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Bioactive Secondary Metabolites Produced by *Streptococcus pyogenes*, Epidemiology, Biofilm formation and Virulence factors

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Abstract: Metabolites are small molecules participating in metabolic reactions, which are necessary for cellular function, maintenance and growth. Typically, metabolites range from 50 to 1500 Da, while their concentrations span several orders of magnitude. The metabolome is highly dynamic, time-dependent, and metabolites are sensitive to many environmental conditions. Streptococcus pyogenes has several virulence factors that enable it to attach to host tissues, evade the immune response, and spread by penetrating host tissue layers. A carbohydrate-based bacterial capsule composed of hyaluronic acid surrounds the bacterium, protecting it from phagocytosis by neutrophils. In addition, the capsule and several factors embedded in the cell wall, including M protein, lipoteichoic acid, and protein F (SfbI) facilitate attachment to various host cells. Several of the discriminant compounds, notably pyrazine compounds, are normally absent in healthy human exhaled breath but can be found in the Streptococcus pyogenes cultures. That these compounds are typically absent suggests that a number of these candidate biomarkers are not typically produced by other members of the oropharyngeal microbial community.

Keywords: Streptococcus pyogenes, Secondary metabolites, Antibacterial, GC/MS.

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Introduction

Intake of food, excretion, inhalation, and secondary metabolites (such as medicines, flavourings, and recreational drugs) are just a few examples of the many ways in which metabolites interact with the environment. The gut microbiota and other organs can further process these metabolites. There isn't a single technique that can capture and analyse the whole metabolome simultaneously because metabolites are chemically extremely different in terms of polarity, charge, pKa, solubility, volatility, stability, and reactivity. In order to isolate and measure particular types of metabolites, a plethora of extraction techniques were devised. Gas chromatography (GC), liquid chromatography (LC), or capillary electrophoresis (CE) online connected to a mass spectrometer (MS), and nuclear magnetic resonance (NMR) spectroscopy are the most used methods for investigating the metabolome [1]. However, due to chemical and contextual diversity, detecting all metabolites in a biological sample is an ambitious aim [1, 2]. Metabolite concentrations span a wide range of magnitudes, adding to the metabolome's complexity and chemical variety, making identification a difficulty. Not only are there many distinct kinds of devices, each with its own unique way of working and collecting data, but there are also many different kinds of data processing programmes. Different fragmentation methods, including collision induced dissociation (CID), higher energy collision dissociation (HCD), electron transfer dissociation (ETD), and pulsed-Q dissociation (PQD) [4-7], as well as collision energy, resolution, targeted, and non-targeted LC-MS instrument settings, can result in varying fragment species and intensities. It is so challenging to employ or construct spectral databases that are particular to metabolites for the purpose of compound identification. Although it takes a lot of work, one of the best solutions is to develop an instrument-specific MRM method for a set of determined metabolites. The benefit of employing a screening strategy of rotenone-treated HeLa cells [9] to analyse complicated materials was proven by utilising up to three MRMs and their MRM ion ratios, which are stable properties between transitions [8]. The transitions were required to co-elute at the same retention time with identical peak forms in this work, which involved developing an MRM method employing pure chemicals. Inosine 51-monophosphate is one example of a compound for which MRM ion ratios are crucial for preventing false-positive identifications [10]. Additionally, a third transition was shown to be necessary by Schürmann and colleagues [11]. Their research showed that the MRMs of two sebuthylazine transitions were supported by ions produced by a matrix chemical that interfered with elution. Such a result would have been a false-positive in accordance with EU directive 2002/657/EC, which governs the verification of probable positive infections. The relative significance of adopting particular transitions for chemical identification has not been adequately addressed thus far. Impure Q1 isolation, co-eluting isobaric compounds, inaccurate database entries, and incorrect peak selecting are some of the possible causes of fragment mis-assignments. In order to simplify the data into a comprehensible set of signals, non-targeted metabolomics must be coupled with modern chemometric techniques [12]. Alonso and colleagues provide a comprehensive overview of analytical methods and necessary advancements in non-targeted metabolomics. A METLIN search for the exemplary parent mass of 136 Da yields 131 isobaric, distinct metabolites, a few of which share nearly identical fragment spectra due to their extremely similar structures. Thus, a ranked list is generated using similarity scores, and cutoff values are required for identity verification. Whether or not this is adequate, and the exact number of false-positive results, are up for debate. There is a lot of work going into improving spectrum databases, but having access to a comprehensive set of reference metabolite spectra is still a must for developing reliable automatic identification methods. One of the most important processes is target identification, hence in silico target identification approaches like CSNAP (Chemical Similarity Network Analysis Pulldown) [13] are utilised. These methods include chemical similarity database searches. For a long time, validation criteria were lacking, while there are several ways for deciphering and interpreting unknown peaks. In 2005, the Metabolomics Standards Initiative (MSI) came up with a set of criteria and minimal requirements for validating metabolite identification. This was done to facilitate the effective use, sharing, and reuse of data. Metabolomics data and metadata are being developed via the "COordination of Standards in MetabOlomicS" (COSMOS) effort, which is also creating strong data infrastructures.

A member of the Streptococcus genus, Streptococcus pyogenes is an aerotolerant, Gram-positive bacterium. The extracellular bacteria in question are composed of spherical cells called cocci, which do not produce spores and are not motile. These cells often form chains. They are a rare but potentially harmful component of the skin microbiota that can infect people with Group A streptococcal infections, making them clinically significant. The Lancefield group A antigen is most commonly found in Staphylococcus pyogenes, also known as group A streptococcus (GAS). But group A antigen can also be found in Streptococcus anginosus and Streptococcus dysgalactiae. The usual growth

pattern of group A streptococci on blood agar is the formation of tiny (2-3 mm) zones of beta-hemolysis, which involve the total degradation of red blood cells. Since this is the case, GABHS is another name for the same type of streptococcus.[12]

