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Expanding, integrating, sensing and responding: the role of primary metabolism in specialised metabolite production

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Producing specialised metabolites such as antibiotics, immunosuppressives, anti-cancer agents and anti-helminthics draws on primary metabolism to provide the building blocks for biosynthesis. The growth phase-dependent nature of production means that producing organisms must deal with the metabolic conflicts of declining growth rate, reduced nutrient availability, specialised metabolite production and potentially morphological development. In recent years, our understanding of gene expansion events, integration of metabolic function and gene regulation events that facilitate the sensing and responding to metabolite concentrations has grown, but new data are constantly expanding our horizons. This review highlights the role evolutionary gene or pathway expansion plays in primary metabolism and examine the adoption of enzymes for specialised metabolism. We also look at recent insights into sensing and responding to metabolites.

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Introduction

All organisms on earth are comprised of chemicals. Bacteria are no different. Indeed, there is a highly conserved collection of chemicals, the products of central metabolic pathways, which are shared by most bacteria. Remarkably these 'primary metabolites' number only several hundred compounds in most bacteria. Historically, the separation of 'primary' and 'secondary' metabolism was widely adopted across biology, yet the implication of this is that secondary metabolism is less important than primary metabolism [1,2]. This view has been changing recently and reflects that 'secondary metabolites' have specialised roles in the lifecycle of an organism and reflect adaptive functions in specific niches. It is these specialised metabolites that contribute to the huge structural and chemical diversity, we see in the natural products of micro-organisms and in particular the Actinobacteria. These metabolites expand the functional capability of these organisms in the natural environment and it is this functionality that we have been able to exploit as clinically useful drug-molecules such as antibiotics, immunosuppressives and anticancer agents. The role precursor supply plays in the production of specialised metabolites is often ignored. Here, we will discuss the role of primary metabolism in specialised metabolism in Actinobacteria and how expanding genetic repertoires, integrating functionality and sensing and responding to metabolite concentrations affects and facilitates the production of specialised metabolites.

The production of complex specialised metabolites is by dedicated biosynthetic gene clusters (BGCs) and is growth phase dependent, often being triggered by the limitation of a particular nutrient. In Actinobacteria such as *Streptomyces* production is intimately linked with morphological development [3,4•,5]. The biosynthetic precursors of all specialised metabolites are supplied from pools of primary metabolites, directly linking primary and specialised metabolism [4•,5,6•]. Drawing on these pools of primary metabolites for specialised metabolite production is likely to create significant metabolic conflict in producing organisms, where declining extracellular nutrients may limit intracellular processes, such that complex regulatory systems are required to ensure cellular integrity [4•]. Understanding the molecular mechanisms of how organisms cope with this is fundamental to our continued exploitation of the specialised metabolism of Actinobacteria.

Expanding

Genome analysis of *Streptomyces* reveals the presence of multiple genes predicted to encode identical primary metabolic enzyme functions [4•]. This is often referred to as redundancy, hypothesised to provide robustness and evolvability in metabolism [7,8]. 'Redundancy', however, is a misleading term, suggesting non-essentiality, with the large genomes of specialised metabolite producing organisms providing multiple routes to many metabolic intermediates. This may be adaptive under certain

