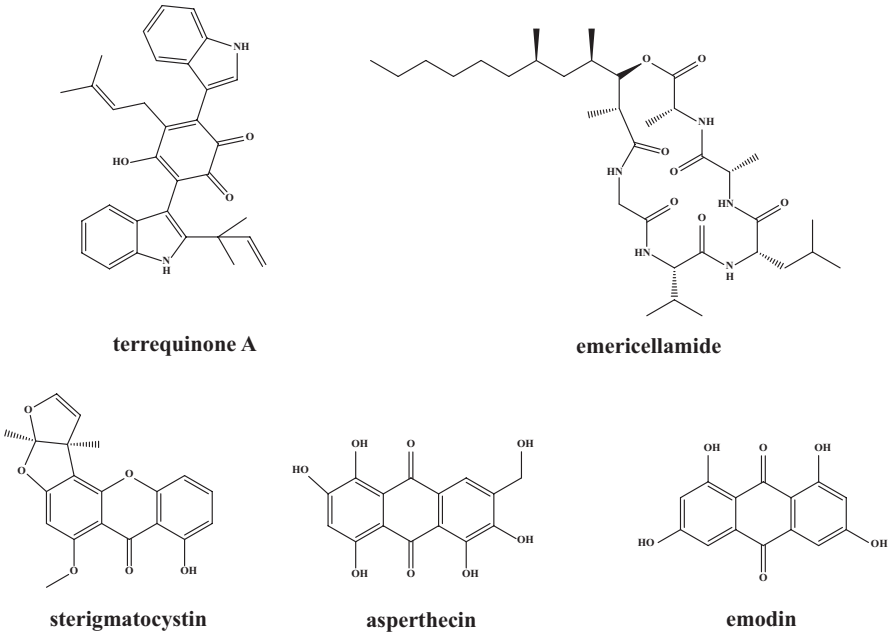


Fig. 2.9 Chemical structure of biomarkers produced from *A. nidulans* during fungal development in the dark or light

biomarkers for the genus *Aspergillus* during fungal development in the dark or light conditions.

Oxygen availability may also alter the metabolome composition of a microorganism and for that reason Fei et al. (2016) used hydrophilic interaction chromatography (HILIC–TOF–MS) to assess the effect of oxygen availability on the intra and extracellular metabolome of *Streptococcus intermedius* strain B196. Oxygen depletion enhanced pyrimidine and purine metabolism and the central carbon metabolism. This microorganism showed high plasticity, especially under anaerobic growth conditions, in which *S. intermedius* adaptation to oxygen depletion involved the re-modelling of the cellular membrane composition. Alterations in cellular membrane composition, especially fatty acids and phospholipids, are an important adaptation mechanism present several microorganisms (Suutari and Laakso 1994) and may also be targeted for biomarker discovery in response to oxygen availability.

Nuclear magnetic resonance spectroscopy (^{1}H NMR) was applied to evaluate the effect of salt stress on the intracellular metabolome of four halophilic bacterial isolates. *Halomonas hydrothermalis*, *Bacillus aquimaris*, *Planococcus maritimus* and *Virgibacillus dokdonensis* were isolated from a saltern region in India. Metabolites involved in the glycolytic pathway, pentose phosphate pathway and citric acid cycle showed a salt-dependent increase. These are main energy-generating pathways, which may explain the fact that cellular homeostasis was favored over growth (Joghee and Jayaraman 2014). Alves et al. (2016) evaluated the effect of