Because pus is produced by several bacterial diseases, the species name is derived from two Greek words that indicate "a chain" (streptos) of berries (coccus, which is Latinized from kokkos) and pus-forming (genes). Catalase testing is the gold standard for distinguishing between Streptococcus spp. and Staphylococcus spp. In contrast to streptococci, staphylococci are catalase positive.in [13] One way to culture S. pyogenes is using fresh blood agar plates. Since Streptococcus pyogenes will result in a positive PYR test, it can be distinguished from other beta-hemolytic streptococci that share physical similarities, such as S. dysgalactiae subsp. esquismilis.[6]

Every year, almost 700 million people get GAS infections. There is less than a 1% fatality rate for these infections generally, but a 25% fatality rate for severe and invasive cases, which affect more than 650,000 patients.[4] Because sepsis and mortality might occur from a delayed diagnosis, prompt treatment is of the utmost importance.[5][10]

Epidemiology Typical sites of S. pyogenes colonisation include the epidermis, vaginal mucosa, rectum, and throat. A small percentage of healthy persons have carriage in the throat, vagina, or rectal area. Such a carrying rate ranges from 2% to 17% in otherwise healthy children. Inhalation of respiratory droplets, skin-to-skin contact, touching a contaminated surface, object, or dust, or, less frequently, ingesting contaminated food are the four ways this bacterium can be transmitted. Streptococcal pharyngitis, rheumatic fever, scarlet fever, and rheumatic heart disease are just a few of the disorders that can be caused by these germs. While most occurrences of pharyngitis are caused by viruses, gram-negative bacteria (GAS) account for around 15–30% of cases in children and streptococcal bacteria for roughly 5–20% in adults. Due to exposures in schools and nurseries as well as a result of poorer host immunity, the number of pharyngitis cases in children is higher than in adults. Streptococcal pharyngitis is more common in seasonal countries from the end of winter into the beginning of spring because so many people breathe in the same air during that time. Autumn is when we see the fewest reported cases of disease.[17[In industrialised nations, invasive Streptococcus pyogenes infections are commonly linked to the MT1 (metabolic type 1) clone. Both the incidence and mortality of S. pyogenes were high before penicillin became widely available, but they had begun to decline before then. This proves that environmental factors are involved in S. pyogenes infections. Developed nations have an incidence of 2-4 cases per 100,000 people, while underdeveloped countries have an incidence of 12–83 cases per 100,000 people. The prevalence of S. pyogenes infection is higher in newborns and the elderly, and is less common in women. S. pyogenes infection occurs in 17–25% of cases in individuals with preexisting conditions such diabetes, cancer, heart disease, acute trauma, surgical incisions, or viral respiratory infections (such as influenza). In most cases, a secondary infection with GAS occurs within a week of an influenza diagnosis. An infection with chickenpox occurs in 14–16% of cases of S. pyogenes in children. Severe infection of soft tissues is the typical symptom of S. pyogenes in children, which often appears 4 to 12 days after a chickenpox diagnosis. In youngsters, the chance of contracting Staphylococcus pyogenes increases 40 to 60 times within the first two weeks after contracting chickenpox. But 20-30% of S. pyogenes infections in adulthood happen in people who don't have any known risk factors. Children, for whom no risk factors have been identified, have a greater frequency of S. pyogenes infection (50–80%). There were 49 cases of scarlet fever per 100,000 people in 2014, up from the average 4 cases per 100,000 in the UK. People living in poverty in underdeveloped nations are more likely to experience rheumatic fever and rheumatic heart disease (RHD) two to three weeks following a throat infection. Rheumatic fever and RHD had an average incidence of 19 per 100,000 people worldwide between 1967 and 1996, with a peak incidence of 51 per 100,000 people.[7[Two to four percent of all S. pyogenes infections are maternal infections, which often occur in the latter stages of pregnancy, between thirty weeks of gestation and four weeks after giving birth. The likelihood of S. pyogenes infections increases by a factor of 20 to 100 as a result of this. The symptoms that patients may experience include pneumonia, septic arthritis, necrotizing fasciitis, and sepsis of the genital tract. In the 1930s, researchers at London's Queen Charlotte's Hospital found that such infections did not typically originate in the vagina. Contrarily, maternal S. pyogenes infection was more commonly seen in the neck and in people who were in close proximity to carriers.[7[

Serotyping

In 1928, Rebecca Lancefield published a method for serotyping S. pyogenes based on its cell-wall polysaccharide,[8] a virulence factor displayed on its surface.[9] Later, in 1946, Lancefield described the serologic

classification of S. pyogenes isolates based on components of their surface pili (known as the T-antigen)[10] which are used by bacteria to attach to host cells.[11] As of 2016, a total of 120 M proteins are identified. These M proteins are encoded by 234 types emm gene with greater than 1,200 alleles.[9]

Lysogeny

All strains of S. pyogenes are polylysogenized, in that they carry one or more bacteriophage on their genomes.[12] Some of the 'phages may be defective, but in some cases active 'phage may compensate for defects in others.[13] In general, the genome of S. pyogenes strains isolated during disease are >90% identical, they differ by the 'phage they carry.[14]

Virulence factors

S. pyogenes is able to infect humans by attaching to their tissues, escaping their immune systems, and then spreading through the layers of their tissues.[15] Hyaluronic acid forms a carbohydrate-based bacterial capsule that encases the bacterium, shielding it from neutrophil phagocytosis.[2] Furthermore, attachment to different host cells is facilitated by the capsule and a number of components embedded in the cell wall, such as M protein, lipoteichoic acid, and protein F (SfbI).[16] Additionally, M protein binds to host complement regulators to prevent opsonization via the alternative complement pathway. Additionally, certain serotypes have a M protein that can bind to fibrinogen and hinder opsonization.[2] Antibodies generated by the immune system that target the M protein engulf the bacteria for phagocyte engulfment, making the M protein the pathogen's Achilles' heel. Because each strain's M protein is distinct, it is possible to employ identification to confirm the infection's strain in a clinical setting.[17]