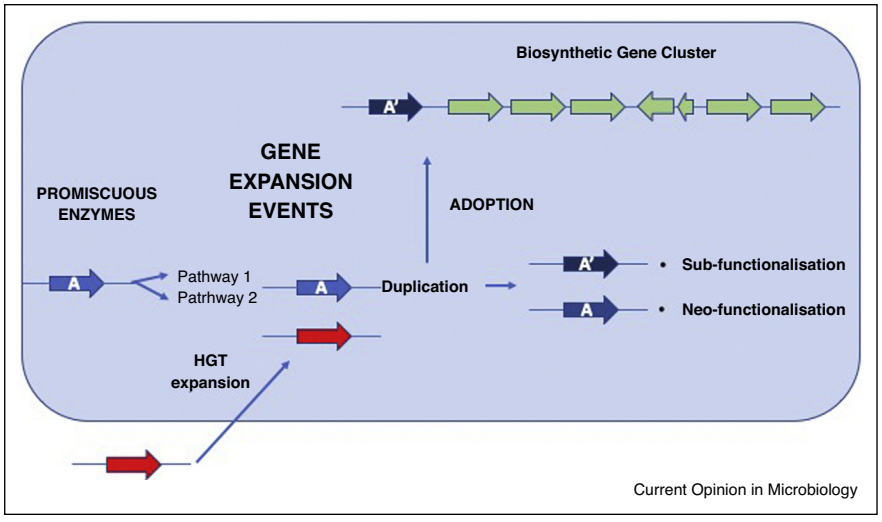
environmental conditions; therefore, 'contingency', 'metabolic flexibility' or 'enzyme expansion' may be more suitable terms [9,10]. This would also provide a framework to account for the 'moonlighting' enzymes, with catalytic promiscuity that facilitates the diversification of metabolism, which may have enabled the extensive specialised metabolism in bacteria to evolve [11,12,13••] (Figure 1).

Extensive primary metabolic enzyme expansion was recently surveyed across the whole Actinobacterial phylum using a phylogenomic approach to identify primary metabolic gene duplication events in the so called, metabolically 'talented' strains, that is, those strains that have extensive specialised metabolism [4•]. The conventional view is that gene duplication is the main source of gene expansion events in bacterial genomes. Following duplication, genes are thought to diverge by neo-functionalisation (gain of new functions by duplicates) or subfunctionalisation, where duplicates undergo complementary degeneration such that both copies are required to complement the function of the ancestral gene. Recently, it has been shown that horizontal gene transfer (HGT) plays the predominant role in gene expansion in bacteria and may be subject to similar divergence events [9] (Figure 1). Remarkably, in *Streptomyces* only two central carbon metabolic enzymes have been expanded through duplication, despite the extensive gene expansion events observed. These are phosphofructokinase (*pfk*), with two of the three genes arising from a duplication event [14] and pyruvate kinase (*pyk*) [4•]. A further 12 enzymes (a total of 48 genes) from glycolysis, TCA cycle, and amino acid metabolism exhibit gene expansion but a phylogenetic analysis revealed that the majority of these had expanded via HGT. It is generally considered that gene expansion events increase genetic robustness to mutation facilitating evolutionary innovation [8], adaptation and ultimately increase strain fitness [4•]. Detailed genetic and biochemical analysis of both *pfk* and *pyk* revealed that deletion mutants may exhibit antibiotic overproduction phenotypes. These data suggest that perturbation of the fine balance between duplicates in primary metabolism can affect precursor supply and stress responses, with each duplicate having a distinct physiological role. Moreover, in the case of *pyk*, the distinct physiological roles for each duplicate is achieved by allosteric regulation and substrate affinity, rather than through developmental expression or activation under specific environmental conditions [4•].

Application of genome scale metabolic modelling to these expanded gene families often misses the nuances of enzyme expansion events, as pathway flux is often combined in a single output and does not reflect the fine levels of control for each gene product. In an age of genome scale studies, this tells us that there is still a need for reductionist, single enzyme biochemical studies to really elucidate biological function.

