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RESEARCH ARTICLE

Consequences of the deletion of the major specialized metabolite biosynthetic pathways of *Streptomyces coelicolor* on the metabolome and lipidome of this strain

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Abstract

Chassis strains, derived from *Streptomyces coelicolor* M145, deleted for one or more of its four main specialized metabolites biosynthetic pathways (CPK, CDA, RED and ACT), in various combinations, were constructed for the heterologous expression of specialized metabolites biosynthetic pathways of various types and origins. To determine consequences of these deletions on the metabolism of the deleted strains comparative lipidomic and metabolomic analyses of these strains and of the original strain were carried out. These studies unexpectedly revealed that the deletion of the peptidic clusters, RED and/or CDA, in a strain deleted for the ACT cluster, resulted into a great increase in the triacylglycerol (TAG) content, whereas the deletion of polyketide clusters, ACT and CPK had no impact on TAG content. Low or high TAG content of the deleted strains was correlated with abundance or paucity in amino acids, respectively, reflecting high or low activity of oxidative metabolism. Hypotheses based on what is known on the bio-activity and the nature of the precursors of these specialized metabolites are proposed to explain the unexpected consequences of the deletion of these pathways on the metabolism of the bacteria and on the efficiency of the deleted strains as chassis strains.

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INTRODUCTION

Bio-active specialized metabolites are produced by numerous microorganisms (Berdy, 2005). The production of these metabolites is usually triggered in conditions of nutritional limitation and their production coincides with phases of growth slowdown or arrest (Bibb, 2005). The production of these metabolites in these specific conditions raises the question of their contribution to the regulation of the metabolism and of the growth rate of the producing micro-organisms in these peculiar circumstances. Such questions were over-looked by the prevalence of current dogma stating that these metabolites constitute primarily weapons to kill or limit growth of other micro-organisms present in the same ecological niche as the producer and would thus confer a competitive advantage to the latter (Subirats et al., 2023). This conception is not wrong, it is simply incomplete. Indeed, considering that these bio-active specialized metabolites are produced in specific growth conditions, they are likely to have peculiar functions impacting primarily the biological systems of their producers in the context of their production. Nevertheless, when excreted, these metabolites could have similar biological effects on other micro-organisms present in the surroundings of the producer conferring competitive advantages to the latter (Hashem & Van Impe, 2021). Besides their bio-activity, the biosynthesis of these metabolites that involves the incorporation of specific precursors stemming from the primary metabolism may also fulfil a specific function in the cellular metabolism of the producer, in the condition where they are produced.

In an attempt to determine the impacts of the biosynthesis of the specialized metabolites on the metabolism and the physiology of the producing bacteria that could be either linked to precursors consumption or bioactivity, we carried out a comparative analysis of the lipidome and metabolome of the antibiotic producer *S. coelicolor* M145 (SC) with that of derivatives of this strain deleted for one or more of its 4 major specialized metabolic pathways, in various combinations. These clusters are the Cryptic PolyKetide/Coelimycin (CPK, precursors: ala, N-acetylcysteine and acetylCoA) (Gomez-Escribano et al., 2012), the peptidic Calcium Dependent Antibiotic (CDA, precursors: asp, asn, gly, hydrophenylgly, glu, methylglu, ser, thr, trp and acetyl-CoA) (Hopwood & Wright, 1983), the hybrid peptidic-polyketide undecylprodigiosin (RED, precursors: pro, gly, ser and acetylCoA) (Takano et al., 1992) and the polyketide actinorhodin (ACT, precursors derived from acetylCoA) (Okamoto et al., 2009). The deleted strains used in this study were constructed by JP Gomez-Escribano in MJ Bibb's group (Gomez-Escribano & Bibb, 2011) and included M1141 (Δ ACT), M1142 (Δ ACT, Δ RED), M1144 (Δ ACT, Δ RED, Δ CPK), M1148 (Δ ACT, Δ RED, Δ CDA, Δ RED, Δ CPK) and M1146 (Δ ACT, Δ RED, Δ CDA, Δ CPK).

These biosynthetic pathways constitute potentially carbon and nitrogen sinks whose elimination in the chassis strains was expected to be beneficial for the expression of heterologous specialized metabolites biosynthetic gene clusters of various types and origins.

In this issue, we compared the lipidome and the metabolome of the original strain with that of the deleted strains to determine the consequences of these deletions on the cellular metabolism. The outcomes of these studies were rather unexpected since it revealed that the deletion of the CDA and RED clusters, directing the biosynthesis of peptidic or of hybrid peptidic-polyketide, in a Δ ACT background, resulted into high triacylglycerol (TAG) content and paucity in amino acids, whereas the deletion of the clusters directing the biosynthesis of the polyketides, ACT and CPK, had no impact on TAG content but resulted into high amino acid content. The unexpected metabolic features of the deleted strains are discussed and hypothesis, based on what is known on the bio-activity and biosynthesis of these specialized metabolites, are proposed to explain the consequences of these deletions on the cellular metabolism as well as on the efficiency of these strains as chassis strains.