Name	Description
Streptolysin O	**An exotoxin, one of the bases of the organism's beta-hemolytic property, streptolysin O causes an immune response and detection of antibodies to it; antistreptolysin O (ASO) can be clinically used to confirm a recent infection. It is damaged by oxygen.**
Streptolysin S	**A cardiotoxic exotoxin, another beta-hemolytic component, not immunogenic and O2 stable: A potent cell poison affecting many types of cell including neutrophils, platelets, and subcellular organelles.**
Streptococcal pyrogenic exotoxinA (SpeA)	**Superantigens secreted by many strains of S. pyogenes: This pyrogenic exotoxin is responsible for the rash of scarlet fever and many of the symptoms of streptococcal toxic shock syndrome, also known as toxic shock like syndrome (TSLS).**
Streptococcal pyrogenic exotoxin C (SpeC)	
Streptococcal pyrogenic exotoxin B (SpeB)	**A cysteine protease and the predominant secreted protein. Multiple actions, including degrading the extracellular matrix, cytokines, complement components, and immunoglobulins. Also called streptopain.[18]**
Streptokinase	**Enzymatically activates plasminogen, a proteolytic enzyme, into plasmin, which in turn digests fibrin and other proteins**
Hyaluronidase	**Hyaluronidase is widely assumed to facilitate the spread of the bacteria through tissues by breaking down hyaluronic acid, an important component of connective tissue. However, very few isolates of S. pyogenes are capable of secreting active hyaluronidase due to mutations in the gene that encodes the enzyme. Moreover, the few isolates capable of secreting hyaluronidase do not appear to need it to spread through tissues or to cause skin lesions.[19] Thus, the true role of hyaluronidase in pathogenesis, if any, remains unknown.**
Streptodornase	**Most strains of S. pyogenes secrete up to four different DNases, which are sometimes called streptodornase. The DNases protect the bacteria from being trapped in neutrophil extracellular traps (NETs) by digesting the NETs' web of DNA, to which are bound neutrophil serine proteases that can kill the bacteria.[20]**
C5a peptidase	**C5a peptidase cleaves a potent neutrophil chemotaxin called C5a, which is**

	produced by the complement system.[21] C5a peptidase is necessary to minimize the influx of neutrophils early in infection as the bacteria are attempting to colonize the host's tissue.[22] C5a peptidase, although required to degrade the neutrophil chemotaxin C5a in the early stages of infection, is not required for S. pyogenes to prevent the influx of neutrophils as the bacteria spread through the fascia.[23]
Streptococcal chemokine protease	**The affected tissue of patients with severe cases of necrotizing fasciitis are devoid of neutrophils.[24] The serine protease ScpC, which is released by S. pyogenes, is responsible for preventing the migration of neutrophils to the spreading infection. ScpC degrades the chemokine IL-8, which would otherwise attract neutrophils to the site of infection.[22][23]**

Biofilm formation

Biofilms are a way for S. pyogenes, as well as other bacterial cells, to communicate with each other. In the biofilm gene expression for multiple purposes (such as defending against the host immune system) is controlled via quorum sensing.[31] One of the biofilm forming pathways in GAS is the Rgg2/3 pathway. It regulates SHP's (short hydrophobic peptides) that are quorum sensing pheromones a.k.a. autoinducers. The SHP's are translated to an immature form of the pheromone and must undergo processing, first by a metalloprotease enzyme inside the cell and then in the extracellular space, to reach their mature active form. The mode of transportation out of the cell and the extracellular processing factor(s) are still unknown. The mature SHP pheromone can then be taken into nearby cells and the cell it originated from via a transmembrane protein, oligopeptide permease.[31] In the cytosol the pheromones have two functions in the Rgg2/3 pathway. Firstly, they inhibit the activity of Rgg3 which is a transcriptional regulator repressing SHP production. Secondly, they bind another transcriptional regulator, Rgg2, that increases the production of SHP's, having an antagonistic effect to Rgg3. SHP's activating their own transcriptional activator creates a positive feedback loop, which is common for the production for quorum sensing peptides. It enables the rapid production of the pheromones in large quantities. The production of SHP's increases biofilm biogenesis.[31] It has been suggested that GAS switches between biofilm formation and degradation by utilizing pathways with opposing effects. Whilst Rgg2/3 pathway increases biofilm, the RopB pathway disrupts it. RopB is another Rgg-like protein (Rgg1) that directly activates SpeB (Streptococcal pyrogenic exotoxin B), a cysteine protease that acts as a virulence factor. In the absence of this pathway, biofilm formation is enhanced, possibly due to the lack of the protease degrading pheromones or other Rgg2/3 pathway counteracting effects.[31]

Disease

S. pyogenes is the cause of many human diseases, ranging from mild superficial skin infections to life-threatening systemic diseases.[2] Infections typically begin in the throat or skin. The most striking sign is a strawberry-like rash. Examples of mild S. pyogenes infections include pharyngitis (strep throat) and localized skin infection (impetigo). Erysipelas and cellulitis are characterized by multiplication and lateral spread of S. pyogenes in deep layers of the skin. S. pyogenes invasion and multiplication in the fascia can lead to necrotizing fasciitis, a life-threatening condition which requires prompt surgical intervention to reduce morbidity and mortality.[32][33] The bacterium is found in neonatal infections.[34] Infections due to certain strains of S. pyogenes can be associated with the release of bacterial toxins. Throat infections associated with release of certain toxins lead to scarlet fever. Other toxigenic S. pyogenes infections may lead to streptococcal toxic shock syndrome, which can be life-threatening.[2]

S. pyogenes can also cause disease in the form of post-infectious "non-pyogenic" (not associated with local bacterial multiplication and pus formation) syndromes. These autoimmune-mediated complications follow a small percentage of infections and include rheumatic fever and acute post-infectious glomerulonephritis. Both conditions appear several weeks following the initial streptococcal infection. Rheumatic fever is characterized by inflammation of the joints and/or heart following an episode of streptococcal pharyngitis. Acute glomerulonephritis, inflammation of the renal glomerulus, can follow streptococcal pharyngitis or skin infection.[citation needed]

This bacterium remains acutely sensitive to penicillin. Failure of treatment with penicillin is generally attributed to other local commensal organisms producing β-lactamase, or failure to achieve adequate tissue levels in the pharynx. Certain strains have developed resistance to macrolides, tetracyclines, and clindamycin. There is a polyvalent

inactivated vaccine against several types of Streptococcus including S. pyogenes called " vacuna antipiogena polivalente BIOL" it is recommended an administration in a series of 5 weeks. Two weekly applications are made at intervals of 2 to 4 days. The vaccine is produced by the Instituto Biológico Argentino.[35]

Secondary Metabolites

All of an organism's biological reactions put together make up its metabolism. Typically, metabolites are tiny molecules that serve as metabolic intermediates or end products. The term "secondary" was first used by A. Kossel in 1891 to describe metabolites that are not essential to an organism's survival, as opposed to the primary metabolites that are present in all cells that may divide. Although they are byproducts of primary metabolism, secondary metabolites are not structural components of the living thing. It differs from primary metabolites in that its absence does not instantly kill an organism, but it does make survival more difficult. Its synthesis and existence are seen in phylogenetically disadvantaged organisms [34].