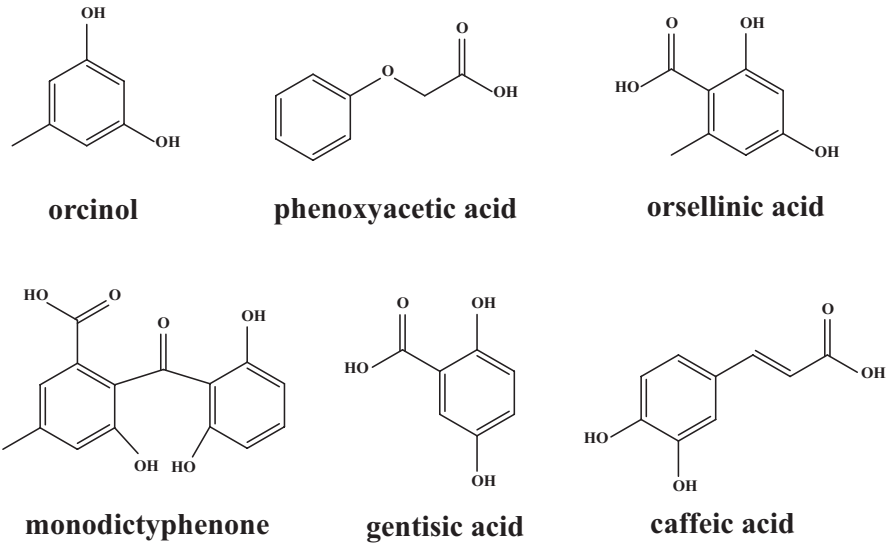


Fig. 2.10 Chemical structure of possible biomarkers produced from *A. nidulans* during fungal development in the presence of ionic liquids' stimulants cholinium chloride and 1-ethyl-3-methylimidazolium chloride

ionic liquids' stimuli (cholinium chloride or 1-ethyl-3-methylimidazolium chloride) on the extracellular metabolome of *Aspergillus nidulans*. ^{1}H NMR analyses revealed that both ionic liquids stimulated production of acetyl-CoA. Acetyl-CoA is an important precursor for secondary metabolites and non proteinogenic amino acids. Ionic liquids stimulated the production of orcinol, phenoxyacetic acid, orsellinic acid, monodictyphenone, gentisic acid, and caffeic acid (Fig. 2.10) (Alves et al. 2016). Taken together, these studies highlights the importance of metabolomics for strain enhancement and phenotypic analysis of microorganisms unfavorable conditions.

2.1.5.2 Metabolic Engineering

Metabolic engineering approaches aim at developing microbial cell factories to modulate metabolic pathways for metabolite over production or to improve cellular properties optimizing genetic and regulatory processes within cells (Chae et al. 2017; Lian et al. 2018). Metabolomics along with systems biology, synthetic biology and evolutionary engineering methodologies have allowed a fast and impressive advancing of metabolic engineering to rewire cellular metabolism.

Saccharomyces cerevisiae is a single-celled eukaryote commonly used for metabolic engineering research. The fact that the genome of this microorganism has been fully sequenced and is easily manipulated makes it an attractive model species for metabolic engineering approaches. Kim et al. (2016a) used GC-TOF-MS to investigate the intracellular metabolome of *S. cerevisiae* in order to identify possible ethanol tolerance mechanisms. For that, an ethanol-tolerant mutant yeast iETS3 was

constructed and its metabolome was compared to the wild-type S. *cerevisiae* BY4741. Several metabolites from the central carbon and nitrogen metabolism were identified including amines, amino acids, fatty acids, organic acids, phosphates, sugars and sugar alcohols. Principal component and hierarchical clustering analyses showed a clear separation of the metabolite profiles of iETS3 and BY4741. Metabolites involved in cell membrane composition, glutamate and trehalose metabolism were identified as possible biomarkers for ethanol tolerance in *S. cerevisiae* (Kim et al. 2016a).