EXPERIMENTAL PROCEDURES

Strains and culture conditions

The strains used in this study were all derived from *S. coelicolor* M145 (SC) (Bentley et al., 2002) and were a generous gift of JP Gomez-Escribano and MJ Bibb (Gomez-Escribano & Bibb, 2011). These strains include M1141 (Δ ACT), M1142 (Δ ACT, Δ RED), M1144 (Δ ACT, Δ RED, Δ CPK), M1148 (Δ ACT, Δ RED, Δ CDA) and M1146 (Δ ACT, Δ RED, Δ CDA, Δ CPK). For all experiments, including the establishment of growth curves, these strains were grown in quadruplicates on agar plates of the modified R2YE medium devoided of sucrose and with no K_2HPO_4 added (condition of phosphate limitation). This medium is thought to be limited both in nitrogen and phosphate. Its main nitrogen sources are proline ($3g\ L^{-1}$), a mixture of casamino acids Difco ($0.1g\ L^{-1}$) and yeast extract ($5g\ L^{-1}$), whereas free Pi ($1\ mM$ as determined by Pi blue test from Gentaur, France) originates mainly from yeast extract. 10^6 spores of the strains were plated on the surface of cellophane disks (Focus Packaging & Design Ltd, Louth, United Kingdom) laid down on the surface of solid R2YE plates. The plates were incubated for 48 h or 72 h at $28^\circ C$, in obscurity. To establish growth curves, mycelium from three, 5 cm diameter plates, was scrapped off the cellophane disks with a spatula at defined time points throughout growth. The collected wet biomass was washed twice with deionized water,

lyophilized and weighted to establish growth curves as mg of dry cell weight (DCW) per plate.

Construction of the plasmids to over-express putative lipases encoding genes, *sco3222* and *sco3219*, present in the CDA cluster of *S. coelicolor* M145

To over-express putative lipases encoding genes, *sco3219* and *sco3222*, present in the CDA cluster of *S. coelicolor* M145, the coding sequence of these genes were amplified by PCR using the primers *sco3222-BamHI-5'* (5'-TATGGATCCgaagggagcggacatATGCGCACCACCACCAGG-3') and *sco3222-NotI-3'* (3'-TATGCGGCCGCTCAGCCGAAGATCTTGACGG-5') for *sco3222* and the primers *sco3219_XhoI_5'* (5'-TTATCTCGAGgagaagggagcggacatATGCCGAACGTGAGGGAAGC-3') and *sco3219-NotI-3'* (3'-TATAGCGGCCGCTCAGCGTCCGTGGGTGAC-5') for *sco3219*. Genomic DNA of *S. coelicolor* M145 was used as template and the phusion polymerase from New England Biolab (NEB) was used according to manufacturer's recommendations. The PCR fragments resulting from *sco3222* amplification were cut by *BamHI* and *NotI* and cloned into pOSV557 (Juguet et al., 2009) yielding pOSV557::*sco3222*. The PCR fragments resulting from *sco3219* amplification were cut by *XhoI* and *NotI* and cloned into pOSV557 yielding pOSV557::*sco3219*. In these constructs the expression of the genes was put under the control of the strong *ermE* promoter (Bibb et al., 1985). These plasmids were conjugated into SC to generate the strains SC/pOSV557::*sco3222* and SC/pOSV557::*sco3219*. The total lipid content and extracellular ACT production of these strains was compared with that of the control strain containing the empty plasmid, SC/pOSV557.

Lipidomic analysis

All strains were grown for 72h on modified R2YE medium and their lipidic content was analysed either by Fourier Transformed InfraRed Spectroscopy (FTIRS, Figure S2) (Deniset-Besseau et al., 2014; Millan-Oropeza, Rebois, et al., 2017) or by LC/MS² (Figures 2 and 5) as described previously (Lejeune et al., 2021).

Metabolomic analysis

All strains were grown for 48h on modified R2YE medium and their content in various metabolites was analysed in GC/MS as described previously (Apel et al., 2023).