Many of the intermediates in primary metabolism are also intermediates in secondary metabolism, making it difficult to tell the two apart. Although they are often thought of as a byproduct of primary metabolites, amino acids are actually secondary metabolites as well. As previously noted, sterols are secondary metabolites that play an essential role in a variety of cellular structures. According to [35], the fact that an intermediate is mosaic-shaped suggests that primary and secondary metabolism share a same metabolic pathway.

Inactive primary metabolism can be diverted into the secondary metabolites, which operate as a buffer zone for excess carbon and nitrogen. When needed, the stored carbon and nitrogen can be converted back to their original metabolite form through the metabolic breakdown of secondary metabolites. Growth, tissue differentiation, and development within the cell or organism, as well as external forces, impact the activities of primary and secondary metabolism, which are dynamic and delicately balanced.

Microbes' Secondary Metabolite Production

Metabolism is a continuous and collective biochemical activity that happens in all living things, whether they have one cell or many. Catabolism and anabolism are the two main categories into which biochemical processes fall. Byproducts of these metabolic pathways, which are called metabolites, are utilised to create intermediates and substrates for other metabolic pathways.

Several biological features exhibited by metabolites are important in the pharmacological, nutritional, and agricultural fields. Primary and secondary metabolites are two categories that these molecules fall into according to their metabolic processes and functional characteristics. Metabolites such as amino acids, pyruvate, citric acid, and lactic acid are essential for the biochemical and physiological processes carried out by living cells. Secondary metabolites, on the other hand, aren't required for cell proliferation but instead help the organism stay alive in tough situations.

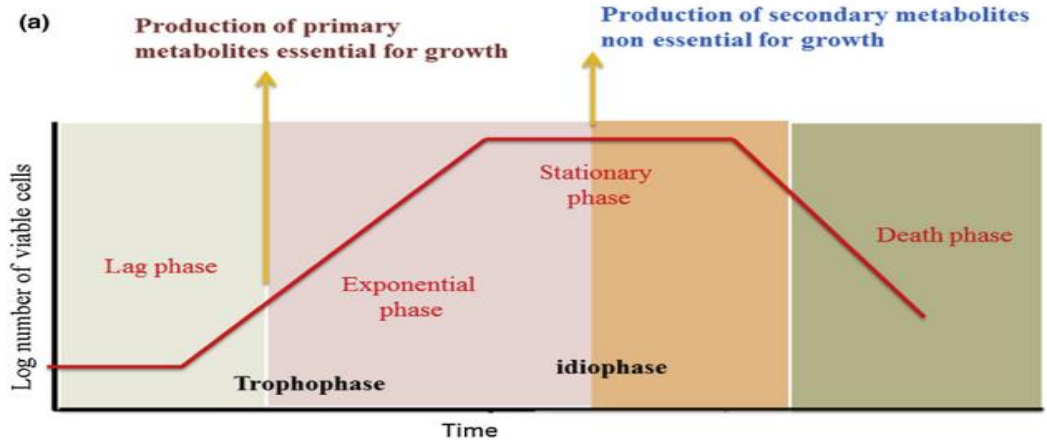
Bacterial secondary metabolite synthesis is the main topic of this chapter (Table 1). During their late and stationary phases of growth, the bacteria that produce secondary metabolites create these complex and bioactive compounds (Figure 1). When development conditions are restricted, environmental stress levels are high, or nutrients are depleted, the body responds by producing secondary metabolites. Many different kinds of organisms, including bacteria, fungus, plants, and marine creatures, produce secondary metabolites. Pesticides, anticancer agents, pigments, sex hormones, metal-transporting agents, antibacterial agents, immunomodulating agents, immunosuppressants, receptor agonists, and antagonists are all within these organisms' biological capabilities.

Enzymes work alone or in complexes to catalyse processes in the secondary metabolic pathway. A systematic metabolic pathway leads to the synthesis of secondary metabolites from intermediate or end-products of a primary metabolic pathway.

Table 1. Biochemical and physiological properties of primary and secondary metabolites.

Primary metabolites	**Secondary metabolites**
• Small molecules	• Small molecules

• Produces few intermediates or end-products • End-products are building blocks for macromolecules. • Essential for growth and cell viability • Known physiological function • Composed of simple chemical structure • End-products are used for Coenzyme synthesis • Production occurs at log phase • Primary metabolites are used in food and feed industry • Provides the energy for cellular activities	• Produces array of molecules • Synthesize new compounds • Not vital for the cell growth • Analysis of physiological function is difficult • Products of complex unusual chemical structure • End-products are used an antibacterial agent • Production occurs at late and dormant phase • Secondary metabolites are used in food, cosmetic, agricultural and farming industry • Protects the organisms under various harsh environment



