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6 Expanding, integrating, sensing and responding: the role of Primary metabolism in specialised
7 metabolite production
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Abstract

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/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.

Highlights

* The building blocks for specialised metabolite production are derived from primary metabolism and their supply is a key, yet often overlooked, component of the regulation of biosynthesis.

* Gene duplication and horizontal gene transfer have played a central role in the evolution and adaption of metabolism for the production of specialised metabolism. This can either be via adaptive expansion across the genome or specifically located in biosynthetic gene clusters. The adoption of primary metabolic enzymes into existing gene clusters may also facilitate the evolution of new specialised metabolites.

* Advances in ‘omics technologies have enabled us to use the wealth of genomic, transcriptomic and metabolomic data to model metabolism and survey metabolic capabilities, yet detailed reductionist approaches studying kinetics are often still required to fully elucidate the physiological role played an enzyme.

57 **Introduction**

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

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

84 of how organisms cope with this is fundamental to our continued exploitation of the specialised
85 metabolism of Actinobacteria.
86
87 **Expanding**
88 Genome analysis of *Streptomyces* reveals the presence of multiple genes predicted to encode
89 identical primary metabolic enzyme functions [4]. This is often referred to as redundancy,
90 hypothesised to provide robustness and evolvability in metabolism [7,8]. 'Redundancy',
91 however, is a misleading term, suggesting non-essentiality, with the large genomes of
92 specialised metabolite producing organisms providing multiple routes to many metabolic
93 intermediates. This may be adaptive under certain environmental conditions, therefore
94 'contingency', 'metabolic flexibility' or 'enzyme expansion' may be more suitable terms [9,10].
95 This would also provide a framework to account for the 'moonlighting' enzymes, with catalytic
96 promiscuity that facilitates the diversification of metabolism, which may have enabled the
97 extensive specialised metabolism in bacteria to evolve [11-13] (**Fig. 1**).
98 Extensive primary metabolic enzyme expansion was recently surveyed across the whole
99 Actinobacterial phylum using a phylogenomic approach to identify primary metabolic gene
100 duplication events in the so called, metabolically 'talented' strains, i.e. those strains that have
101 extensive specialised metabolism [4]. The conventional view is that gene duplication is the
102 main source of gene expansion events in bacterial genomes. Following duplication genes are
103 thought to diverge by neo-functionalisation (gain of new functions by duplicates) or sub-
104 functionalisation, where duplicates undergo complementary degeneration such that both
105 copies are required to complement the function of the ancestral gene. Recently it has been
106 shown that horizontal gene transfer (HGT) plays the predominant role in gene expansion in
107 bacteria and may be subject to similar divergence events [9] (**Fig. 1**). Remarkably, in
108 *Streptomyces* only two central carbon metabolic enzymes have been expanded through
109 duplication, despite the extensive gene expansions observed. These are phosphofructokinase
110 (*pfk*), with two of the three genes arising from a duplication event [14] and pyruvate kinase
111 (*pyk*) [4]. A further 12 enzymes (a total of 48 genes) from glycolysis, TCA cycle, and amino

112 acid metabolism exhibit gene expansion but phylogenetic analysis reveals that the majority of
113 these had expanded via HGT. It is generally considered that gene expansion events increase
114 genetic robustness to mutation facilitating evolutionary innovation [8], adaptation and
115 ultimately increase strain fitness [4]. Detailed genetic and biochemical analysis of both *pfk* and
116 *pyk* indicated that deletion mutants may exhibit antibiotic overproduction phenotypes. These
117 data suggest that perturbation of the fine balance between duplicates in primary metabolism
118 can affect precursor supply and stress responses, with each duplicate having distinct
119 physiological roles. Moreover, in the case of *pyk,* the distinct physiological roles for each
120 duplicate is achieved by allosteric regulation and substrate affinity, rather than through
121 developmental expression or activation under specific environmental conditions [4].