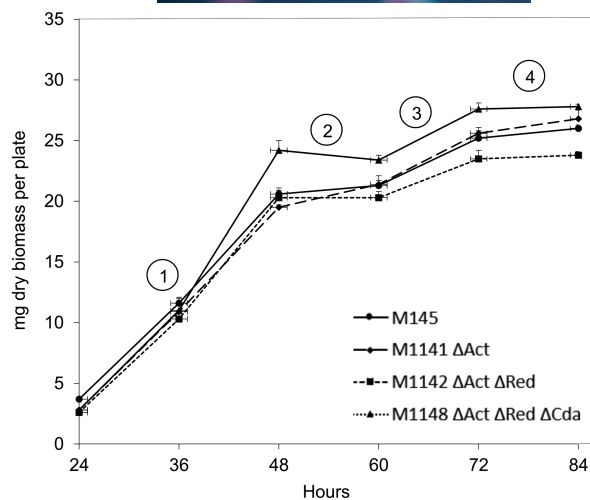


FIGURE 1 Growth curves of the strain *S. coelicolor* M145 (continuous black line with black circles), M1141 (Δ ACT) (large and black dashes line with black lozenges), M1142 (Δ ACT, Δ RED) (small and black dashes line with black squares) and M1148 (Δ ACT, Δ RED, Δ CDA) (dotted black line with black triangles) grown, in quadruplicate, on agar plates (5 cm diameter) containing 8 mL of modified R2YE medium (devoided of sucrose and with no K_2HPO_4 added, condition of Pi limitation) and containing 50 mM glucose as main carbon source, at 28°C, in obscurity.

Assay of extracellular ACT production

Extracellular ACT production was assayed as described in Dulermo et al. (2023).

RESULTS

Growth curves of the original strain *S. coelicolor* M145 and derived deleted strains

Growth curves of the original strain *S. coelicolor* M145 (SC) and of the deleted strains M1141, M1142 and M1148 were established to determine whether these deletions had any impact on growth. As shown in Figure 1, the results demonstrate that the growth curves of the four strains had the typical quadriphasic structure of *Streptomyces* growth curves in this specific medium. Growth curves start with an exponential growth phase (ph1) ending by a short phase of growth arrest (ph2) called the “transition phase” (Puglia et al., 1995) that is followed by a second phase of slower growth (ph3) that precedes the entry into stationary phase (ph4). In this solid R2YE medium limited in phosphate and nitrogen, the biosynthesis of the specialized metabolites biosynthetic clusters of SC occurs in an immutable order. The biosynthesis of CPK (Bednarz et al., 2019; Pawlik et al., 2007) and CDA (Gomez-Escribano et al., 2012) occurs before or during the “transition phase” (ph2) (Puglia et al., 1995); that of RED occurs in ph3 and that of ACT in stationary phase (ph4) (Takano et al., 1992).