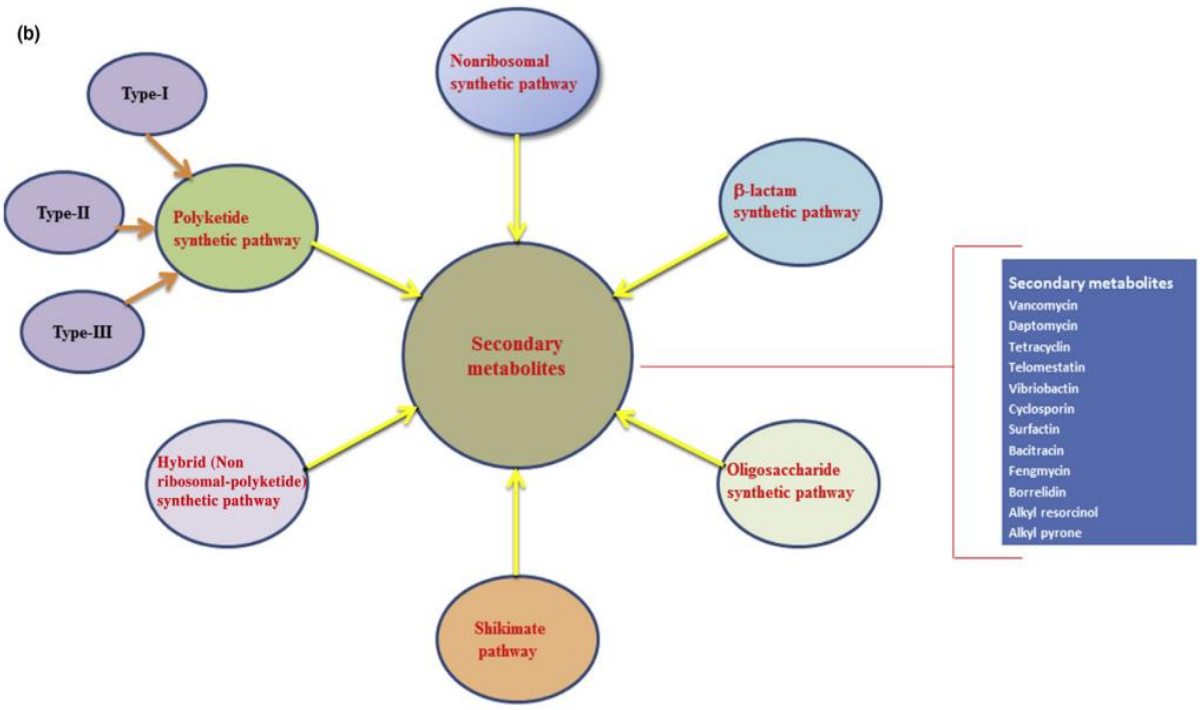


Figure 1. (a) Various phases of bacterial growth and production of metabolites. The primary metabolites production generally occurs at the late lag phase and middle of exponential phase. The secondary metabolites production occurs at the end of the stationary phase and during the persistent phase. **(b)** Various pathways responsible for the assembly of secondary metabolites.

Secondary Metabolites of Microorganisms

Microbial secondary metabolites are low molecular mass products with unusual structures. The structurally diverse metabolites show a variety of biological activities like antimicrobial agents, inhibitors of enzymes and antitumors, immune-suppressives and antiparasitic agents [35,36], plant growth stimulators, herbicides, insecticides, antihelmintics, etc. They are produced during the late growth phase of the microorganisms. The secondary metabolite production is controlled by special regulatory mechanisms in microorganisms, as their production is generally repressed in logarithmic phase and depressed in stationary growth phases. The microbial secondary metabolites have distinctive molecular skeleton which is not found in the chemical libraries and about 40% of the microbial metabolites cannot be chemically synthesized.

Features of microbial secondary metabolites

- The principle and process of natural fermentation product synthesis can be successfully scaled up and employed to maximize its application in the field of medicine, agriculture, food, and environment.
- The metabolite can serve as a starting material for deriving a product of interest, extended further through chemical or biological transformation.
- New analog or templates in which secondary metabolite serve as lead compounds will lead discovery and design of new drugs.

Applications of Microbial Secondary Metabolites

Antibiotics:

The discovery of penicillin initiated the researchers for the exploitation of microorganisms for secondary metabolite production, which revolutionized the field of microbiology [37]. With the advent of new screening and isolation techniques, a variety of β-lactam-containing molecules [38] and other types of antibiotics have been identified. About 6000 antibiotics have been described, 4000 from actinobacteria. In the prokaryotic group, unicellular bacteria Bacillus and Pseudomonas species are the most recurrent antibiotic produc ers. Likewise in eukaryotes, fungi are dominant antibiotic producers next to plants. In the recent years, myxobacteria and cyanobacteria species have joined these distinguished organisms as productive species.

The pharmaceutical product, especially anti-infective derivatives comprise 62% antibacteri als, 13% sera, immunoglobulins, and vaccines, 12% anti-HIV antivirals, 7% antifungals, and 6% nonHIV antivirals. There are over 160 antibiotics. Streptomyces hygroscopicus with over 200 antibiotics, Streptomyces griseus with 40 antibiotics, and Bacillus subtilis with over 60 com pounds are the major contributors to the antibiotic market [39].

Antitumor agents

Natural product and its derivatives account for more than 60% of anticancer formula tions. Actinobacteria derived antineoplastic molecules currently in use are actinomycin D, anthracyclines (daunorubicin, doxorubicin, epirubicin, pirarubicin, and valrubicin), bleomy cin, mitosanes (mitomycin C), anthracenones (mithramycin, streptozotocin, and pentostatin), enediynes (calicheamicin), taxol, and epothilones [40]. Taxol is the nonactinobacterial molecule derived from plant Taxus brevifolia and endophytic fungi Taxomyces andreanae and Nodulisporium sylviforme. It interferes with microtubule break down, an essential event leading to cell division, thereby inhibiting rapidly dividing cancer cells. It is effective against breast and advanced form Kaposi's sarcoma. It is also found to exhibit antifungal activity against Pythium, Phytophthora, and Aphanomyces.[41]

Pharmacological and nutraceutical agents

One huge success was the discovery of the fungal statins, including compactin, lovastatin, pravastatin, and others which act as cholesterol-lowering agents. Lovastatin is produced by A. terreus. Of great importance in human

medicine are the immunosuppressants such as cyclo sporin A, sirolimus (rapamycin), tacrolimus, and mycophenolate mofetil. They are used for heart, liver, and kidney transplants and were responsible for the establishment of the organ transplant field. Cyclosporin A is made by the fungus Tolypocladium niveum. Mycophenolate mofetil is a semisynthetic product of the oldest known antibiotic, mycophenolic acid, and is also made by a fungus. Sirolimus and tacrolimus are products of streptomycetes [42].