As outlined above, gene duplications may contribute less to gene expansion events in Actinobacteria than previously thought and little consideration has been given to the role of HGT in metabolic gene expansion [10,15]. Conventional thoughts on biological innovation are that orthologs exhibit conserved functionality and paralogs

Figure 1



Gene expansion events. Gene A encodes for a primary metabolic enzyme. It may have promiscuous function, expanding its role in metabolism by acting in multiple pathways (Pathway 1 and Pathway 2). Its functionality can be expanded by HGT, gene duplication followed by neofunctionalisation or subfunctionalisation in primary metabolism or the gene duplicate may be adopted directly into a specialised metabolite cluster.

tend to diverge in functionality. Integrating HGT into studies of gene expansion events has been difficult to implement. However, it has recently been shown that horizontally acquired genes for metabolism may enable the evolution of existing metabolic functions and diversification of substrate specificity which may be more common that previously appreciated [10]. Using a combination of approaches spanning phylogenetics, structural biology, biochemistry and bioinformatics Cruz-Morales *et al.* [13••] showed that acquisition of key metabolic function may be shaped by positive selection, narrowing substrate specificity of ancient, promiscuous genes, which exhibit highly conserved structural features [10]. In *Streptomyces* the clustering of histidine and tryptophan genes on the chromosome reflects the lack of a *trpF* gene, with the dual (promiscuous) functional enzyme PriA (a ~50% homolog of *hisA*) enabling biosynthesis of both histidine and tryptophan [16]. These studies suggest that despite gene expansion events being common, convergent evolutionary processes are supporting primary metabolic diversity in Actinobacteria.

Whilst it is common that HGT can expand pathways in primary metabolism concomitantly increasing robustness and adaptive function, there are increasing examples of gene co-option/adoption directly into specialised metabolite biosynthetic clusters (Figure 1). Expanding and exploiting evolutionary guided approaches to understand specialised metabolite production have yielded some insight into this process. Using a database of 'precursor supply central metabolic pathway' (PSCP) enzymes, Cruz-Morales *et al.* [13••,17] were able to demonstrate the repurposing of primary metabolic enzymes into the BGCs for specialised metabolites. Identification of a homologue of *aroA*, the 3-phosphoshikimate-1-carboxyvinyltransferase usually involved in aromatic amino acid biosynthesis, associated with a two gene polyketide synthase (PKS) gene system [13••] suggested that there may be interplay between the enzymes. Synteny between *Streptomyces coelicolor* and *Streptomyces lividans* further suggested that there may be a functional linkage between the PKS genes and a cluster of genes required for phosphonate biosynthesis, located in proximity to some arsenate resistance genes suggesting that an arseno-organic metabolite may be the product of the pathway. Functional analysis of the cluster using genetics and biochemistry confirmed the presence of an arseno-organic molecule in the supernatant of *S. coelicolor* and *S. lividans* which was lost when a deletion mutant of the *aroA* homologue was constructed. The conceptual loop for this so called 'EvoMining' approach was closed with the widespread identification of similar BGCs in other *Streptomyces* species [13••]. Further examples of pathway specific precursor supply enzymes have recently been shown for the polyketide immunosuppressive FK506, with the identification of a pathway specific crotonyl-CoAcarboxylase/reductase in *Streptomyces tsukubaensis* [18•]. Two homologous *ccr* genes were identified, with *ccr1* being located within the ethylmalonyl-CoA biosynthetic operon, with a second copy *allR* being located on the fringes of the FK506 BGC. Deletion of *ccr1* results in a strain that cannot grow using acetate as a sole carbon source, complementation of the ethylmalonyl-CoA biosynthetic operon with *allR*, creating a chimeric operon, is unable to support FK506 production. These data reinforce the idea that there are distinct functional roles for duplicate genes and there is likely physiological partitioning.