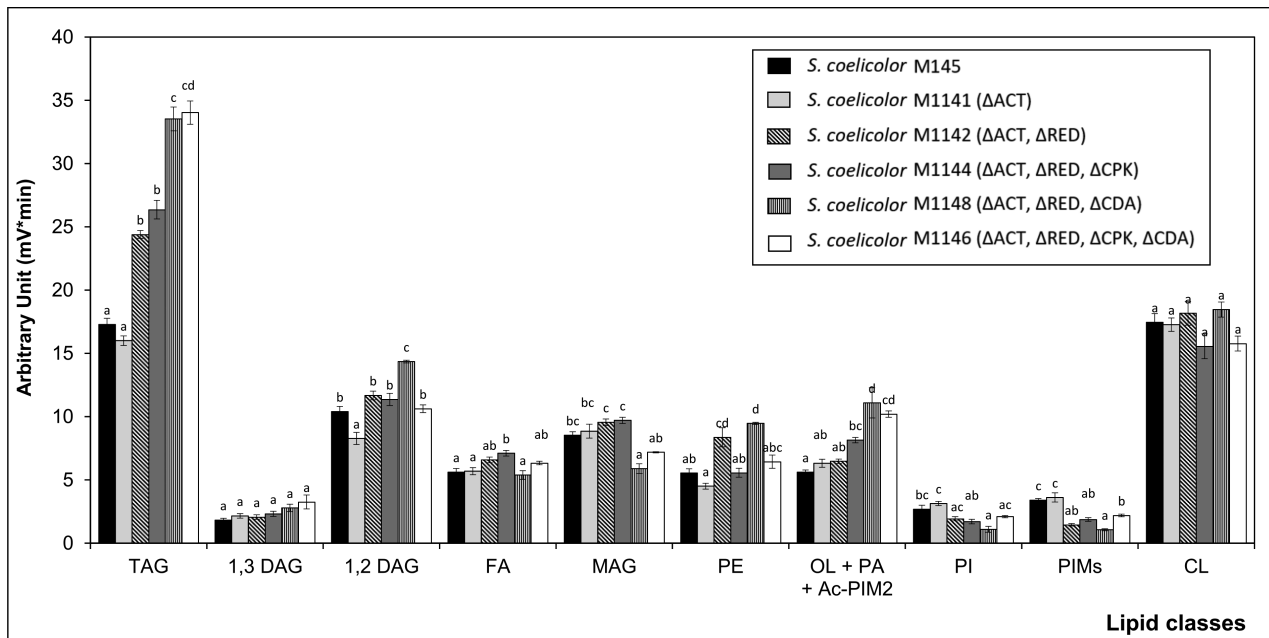


FIGURE 2 Lipid classes, as determined by LC/Corona-CAD analysis, present in *S. coelicolor* M145 (black histograms), M1141 (Δ ACT, light grey histograms), M1142 (Δ ACT, Δ RED) (hatched histograms); M1144 (Δ ACT, Δ RED, Δ CPK) (dark grey histograms); M1148 (Δ ACT, Δ RED, Δ CDA) (histograms with vertical lines); and M1146 (Δ ACT, Δ RED, Δ CPK, Δ CDA) (white histograms). The strains were grown, in quadruplicates, on modified solid R2YE medium limited in Pi (1 mM) for 72h at 28°C. Lipid classes from left to right are Triacylglycerol (TAG); 1,3- and 1,2-Diacylglycerol (DAG); Fatty acids (FA); Monoacylglycerol (MAG); Phosphatidylethanolamine (PE); ornithine lipids (OL), phosphatidic acid (PA) and acetylated phosphatidylinositol mannoside-2 (Ac-PIM2) that are co-eluting, phosphatidylinositol (PI), phosphatidylinositol-mannosides (PIMs) and cardiolipin (CL). Means values are shown as histograms with error bars representing standard error. Means sharing a letter are not significantly different ($p > 0.05$; Tukey-adjusted comparisons).

Data of Figure 1 indicate a slightly higher biomass yield of M1141 (Δ ACT) compared with M145 in ph4, a slower growth rate of M1142 (Δ ACT, Δ RED) compared with M1141 (Δ ACT) and M145 in ph3 and ph4 and a significantly higher biomass yield of M1148 (Δ ACT, Δ RED, Δ CDA) compared with M1142 (Δ ACT, Δ RED), from the end of ph 1 onwards.

Lipidomic analysis in LC/MS²

LC-MS² analysis was used to assess the consequences of the deletion of the various biosynthetic pathways on the lipidic content of the deleted strains. This analysis provided the content of these strains in neutral storage lipids of the TriAcylGlycerol family (TAG) and in membranous phospholipids including Phosphatidyl Ethanolamine (PE), Phosphatidyl Inositol (PI), Phosphatidyl Inositol Mannoside (PIMs) whose number of mannosides could not be determined and cardiolipins (CL) (Figure 2). Unfortunately Acetylated Phosphatidyl Inositol Mannoside-2 (Ac-PIM2), phosphatidic acid/PA as well as ornithine lipids (OL) that are phosphate-free lipids were co-eluted in our conditions. OL is within bracket in Figures 2 and 5 since it is present in very low amounts in SC (Sonia Abreu, personal communication). Biosynthetic intermediates

of the lipids mentioned above including, fatty acids; 1,2-DiAcylGlycerol (DAG) and 1,3-DAG, were also detected and shown in Figure 2.

The results of Figure 2 indicated that the deletion of the ACT cluster alone in M1141 or of the CPK cluster in M1142 (Δ ACT, Δ RED) and M1148 (Δ ACT, Δ RED, Δ CDA) yielding M1144 (Δ ACT, Δ RED, Δ CPK) and M1146 (Δ ACT, Δ RED, Δ CDA, Δ CPK), respectively, had no impact on the TAG and PE content of these strains (Figure 2). In contrast the deletion of the RED and/or CDA clusters in a strain already deleted for the ACT cluster had a clear positive impact on the TAG and PE content (to a lesser extent). These results were unexpected since the biosynthesis of polyketides and TAG requiring the same precursor, acetylCoA, the deletion of these clusters was predicted to result into an acetyl-CoA surplus that could be stored as TAG but this was obviously not the case.

Interestingly, the deletion of the ACT and RED clusters in M1142 and of the ACT, RED and CDA clusters in M1148 was correlated with a lower content in PI and PIMs than in M1141 (Δ ACT) and M145 (Figure 2). This suggested a link between the disappearance of these specific phospholipids and the accumulation of TAG and PE that will be commented in the light of the comparative metabolomic analyses of the following section.

Comparative metabolomic analysis of the strains in GC/MS

Since some specific primary metabolites are used as precursors for the biosynthesis of the specialized metabolites under study, targeted metabolomics analysis of the original and the deleted strains was carried out by GC–MS. Resulting metabolites quantifications were subjected to un-supervised principal component analysis (PCA, [Figure S1](#)) to obtain an overview of the metabolic differences between genotypes. PCA showed complete separation between the original strain *S. coelicolor* M145 and the deleted strains. Interestingly, M1141 (Δ ACT) replicates were clearly separated from M1142 (Δ ACT, Δ RED) and M1148 (Δ ACT, Δ RED, Δ CDA) replicates that both carry deletions of the RED and ACT clusters.