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

127 As outlined above, gene duplications may contribute less to gene expansion events in
128 Actinobacteria than previously thought and little consideration has been given to the role of
129 HGT in metabolic gene expansion [10,15]. Conventional thoughts on biological innovation are
130 that orthologs exhibit conserved functionality and paralogs tend to diverge in functionality.
131 Integrating HGT into studies of gene expansion events has been difficult to implement.
132 However, it has recently been shown that horizontally acquired genes for metabolism may
133 enable the evolution of existing metabolic functions and diversification of substrate specificity
134 which may be more common that previously appreciated [10]. Using a combination of
135 approaches spanning phylogenetics, structural biology, biochemistry and bioinformatics Cruz-
136 Morales et al., [13] showed that acquisition of key metabolic function may be shaped by
137 positive selection, narrowing substrate specificity of ancient, promiscuous genes, which exhibit
138 highly conserved structural features [10]. In *Streptomyces* the clustering of histidine and

139 tryptophan genes on the chromosome reflects the lack of a *trpF* gene, with the dual
140 (promiscuous) functional enzyme PriA (a ~50% homolog of *hisA*) enabling biosynthesis of
141 both histidine and tryptophan [16]. These studies suggest that despite gene expansion events
142 being common, convergent evolutionary processes are supporting primary metabolic diversity
143 in Actinobacteria.
144 Whilst it is common that HGT can expand pathways in primary metabolism concomitantly
145 increasing robustness and adaptive function, there are increasing examples of gene co-
146 option/adoption directly into specialised metabolite biosynthetic clusters (**Fig. 1**). Expanding
147 and exploiting evolutionary guided approaches to understand specialised metabolite
148 production has yielded some insight into this process. Using a database of 'precursor supply
149 central metabolic pathway' (PSCP) enzymes, Cruz-Morales et al., [13,17] were able to
150 demonstrate the repurposing of primary metabolic enzymes into the BGCs for specialised
151 metabolites. Identification of a homologue of *aroA*, the 3-phosphoshikimate-1-
152 carboxyvinyltransferase usually involved in aromatic amino acid biosynthesis, associated with
153 a two gene polyketide synthase (PKS) gene system [13] suggested that there may be interplay
154 between the enzymes. Synteny between *S. coelicolor* and *S. lividans* further suggested that
155 there may be a functional linkage between the PKS genes and a cluster of genes required for
156 phosphonate biosynthesis in proximity to some arsenate resistance genes suggesting that an
157 arseno-organic metabolite may be the product of the pathway. Functional analysis of the
158 cluster using genetics and biochemistry confirmed the presence of an arseno-organic
159 molecule in the supernatant of *S. coelicolor* and *S. lividans* which was lost when a deletion
160 mutant of the *aroA* homologue was constructed. The conceptual loop for this so called
161 'EvoMining' approach was closed with the widespread identification of similar BGCs in other
162 *Streptomyces* species [13]. Further examples of pathway specific precursor supply enzymes
163 has recently been shown for the polyketide immunosuppressive FK506, with the identification
164 of a pathway specific crotonyl-CoA carboxylase/reductase in *S. tsukubaensis* [18]. Two
165 homologous *ccr* genes were identified, with *ccr1* being located within the ethylmalonyl-CoA
166 biosynthetic operon, with a second copy *allR* being located on the fringes of the FK506 BGC.

167 Deletion of *ccr1* results in a strain that cannot grow using acetate as a sole carbon source,
168 complementation of the ethylmalonyl-CoA biosynthetic operon with *allR*, creating a chimeric
169 operon, is unable to support FK506 production. These data reinforce the idea that there are
170 distinct functional roles for duplicate genes and there is likely physiological partitioning.
171