Pichia pastoris is used for process-scale production of recombinant secreted proteins, but often shows low productivity. Tredwell et al. (2017) used recombinant *P. pastoris* strains with varying levels of unfolded protein response (UPR) induction to study cellular stress responses and to identify potential biomarkers of UPR induction. NMR metabolic profiling of the intra- and extracellular metabolome allowed the quantification of 32 metabolites including 15 amino acids (alanine, arginine, asparagine, aspartate, glutamate, glutamine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, serine, tyrosine, and valine), 9 organic acids (benzoate, citrate, formate, fumarate, lactate, malate, succinate, 2-oxoisocaproate, and 3-methyl-2-oxovalerate), glucose, and some other metabolites. The authors suggested that the metabolites identified from both cell extracts and supernatants could be used as potential biomarkers for future high-throughput screening of large numbers of *P. pastoris* clones.

Campylobacter jejuni is a human bacterial pathogen described as one of the most common causes of food poisoning worldwide leading to self-limited diarrheal illness. In some cases, macrolide antibiotics are required for its efficient treatment (Chen et al. 2018; Ranjbar et al. 2017). The resistance gene erm(B) and mutations in rplD and rplV (23S rRNA) has led to the development of macrolide-resistant Campylobacter strains (Wang et al. 2014). Fu et al. (2018) have applied UHPLC-MS/MS to investigate the intracellular metabolome of a susceptible (NCTC 11168) and a resistant (NCTC 11168 with ermB) strain of *C. jejuni*. The resistance gene erm(B) had a deep impact on membrane integrity and stability, what was confirmed by the reduced biofilm formation capability of resistant strain as compared to the susceptible strain. Thirty-six metabolites were identified as potential biomarkers to differentiate the susceptible and resistant *C. jejuni* stain. These metabolites are involved in cell signaling, membrane integrity and stability, and energy-generating pathways. These results highlight important metabolic regulatory pathways associated with resistant of *C. jejuni*.

Microbes often produce metabolites as a survival strategy, especially when growing in harsh environments. Scarce nutrient and water availability may impose competition among microorganisms and, therefore, compelling them to produce metabolites to protect themselves, to control the proliferation of other microorganisms, or to acquire certain advantage when challenged by other microorganisms within a given microbiota. This process is called quorum quenching, which is usually proceeded by the quorum sensing. Quorum sensing is a transcriptional regulatory mechanism by which microorganisms regulate population density through chemical signaling. Molecules secreted by microorganisms are a

form of intra- and interspecies communication that helps bacteria coordinate their behavior (Gökalsın and Sesal 2016; Liu et al. 2016a; Padder et al. 2018). For example, *P. aeruginosa* uses quorum sensing to regulate the production of secreted virulence factors through the *N*-acyl-homoserine lactone (AHL)-dependent quorum sensing system (Davenport et al. 2015). Davenport et al. (2015) used a mutant unable to produce quorum sensing signaling molecules and its wild-type progenitor to investigate their extracellular metabolome throughout the growth curve by GC-MS and UHPLC-MS analyses. Metabolites involved on the central primary metabolism were affected suggesting that the AHL-dependent quorum sensing induces a general reprogramming of the metabolome. The content of metabolites involved in energy-generating pathways, such as citrate, malate, succinate and fumarate (tricarboxylic acid cycle intermediates) was higher in the mutant unable to produce quorum sensing signaling molecules as compared to its wild-type progenitor. Depletion of TCA intermediates might be responsible for the consistent lower growth yield of the wild-type as compared to mutant (Davenport et al. 2015).

2.2 Conclusion and Perspectives

Compared to transcriptomics approaches, metabolomics has the advantage of the relatively smaller number of metabolites, which makes it easier to extract relevant information of a given biological system. Additionally, metabolite composition rapidly responds to cellular activity and external stimuli. Metabolomics is becoming increasingly widespread toward understanding of microbe plasticity due to its unique ability to generate fast and robust functional data. We presented a detailed systematic literature review of the current knowledge, main findings, and perspective toward the understanding of microbe plasticity in microbial metabolomics research. Taken together, these studies demonstrated that metabolomics can be a useful tool for the identification of microbial bioactive metabolites, for the characterization of biofilms and for biomarkers discovery through strain improvement and phenotypic analysis of microorganisms.

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