Comparative analysis of M145 and M1141 (Δ ACT)

The results shown in [Figures 3](#) and [4](#) revealed a contrasted abundance of various metabolites in M145 and M1141 resulting from the deletion of the ACT cluster.

Glucose 6P, isomaltose, myo-inositol, xylose, D-mannitol, glycolic and glyceric acids were more abundant in M1141 (Δ ACT) than in M145 ([Figures 3](#) and [4A](#)). Isomaltose and myo-inositol are two metabolites derived from glucose 6P in a single enzymatic step. Xylose is a metabolite stemming from the Pentose Phosphate Pathway (PPP) whose entry point is glucose 6P. D-mannitol is derived from fructose 6 P, itself derived from glucose 6P. The abundance of these metabolites in M1141 suggests the occurrence of a bottleneck after the generation of fructose 6P at the level of the phosphofruktokinase whose activity might be inhibited by ATP as demonstrated in other bacteria (Bang et al., 1977). ACT was previously proposed to have a negative impact on ATP generation since the production of ACT was shown to be coincidental with an abrupt drop of the intracellular ATP concentration (Esnault et al., 2017). The deletion of the ACT cluster in M1141 would thus result into a higher ATP content than in M145 that might be responsible for this bottleneck as well as for the slightly higher biomass yield of this strain compared with M145. Such bottleneck would reduce the carbon flux through glycolysis and thus the generation of acetylCoA that could have been stored as TAG. At last, the higher abundance of glycolic and glyceric acids that are both oxidation products of glycerol, in M1141 compared with M145, may reduce the availability of glycerol, a molecule necessary for TAG biosynthesis.

Furthermore, nine amino acids (AA) were found more abundant in M1141 (Δ ACT) than in M145 ([Figure 4C](#)). These include serine and isoleucine/leucine (and beta

hydroxyiso valerate a leucine-derived metabolite) synthesized from 3-phosphoglycerate and pyruvate, respectively; tyrosine synthesized from phosphoenolpyruvate (PEP) and erythrose 4-phosphate originating from the Pentose Phosphate Pathway (PPP); proline, citrulline and γ -aminobutyric acid (GABA) derived from glutamate itself derived from α -ketoglutarate as well as methionine and threonine (degraded into threonic acid) derived from aspartate itself derived from oxaloacetate. Amino acids abundance in M1141 indicates that the TCA cycle is active and thus consumes acetylCoA whose availability might result, at least in part, from the absence of ACT biosynthesis. An active TCA cycle generates reduced co-factors whose re-oxidation by the respiratory chain generates oxidative stress (Imlay, 2019). Since ACT was shown to have anti-oxidant properties (Esnault et al., 2017; Millan-Oropeza, Henry, et al., 2017), the deletion of the ACT cluster in M1141 is likely to be correlated with a raise of oxidative stress in this strain. Consistently, the lipidic compounds, lauric acid ($\text{CH}_3-(\text{CH}_2)_{10}-\text{COOH}$) and squalene, that are known to have anti-oxidant properties (Shaheryar et al., 2023; Tian et al., 2023), were more abundant in M1141 than in M145 ([Figures 3](#) and [4B](#)). Their biosynthesis might be triggered by the high oxidative stress resulting for the deletion of the ACT cluster and would thus compensate for the loss of ACT production.

Altogether, these results unexpectedly revealed that the absence of ACT biosynthesis has an indirect negative impact on the glycolytic activity of the strain and a positive one on the activity of the TCA cycle and thus on AA biosynthesis. The biosynthesis of ACT at stationary phase consumes acetylCoA and its bioactivity might impair ATP synthesis. ACT thus possibly contributes to the management of the metabolic consequences of the growth arrest at stationary phase or directly contributes to the growth arrest itself. Interestingly, the specific metabolic features of M1141 (Δ ACT) are predicted to have a positive influence on the biosynthesis of peptidic antibiotics of endogenous or heterologous origin.

Comparative analysis of M1141 (Δ ACT) and M1142 (Δ ACT, Δ RED)

The results shown in [Figures 3](#) and [4](#) revealed a contrasted abundance of various metabolites between M1141 and M1142 that ought to result from the deletion of the RED cluster. RED is an hybrid peptidic-polyketide antibiotic that requires pyruvate and the amino acids, Gly, Ser and Pro for the biosynthesis of its peptidic moiety and acetylCoA for the biosynthesis of its C11 acyl moiety (Takano et al., 1992). The production of prodiginines synthesized by the RED cluster occurs in ph 3 after the “transition phase” (ph 2). Prodiginines are thought to be involved in