Metabolites of probiotic bacteria are considered as a remedy for controlling weight gain, pre venting obesity, increasing satiety, prolonging satiation, reducing food intake, reducing fat deposition, improving energy metabolism, treating and enhancing insulin sensitivity, and treating obesity. Firmicutes and Bacteroidetes are the dominant beneficial bacteria present in the normal human gastrointestinal tract, and the latter was reported in lower numbers in constipation-predominant irritable bowel syndrome patients [43].

Carotenoids of microbial origin are used as food colorant, fish feeds, nutraceuticals, cosmetics, and antioxidants. Food colorant widely used is carotene derived from Blakeslea trispora, Dunaliella salina and lycopene from B. trispora and Streptomyces chrestomyceticus, subsp. rubescens. Astaxanthin produced from Xanthophyllomyces dendrorhous is an approved fish feed. Astaxanthin, lutein, β-carotene, zeaxanthin, and canthaxanthin are used as nutraceuticals due to their excellent antioxidant property. Docosahexaenoic acid (DHA) used in infant formula as nutritional supplements is derived from microalgae Schizochytrium spp. [44].

Enzymes and enzyme inhibitors

Enzymes produced from microorganism have annual sales of US $ 2.3 billion enzymes that find application in detergents (34%), foods (27%), agriculture and feeds (16%), textiles (10%), and leather, chemicals, and pulp and paper (10%). The protease subtilisin used in detergents has an annual sale of $ 200 million. The other major enzymes include glucose isomerase (100,000 tons) and penicillin amidase (60,000 tons). Nitrilase (30,000 tons) and phytase are amounting for $135 million worth of production. Streptomyces glucose isomerase is used to isomerize D-glucose to D-fructose, to make 15 million tons per year of high fructose corn syrup, valued at $1 billion [45].

The most important enzyme inhibitors are clavulanic acid, synthesized by Streptomyces cla vuligerus, the inhibitor of β-lactamases. Some of the common targets for other inhibitors are glucosidases, amylases, lipases, proteases, and xanthine oxidase. Amylase inhibitors prevent absorption of dietary starches into the body, and hence can be used for weight loss.

Agricultural and animal health products

Secondary metabolites find wide applications in the field of agriculture and animal health: kasugamycin and polyoxins are used as biopesticides; Bacillus thuringiensis crystals, nikkomy cin, and spinosyns are used as bioinsecticides; bioherbicides (bialaphos) find application as bioherbicides; ivermectin and doramectin as antihelmintics and endectocides against worms, lice, ticks, and mites; ruminant growth promoters in the form of coccidiostats; plant hormones like gibberellins as growth regulators are the most common application [46].

Production of secondary metabolites from microorganisms

1. Liquid fermentation Batch or fed-batch culture in submerged fermentation is employed for production of secondary metabolites. Inoculum is developed after careful strain improvement of producing organism. Initially, shake flasks culture is employed, and the culture which are in active growth phase are transferred to a small fermenter and later into a larger fermenter with production medium. Several parameters, like medium composition, pH, temperature, and agitation and aeration rate, are controlled [47,48]. An inducer such as methionine is added to cephalosporin fermentations, phosphate is restricted in chlortetracycline fermentation, and glucose is avoided in penicillin or erythromycin fermentation.
2. Solid-state fermentation Solid-state fermentation, defined as a microbial culture that develops on the surface and at the interior of a solid matrix and in the absence of free water, holds an important potential for the production of secondary metabolites [49]. Two types of SSF can be distinguished, depending on the nature of solid phase used: (a) solid culture of one support-substrate phase solid phase and (b) solid culture of two substrate-support phase solid phase. The advantages of solid state fermentation in relation with submerged

fermentation include: energy requirements of the process are relatively low, since oxygen is transferred directly to the microorganism. Secondary metabolites are often produced in much higher yields, often in shorter times, and often sterile conditions are not required.

Biological Interpretation of Results

At this point, a large knowledge gap exists in the translation from changes in metabolite concentration in body fluids to organ biochemistry and (molecular) physiological interpretation [50]. Existing scarce information are usually only available for specific species, organs, or body fluids and cannot be transferred from one another easily. Software tools, such as pathway or enrichment analysis are not taking that into account. What does it actually mean, if metabolite X from a distinct pathway is found to be decreased? On one hand, downstream enzymes could have an increased activity or, on the other hand, the enzyme producing metabolite X could have a decreased activity; both can lead to a decreased level of metabolite X. Enzyme activities can be influenced by their specific product by steric inhibition/feedback-inhibition or activation, and thereby guarantee a balanced homeostasis in the cell. This strategy avoids large metabolic changes with a potential negative effect for the cell. Many metabolites play a role in several pathways and are the product or substrate of many different enzymes or processes. Thus, it is a challenge to pinpoint an altered metabolite to a specific pathway or enzyme. Nevertheless, the pathway information may already give the correct answer, or at least a hint, for a biological question. Changes in metabolite abundances can be mapped to specific pathways, thereby providing mechanistic information of the process under study. Data derived from metabolic profiling can be complemented by genome, proteome, clinical, and environmental data, which supports the discovery of potential biomarkers that would not have been identified with targeted studies alone [51-53].