172 **Integrating**

173 Given the rise in antimicrobial resistant infections, there is an urgent need to discover new
174 antimicrobial drugs and to overproduce existing molecules, which may provide scaffolds for
175 semi-synthesis. Understanding the integration of the pathways that supply precursors to
176 specialised metabolism will facilitate strategies to enhance production of specialised
177 metabolites [4,19]. The wealth of genomic data available enables rapid genome scale
178 modelling (GSM) of strains. Recently a GSM was produced for *S. leeuwenhoekii*, an organism
179 that produces a novel ansamycin-like polyketide called chaxamycin and an additional
180 polyketide, with anti-cancer activity called chaxalactin [20]. GSMs allow the identification of
181 potential metabolic engineering targets to enhance specialised metabolite production. The
182 work of Razmilic et al [20] suggested that the deletion of genes that encode acetyl CoA
183 consuming reactions and increasing the production of acetyl CoA and malonyl CoA and
184 pentose phosphate pathway intermediates may be routes to increasing chaxamycin and
185 chaxalactin. There is a cautionary note relating to these studies, often plasticity and
186 contingency in metabolism means that targets for metabolic engineering frequently do not
187 yield the expected increase in production [21], validated targets may not show up in GSM
188 studies [22,23] or activation of previously silent biosynthetic gene clusters may occur [4].
189 Enhancing supply of primary metabolic substrates can also be achieved through inhibition of
190 key cellular processes – what has been termed a 'metabolic perturbation' approach. Recently
191 the inhibition of fatty acid biosynthesis in model and industrial strains of *Streptomyces* was
192 used as a route to enhancing specialised metabolite production – in some cases achieving
193 titre increases of 40% for polyketides [6,24] – demonstrating that primary metabolism limits
194 the production of specialised metabolites. Remarkably, inhibitors of fatty acid biosynthesis

195 such as ARC2 or Triclosan may not be simply increasing the fatty acid pool for polyketide
196 synthesis through the limitation of fatty acid synthesis but through the increase in pools of
197 certain unsaturated fatty acids [25]. Using a similar 'metabolic perturbation' approach Tanaka
198 et al., [6] hypothesised that disruption of ribosomal activity, through ribosomal inhibiting drugs
199 would positively affect non-ribosomal peptide (NRP) synthesis by increasing the pool of
200 intracellular amino acids that could then be targeted to the NRP machinery rather than protein
201 synthesis. Using sub-inhibitory concentrations of chloramphenicol, these authors showed up
202 to 2-fold increases in NRP specialised metabolites [6] and achieving up to 6-fold increases in
203 cellular amino acid pools.
204

205 **Sensing and Responding**

206 It has long been known that supply of phosphate, nitrogen and carbon can have profound
207 effects on the production of specialised metabolites. In recent years the application of global
208 'omics studies have shed increasing detail on the complexity of these interactions. The
209 negative role played by glucose has long been known, but it is now becoming clear that the
210 concentrations of these extracellular metabolites may down-regulate specialised metabolism,
211 yet the exact molecular mechanism remains elusive [26-28] (**Fig. 2**). In *Streptomyces* the
212 regulation of carbon catabolite repression (CCR) not directed by a phosphotransferase system
213 (PTS) and is directly through glucose kinase (Glk) and its transport through the permease,
214 GlcP [29]. Understanding how integrating the function of a Glk/GlkP system with the PTS for
215 fructose and N-acetyl-glucosamine (GlcNAc) to control global carbon metabolism remains to
216 be elucidated in *Streptomyces* [30-32]. There are still fundamental gaps in our knowledge of
217 how *Streptomyces* regulate specialised metabolism with preferred carbon sources, the role of
218 carbon catabolite repression and how this may be overcome in industrial situations. Recent
219 progress has proposed that there are at least two mechanisms of regulation – one Glk-
220 dependant and one Glk-independent (glucose dependent) [27], with the emphasis being on
221 the second mechanism, where glucose may also stimulate additional carbon transporters
222 [23,27,33], suggesting that there is a wider eco-evolutionary mechanism at play. Whilst the