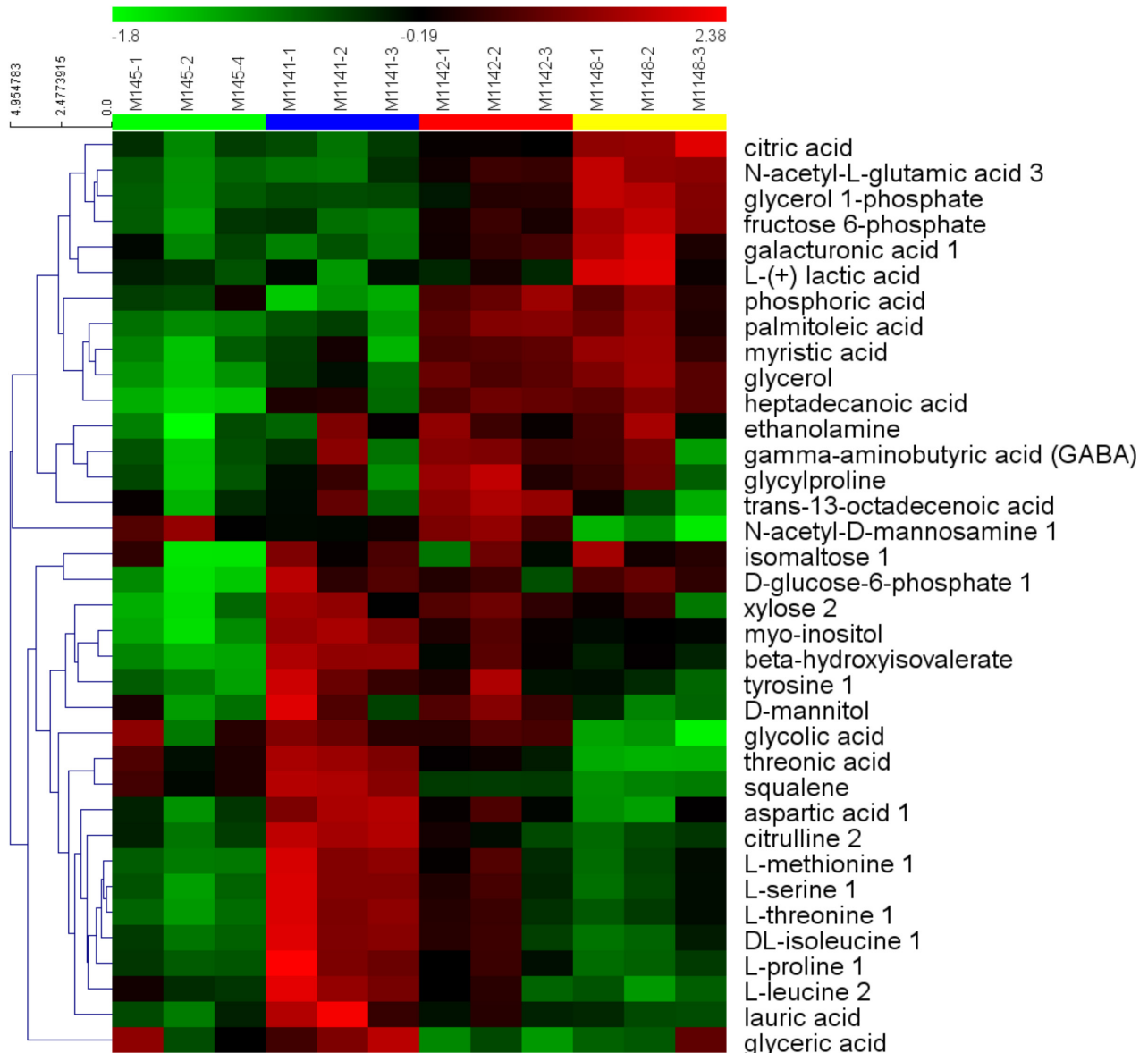


FIGURE 3 Global comparative analysis of the metabolome of *S. coelicolor* M145, M1141 (Δ ACT), M1142 (Δ ACT, Δ RED) and M1148 (Δ ACT, Δ RED, Δ CDA) using GC/MS. Each strain was plated at 10^6 spores per plate, in triplicates, on 9 cm diameter Petri dishes containing 20 mL of modified R2YE medium (devoided of sucrose and with no K_2HPO_4 added, condition of Pi limitation, 1 mM Pi), containing 50 mM glucose as main carbon source and incubated for 48 h at 28°C, in obscurity.

the programmed cell death process of SC (Tenconi et al., 2018) and to contribute to the leakage of the cellular content of dying mycelial filaments that constitutes carbon, nitrogen and phosphate sources for the surviving hyphae (Tenconi et al., 2020).