Integrating

Given the rise in antimicrobial resistant infections, there is an urgent need to discover new antimicrobial drugs and to overproduce existing molecules, which may provide scaffolds for semi-synthesis. Understanding the integration of the pathways that supply precursors to specialised metabolism will facilitate strategies to enhance production of specialised metabolites [4•,19]. The wealth of genomic data available enables rapid genome scale modelling (GSM) of strains. Recently a GSM was produced for *Streptomyces leeuwenhoekii*, an organism that produces a novel ansamycin-like polyketide called chaxamycin and an additional polyketide, with anti-cancer activity called chaxalactin [20]. GSMs allow the identification of potential metabolic engineering targets to enhance specialised metabolite production. The work of Razmilic *et al.* [20] suggested that the deletion of genes that encode acetyl-CoA consuming reactions and increasing the production of acetyl-CoA and malonyl-CoA and pentose phosphate pathway intermediates may be routes to increasing chaxamycin and chaxalactin. There is a cautionary note relating to these studies, often plasticity and contingency in metabolism means that targets for metabolic engineering frequently do not yield the expected increase in production [21], validated targets may not show up in GSM studies [22,23] or activation of previously silent biosynthetic gene clusters may occur [4•].

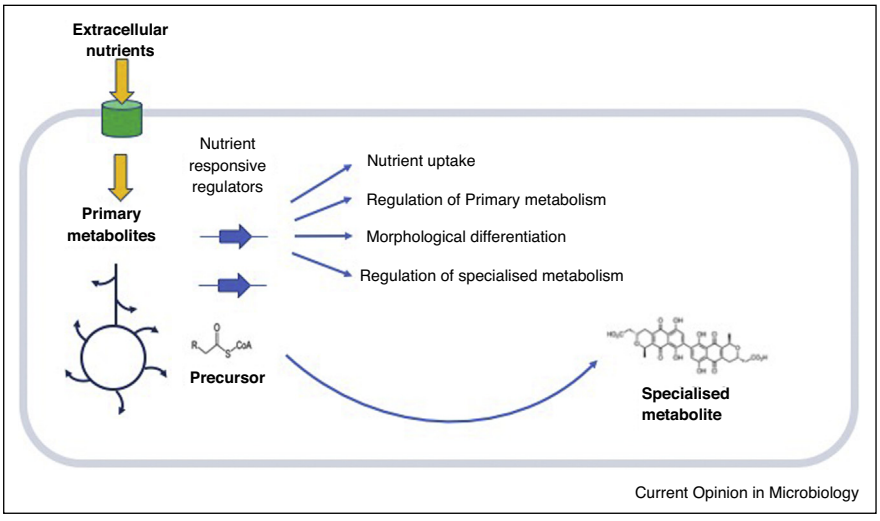
Enhancing supply of primary metabolic substrates can also be achieved through inhibition of key cellular processes — what has been termed a 'metabolic perturbation' approach. Recently, the inhibition of fatty acid biosynthesis in model and industrial strains of *Streptomyces* was used as a route to enhancing specialised metabolite production – in some cases achieving titre increases of 40% for polyketides [6•,24] – demonstrating that primary metabolism limits the production of specialised metabolites. Remarkably, inhibitors of fatty acid biosynthesis such as ARC2 or Triclosan may not be simply increasing the fatty acid pool for polyketide synthesis through the limitation of fatty acid synthesis but through the increase in pools of certain unsaturated fatty acids [25]. Using a similar 'metabolic perturbation' approach Tanaka *et al.* [6•] hypothesised that disruption of ribosomal activity, through ribosomal inhibiting drugs

would positively affect non-ribosomal peptide (NRP) synthesis by increasing the pool of intracellular amino acids that could then be targeted to the NRP machinery rather than protein synthesis. Using subinhibitory concentrations of chloramphenicol, these authors showed up to twofold increases in NRP specialised metabolites [6•] and achieving up to sixfold increases in cellular amino acid pools.