223 use of carbon sources such as glucose and glycerol are favoured in industrial scenarios,
224 where rapid growth and high levels of biomass are desired, the negative effects of such on
225 specialised metabolism suggests that perhaps a deeper understanding of the ecology of
226 specialised metabolite producers may enable novel over production strategies to be
227 developed. The GntR-like regulator, DasR is one metabolite responsive regulator that links
228 primary and specialised metabolism and which is known to bind directly to pathway specific
229 regulators of specialised metabolites [23,32]. The sugar metabolism of streptomycetes is
230 geared towards the amino sugar, GlcNAc rather than glucose [32], reflecting the ecology of
231 an organism that has evolved in an environment that has an abundance of the GlcNAc
232 polymer, chitin. GlcNAc can act as a carbon and nitrogen source in cells, but recent work has
233 shown that DasR may be a central player in how responses to carbon, nitrogen and phosphate
234 availability occur in the cell, affecting global primary metabolism and directly affecting the
235 transcription of specialised metabolite BGCs [34,35]. The phosphate responsive PhoR-PhoP
236 two component regulatory system and the orphan response regulator GlnR act as master
237 regulators of phosphate uptake and global nitrogen metabolism respectively [36-40], with
238 similarity and overlap in their binding sites [5]. PhoR-PhoP and GlnR are both known to have
239 profound effects on specialised metabolite production, providing a regulatory link to well-
240 studied physiological responses, however it is becoming apparent that other, less well studied
241 nutrient sources may also have profound effects on specialised metabolism. A recent example
242 of this is the role played by global sulphur metabolism in specialised metabolite precursor
243 supply affecting the synthesis of albomycin, which contains a sulphur moiety [41].
244 Often overlooked in specialised metabolism is the requirement for maintenance of cofactor
245 supply [21] and redox poise [42] to enable balancing of the substrate oxidation, energy supply
246 and as key allosteric regulators of primary metabolic enzymes. Building on this, the work of
247 Tala et al., [43] has recently shown that a modulator of oxidative stress may act on gene
248 expression in central carbon metabolism. Tala et al., [43] demonstrated that a conserved iron-
249 containing Pirin protein, PirA could affect beta-oxidation pathways, disrupting polyketide
250 precursor supply in *S. ambofaciens*. This action is brought about through the negative

251 regulation of a long chain fatty acid dehydrogenase which catalyses the first step of the beta-
252 oxidation pathway. The identification of these effects of pirin suggests that there is still much
253 to be discovered in the links between primary and specialised metabolism.
254

255 **Summary**

256 There is an intimate link between the biosynthesis of specialised metabolites and the supply
257 of building blocks from primary metabolism. It is clear that producing organisms have invested
258 a huge amount of genome content to link these processes in terms of expanding gene content
259 to play specialised physiological roles, integrating metabolism to enable production to proceed
260 and sensing and responding to the extracellular and intracellular environment to ensure
261 appropriate production. There is still much to be learned about how these systems fully
262 integrate, but it is clear that there is increasing interest in using metabolic engineering and
263 synthetic biology to enable the exploitation of bacterial specialised metabolism.
264

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271

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433 • ** Pirin is a small, iron-containing protein that is redoc active. These authors showed
434 that it regulates the beta-oxidation pathways in *Streptomyces ambofaciens*, and its
435 deletion disrupted production of the polyketide spiramycin. Pirin, modulates the activity
436 of a long-chain fatty acid dehydrogenase, and therefore release of precursors for
437 polyketide synthesis.