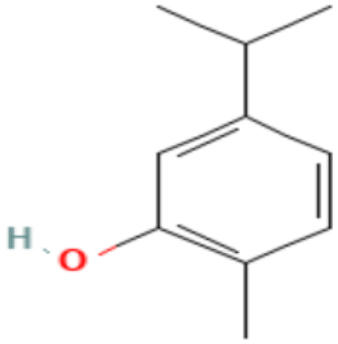
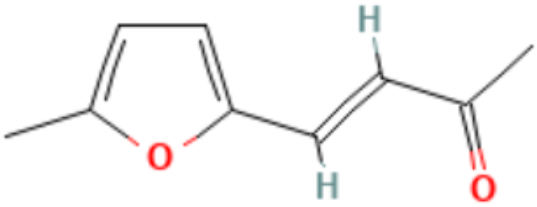
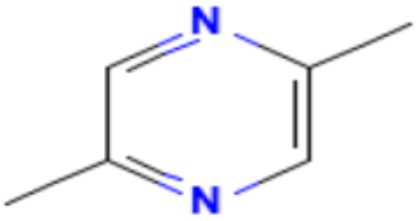
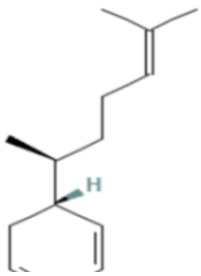
Metabolome profiles have the advantage to detect unknown compounds (shotgun) and alterations on a global scale (shotgun and targeted). As many metabolites usually originate from several different processes, specific methods have to be used for validation, like a knockdown of the according enzyme or isotope-labeled MFA. Many bioinformatics tools are continuously created to address several important questions in the metabolomic field, like interpreting profiling data. MetaboAnalyst (www.metaboanalyst.ca), permits a comprehensive metabolomic data analysis, visualization and interpretation, including complex statistical calculations [54, 55]. Metabolite pathway enrichment analysis (MPEA) was designed for the visualization and biological interpretation of metabolic profiling data at the system level. The tool tests whether metabolites involved in some predefined pathways occur towards the top or bottom of a ranked query compound list. Integrated Molecular Pathway Level Analysis (IMPaLA) [56, 57] is a tool for pathway over-representation and enrichment analysis with expression- and metabolite data. This proves the importance of metabolomics, but on the downside, many of these programs get funding for only a few years and are abandoned thereafter and egress, or even become useless, as file formats and necessary accompanying software changes permanently. Furthermore, most of the programs require specific raw or input data, which are frequently not interchangeable between programs. Biomarker discovery is driven by applying new instrumentation, protocols, and software tools, in order to find novel and specific key metabolic features, which are characteristic for specific pathological conditions, diseases, or cancer. Surprisingly, the clinical breakthrough is still out of sight. Nevertheless, metabolomic key features indicative for diseases such as depression [58], schizophrenia [59],

cardiovascular and coronary artery disease [60], diabetes [61], and cancers, such as liver, ovarian, and breast cancers have been reported [62]. The metabolic level variation between people, as well as within tissues and time points, is huge and dynamic. The genome, epigenome, transcriptome, and proteome states are much more stable compared to the high fluctuating metabolites. The aim in the biomarker field is to find biomarkers, which can precisely detect an early malignancy in order to achieve the best treatment effects and finally the highest survival rates for patients. So far, there were no new approved biomarkers in recent years [63, 64]. The current strategy to find single

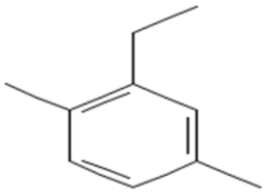
biomarkers for a disease is hampered by high and dynamic fluctuations of metabolites. As every disease not only changes one metabolite, but entire metabolic pathways, we probably should search for differentially regulated pathways or metabolite classes to be the more robust biomarkers in the future. This can be accomplished the best by an integrative approach taking many omics subdisciplines into account [65-68]. Thus, accurate multi-data analyses will be the key to reveal, assess, and track molecular patterns, which reflect disease-perturbed networks [69-73].

The fact that S. pyogenes remains a successful pathogen, despite its susceptibility to most modern therapies, reflects its exquisite ability to adapt its metabolism to exploit a variety of adverse environments and host tissues. Since metabolism is intimately linked to virulence, it is a burgeoning question and a matter of important discussion whether a virulence-promoting alteration to its metabolism comes at too high a cost in fitness and is a non-adaptive side effect of traits required for superficial symptomatic infection [74]. Cumulative evidence has provided unequivocal evidence that diverse metabolic activities enable S. pyogenes to survive successfully in the presence of a variety of stress conditions. The earlier interest in understanding the metabolism of S. pyogenes was driven by a need to investigate the biochemical basis for its growth requirements. The present chapter covers the knowledge obtained from subsequent reports of numerous investigators who used the established biochemical basis of metabolism to understand the underlying mechanisms of virulence as to how S. pyogenes, as a successful pathogen, senses its environment and changes its metabolic status to survive, persist, and proliferate in a broad range of host environments. It is also worth noting that early studies on S. pyogenes metabolism focused on various aspects of the pathogen's carbohydrate and amino acid metabolism. However, its lipid metabolism and membrane transport have received relatively limited attention, despite the fact that these processes play a crucial role in the secretion of many cellular products, including many virulence factors.

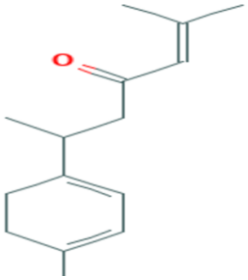
Table 2. Structures of metabolites produced by *Streptococcus pyogenes*

Compound	Structure
Carvacrol	
1-(5(Methyl-2-furanyl)-1-buten-3-one	
Pyrazine, 2,5-dimethyl-	
beta.Sesquiphellandrene	