Interestingly, glucose 6P, isomaltose, xylose, myo-inositol, glyceric acid (Figure 4A), most AA detected (Figure 4C) as well as squalene and lauric acid were less abundant in M1142 than in M1141, whereas trans-13-octadecenoic acid that is also known to have antioxidant properties (Furumoto et al., 2016), was more abundant. The lower abundance of lauric acid and squalene in M1142 than in M1141 suggests a lower level of oxidative stress in M1142 than in M1141. However,

since trans-13-octadecenoic acid was more abundant in M1142 than in M1141, this signals the existence of oxidative stress in this strain although probably at a lower level than in M1141. Indeed, the lower AA content of M1142 compared with M1141 is consistent with a reduced feeding and thus activity of the TCA cycle that is due to the storage of acetylCoA as TAG. This lower activity of the oxidative metabolism is correlated with a lower level of oxidative stress (Imlay, 2019).

The fatty acids, heptadecanoic, myristic and palmitoleic acids (Figure 4B) as well as glycerol and glycerol 1P (to a lesser extent) (Figure 4A) were more abundant in M1142 than in M1141. The high abundance of these fatty acids and of glycerol in

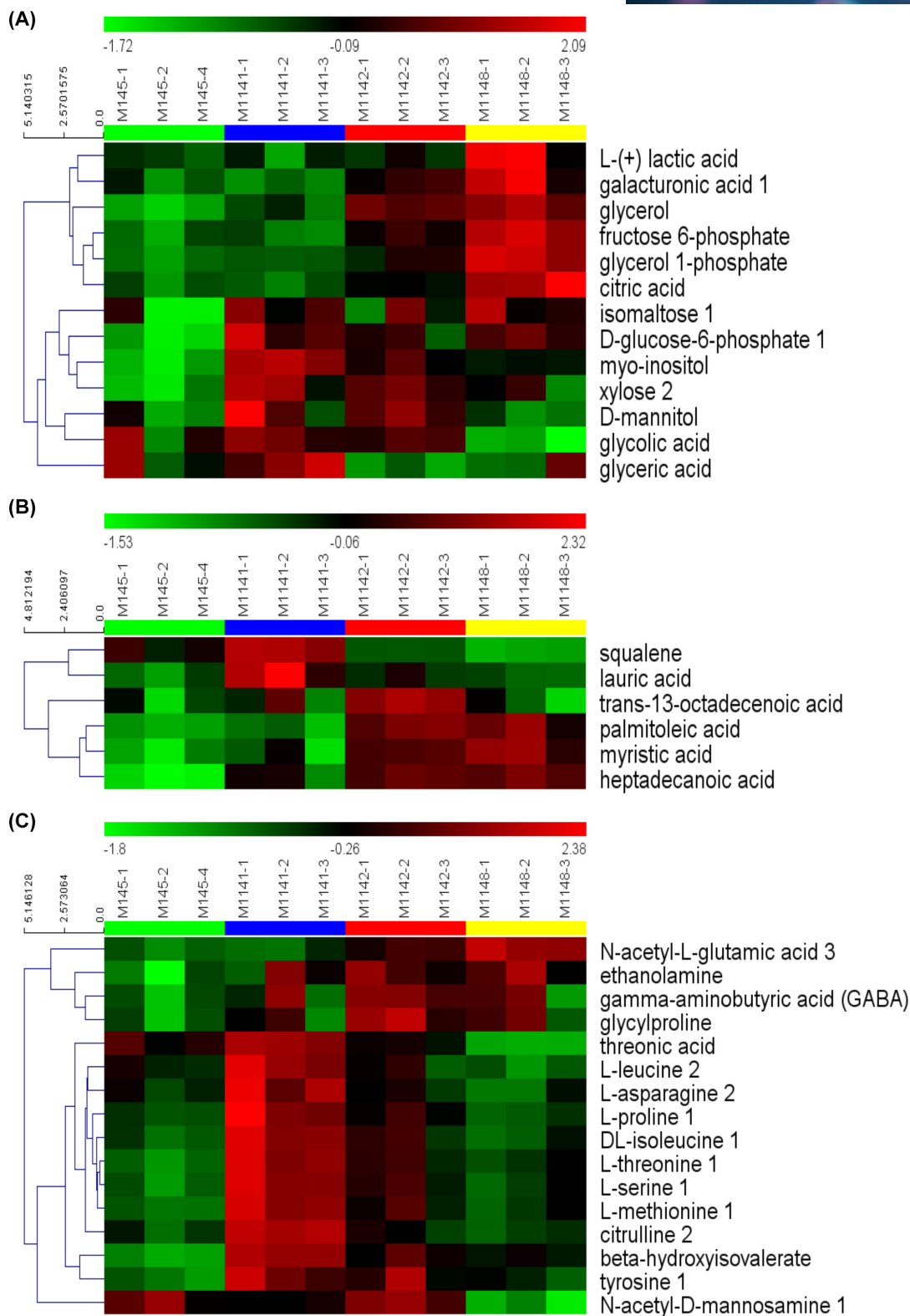


FIGURE 4 Comparative analysis of the classified metabolites mentioned in Figure 3. Metabolites of central carbon metabolism (A) Fatty acids (B) Amino acids and other nitrogen containing compounds (C).