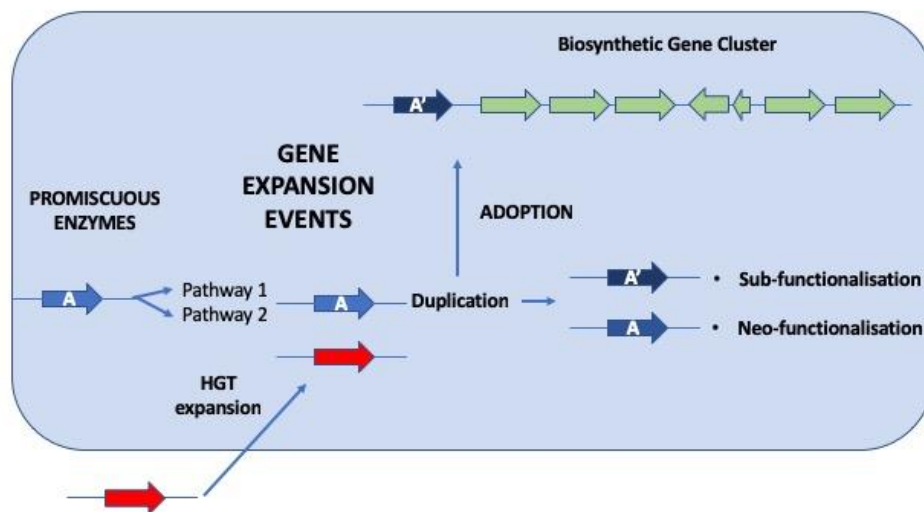
438

439

440 **Figure Legends**

441 **Fig. 1.** **Gene expansion events.** Gene A encodes for a primary metabolic enzyme. It
442 may have promiscuous function, expanding its role in metabolism by acting in
443 multiple pathways (Pathway 1 and Pathway 2). Its functionality can be expanded
444 by HGT, gene duplication followed by neo- or sub-functionalisation in primary
445 metabolism or the gene duplicate may be adopted directly into a specialised
446 metabolite cluster.

Fig. 1.



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449 **Fig. 2** **Integrating metabolic function.** Extracellular nutrient uptake provides the
450 material for interconversion by primary metabolism. This provides the building
451 blocks for cellular function, sensing and responding to these enables the
452 regulation of specialised metabolism and the integration of metabolic function.

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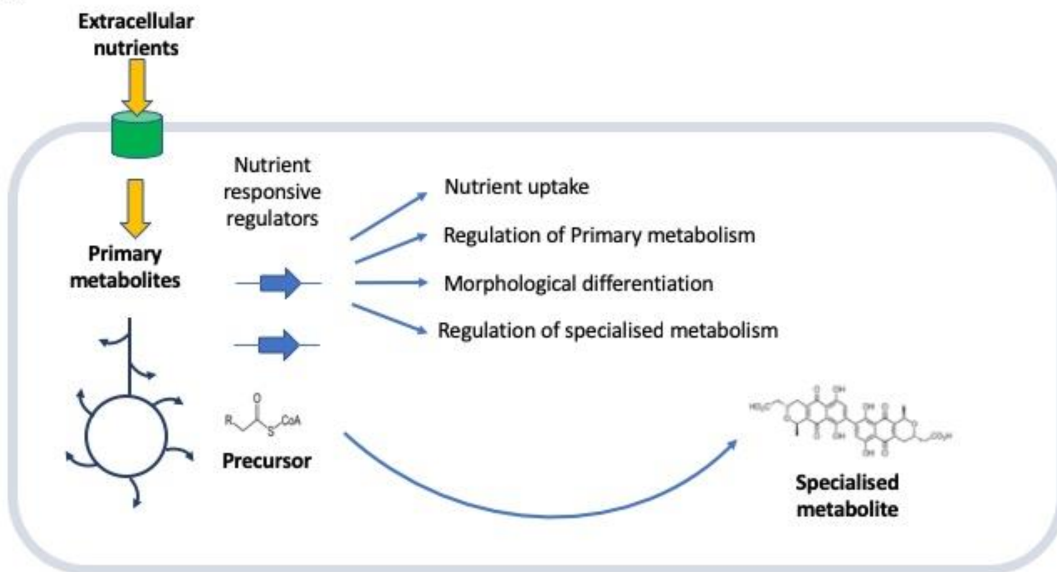

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