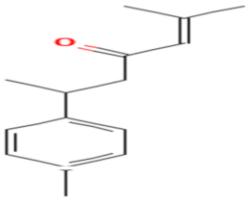
Benzene, 2-ethyl-1,4-dimethyl



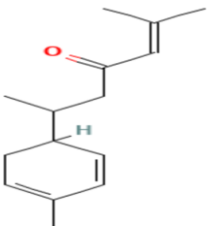
TUMERONE



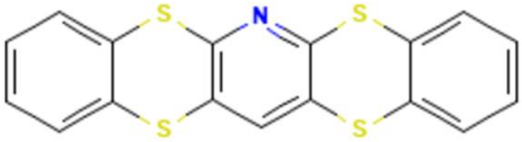
AR-TUMERONE



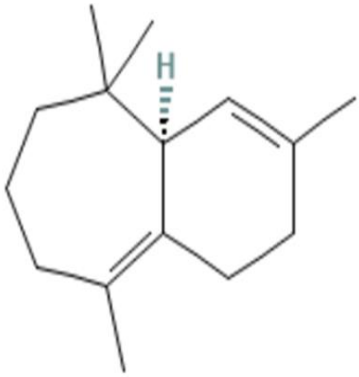
ALPHA.-TUMERONE



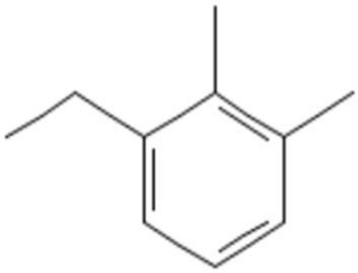
6-Aza-5,7,12,14-tetrathiapentacene



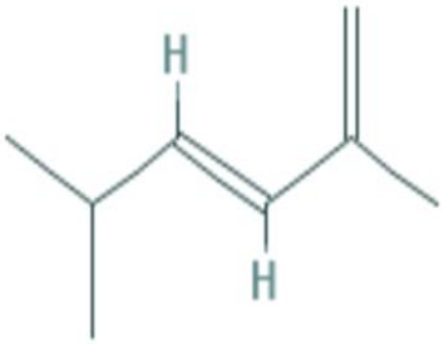
β-HIMACHALENOXIDE



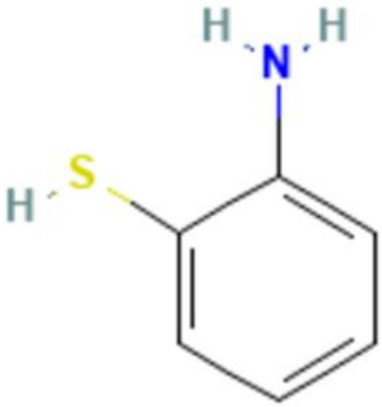
3-Ethyl-o-xylene



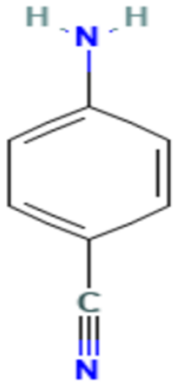
1,3-Hexadiene, 2,5-dimethyl-



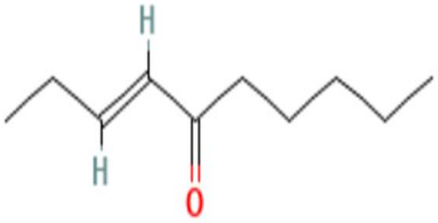
o-Mercaptoaniline



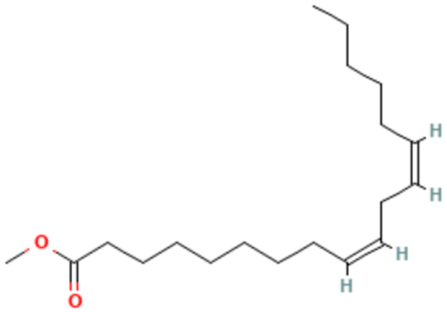
Benzonitrile, 4-amino-



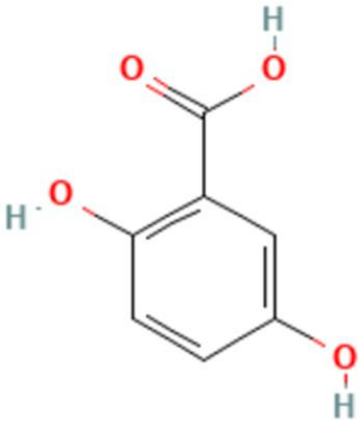
3-Decen-5-one



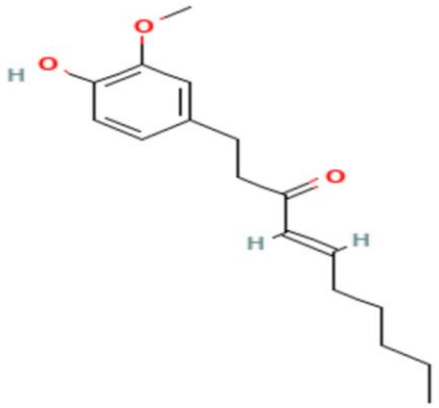
Methyl linoleate



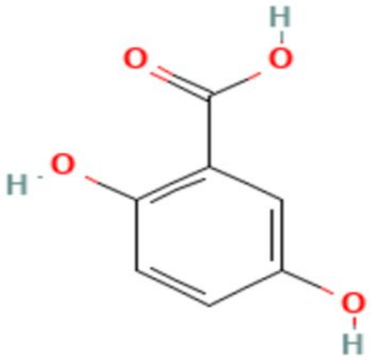
Gentisic acid



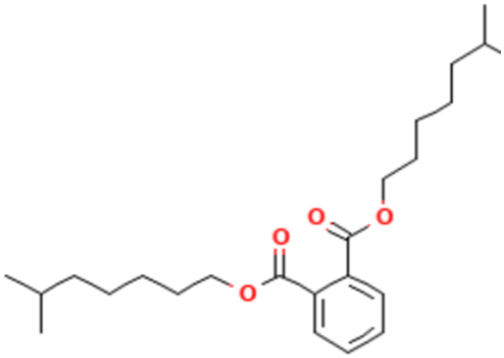
Shogaol



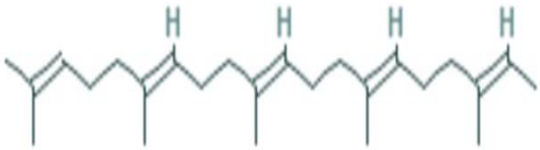
Gentisic acid



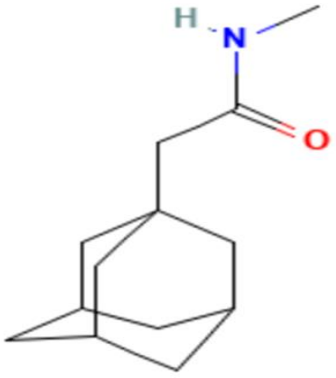
Isooctyl phthalate



2,6,10,14,18-Pentamethyl-eicosapentaene



N-Methyl-1-adamantaneacetamide



Conclusion

Our study provides evidence to pursue an ongoing study of volatile biomarkers in oropharyngeal streptococcal species. Several of the discriminant compounds, notably pyrazine compounds, are normally absent in healthy human exhaled breath but can be found in the *Streptococcus pyogenes* cultures. That these compounds are typically absent suggests that a number of these candidate biomarkers are not typically produced by other members of the oropharyngeal microbial community.

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