Sensing and responding

It has long been known that supply of phosphate, nitrogen and carbon can have profound effects on the production of specialised metabolites. In recent years, the application of global 'omics studies has shed increasing detail on the complexity of these interactions. The negative role played by glucose has long been known, but it is now becoming clear that the concentrations of these extracellular metabolites may downregulate specialised metabolism, yet the exact molecular mechanism remains elusive [26–28] (Figure 2). In *Streptomyces* the regulation of carbon catabolite repression (CCR) not directed by a phosphotransferase system (PTS) and is directly through glucose kinase (Glk) and its transport through the permease, GlcP [29]. Understanding how integrating the function of a Glk/GlkP system with the PTS for fructose and *N*-acetylglucosamine (GlcNAc) to control global carbon metabolism remains to be elucidated in *Streptomyces* [30–32]. There are still fundamental gaps in our knowledge of how *Streptomyces* regulate specialised metabolism with preferred carbon sources, the role of carbon catabolite repression and how this may be overcome in industrial situations. Recent progress has proposed that there are at least two mechanisms of regulation — one Glk-dependant and one Glk-independent (glucose dependent) [27], with the emphasis being on the second mechanism, where glucose may also stimulate additional carbon transporters [23,27,33], suggesting that there is a wider eco-evolutionary mechanism at play. Whilst the use of carbon sources such as glucose and glycerol are favoured in industrial scenarios, where rapid growth and high levels of biomass are desired, the negative effects of such on specialised metabolism suggests that perhaps a deeper understanding of the ecology of specialised metabolite producers may enable novel over-production strategies to be developed. The GntR-like regulator, DasR is one metabolite responsive regulator that links primary and specialised metabolism and which is known to bind directly to pathway specific regulators of specialised metabolites [23,32]. The sugar metabolism of streptomycetes is geared towards the amino sugar, GlcNAc rather than glucose [32], reflecting the ecology of an organism that has evolved in an environment that has an abundance of the GlcNAc polymer, chitin. GlcNAc can act as a carbon and nitrogen source in cells, but recent work has shown that DasR may be a central player in how responses to carbon, nitrogen and phosphate availability occur in the cell, affecting global primary metabolism and directly affecting the transcription of specialised metabolite BGCs [34,35]. The phosphate responsive PhoR-PhoP two component regulatory system and the orphan response regulator GlnR act as master regulators of phosphate uptake and global nitrogen metabolism respectively [36–40], with similarity and overlap in their binding sites [5]. PhoR-PhoP and GlnR are both known to have

Figure 2



Integrating metabolic function. Extracellular nutrient uptake provides the material for interconversion by primary metabolism. This provides the building blocks for cellular function, sensing and responding to these enables the regulation of specialised metabolism and the integration of metabolic function.

profound effects on specialised metabolite production, providing a regulatory link to well-studied physiological responses; however it is becoming apparent that other, less well studied nutrient sources may also have profound effects on specialised metabolism. A recent example of this is the role played by global sulphur metabolism in specialised metabolite precursor supply affecting the synthesis of albomycin, a sulphur moiety containing molecule [41].

Often overlooked in specialised metabolism is the requirement for maintenance of cofactor supply [21] and redox poise [42] to enable balancing of the substrate oxidation, energy supply and as key allosteric regulators of primary metabolic enzymes. Building on this, the work of Tala *et al.* [43••] has recently shown that a modulator of oxidative stress may act on gene expression in central carbon metabolism. Tala *et al.* [43••] demonstrated that a conserved iron-containing Pirin protein, PirA could affect beta-oxidation pathways, disrupting polyketide precursor supply in *Streptomyces ambofaciens*. This action is brought about through the negative regulation of a long chain fatty acid dehydrogenase which catalyses the first step of the beta-oxidation pathway. The identification of these effects of pirin suggests that there is still much to be discovered in the links between primary and specialised metabolism.

Summary

There is an intimate link between the biosynthesis of specialised metabolites and the supply of building blocks from primary metabolism. It is clear that producing organisms have invested a huge amount of genome content to link these processes in terms of expanding gene content to play specialised physiological roles, integrating metabolism to enable production to proceed and sensing and responding to the extracellular and intracellular environment to ensure appropriate production. There is still much to be learned about how these systems fully integrate, but it is clear that there is an increasing interest in using metabolic engineering and synthetic biology to enable the exploitation of bacterial specialised metabolism.

Conflict of interest statement

Nothing declared.

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