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6 Expanding, integrating, sensing and responding: the role of Primary metabolism in specialised

7 metabolite production

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29 **Abstract**

30 Producing specialised metabolites such as antibiotics, immunosuppressives, anti-cancer

31 agents and anti-helminthics draws on primary metabolism to provide the building blocks for

32 biosynthesis. The growth phase-dependent nature of production means that producing

33 organisms must deal with the metabolic conflicts of declining growth rate, reduced nutrient

34 availability, specialised metabolite production and potentially morphological development. In

35 recent years our understanding of gene expansion events, integration of metabolic function

36 and gene regulation events that facilitate the sensing and responding to metabolite

37 concentrations has grown, but new data are constantly expanding our horizons. This review

38 highlights the role evolutionary gene/pathway expansion plays in primary metabolism and

39 examine the adoption of enzymes for specialised metabolism. We also look at recent insights

40 into sensing and responding to metabolites.

41

42 **Highlights**

43 * The building blocks for specialised metabolite production are derived from primary

44 metabolism and their supply is a key, yet often overlooked, component of the regulation of

45 biosynthesis.

46 * Gene duplication and horizontal gene transfer have played a central role in the evolution

47 and adaption of metabolism for the production of specialised metabolism. This can either be

48 via adaptive expansion across the genome or specifically located in biosynthetic gene

49 clusters. The adoption of primary metabolic enzymes into existing gene clusters may also

50 facilitate the evolution of new specialised metabolites.

51 * Advances in 'omics technologies have enabled us to use the wealth of genomic,

52 transcriptomic and metabolomic data to model metabolism and survey metabolic capabilities,

53 yet detailed reductionist approaches studying kinetics are often still required to fully elucidate

54 the physiological role played an enzyme.

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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 [3-5]. The biosynthetic precursors of all specialised metabolites are supplied from pools of primary metabolites, directly linking primary and specialised metabolism [4-6]. 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

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

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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

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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 (**Fig. 1**). Expanding and exploiting evolutionary guided approaches to understand specialised metabolite production has 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 *S. coelicolor* and *S. lividans* further suggested that there may be a functional linkage between the PKS genes and a cluster of genes required for phosphonate biosynthesis 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 has recently been shown for the polyketide immunosuppressive FK506, with the identification of a pathway specific crotonyl-CoA carboxylase/reductase in *S. 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.

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.

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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

265 **Acknowledgements**

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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

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
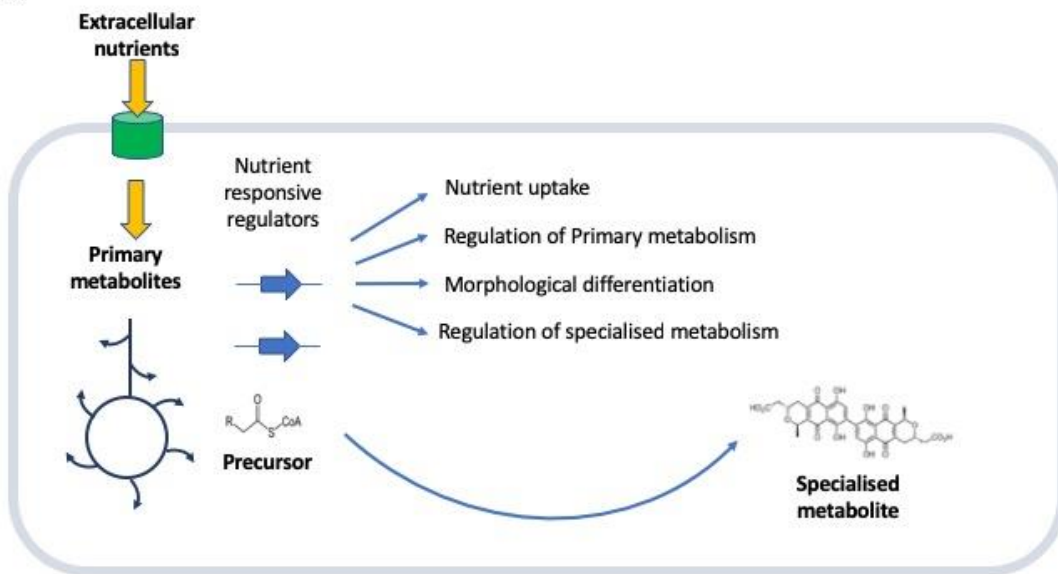
446 metabolite cluster.

Fig. 1.

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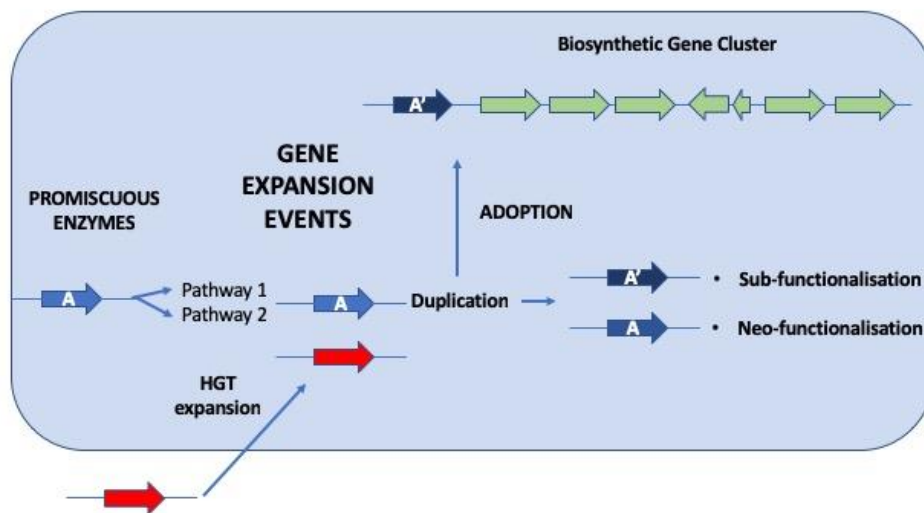
18

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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