M1142 is consistent with the high TAG content of this strain (Figure 2) since glycerol and fatty acids are required for the biosynthesis of these storage lipids. Interestingly, the higher abundance of TAG and PE (to

a lesser extent) and the lower abundance of PI and PIMs in M1442 (and M1148) than in M1141 suggested a link between the disappearance of these specific phospholipids and the accumulation of TAG and PE.

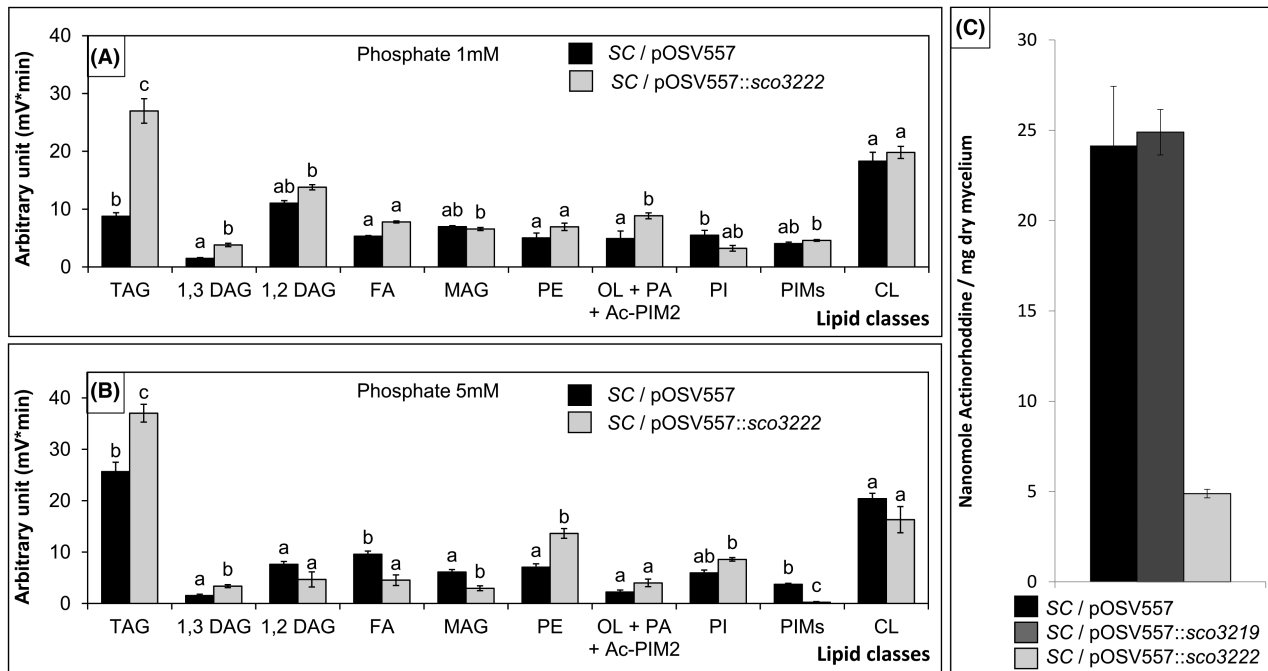


FIGURE 5 Lipid classes, as determined by LC/Corona-CAD analysis, present in *S. coelicolor* M145/pOSV557 containing the empty vector (black histograms) and *S. coelicolor* M145/pOSV557::sco3222 (light grey histograms) grown, in quadruplicates, on modified solid R2YE medium either limited (1 mM) (A) or proficient (5 mM) (B) in Pi, for 72 h at 28°C. Lipid classes from left to right are Triacylglycerol (TAG); 1,3- and 1,2-Diacylglycerol (DAG); Fatty acids (FA); Monoacylglycerol (MAG); Phosphatidylethanolamine (PE); ornithine lipids (OL), phosphatidic acid (PA) and acetylated phosphatidylinositol mannoside-2 (Ac-PIM2) that are co-eluting, phosphatidylinositol (PI), phosphatidylinositol-mannosides (PIMs) and cardiolipin (CL). (C) Extracellular actinorhodin production of *S. coelicolor* M145/pOSV557 (black histogram), *S. coelicolor*/pOSV557::sco3219 (dark grey histogram) and *S. coelicolor*/pOSV557::sco3222 (light grey histogram) grown, in quadruplicates, on modified solid R2YE limited in Pi (1 mM), for 72 h at 28°C. Means values are shown as histograms with error bars representing standard error. Means sharing a letter were not significantly different ($p > 0.05$; Tukey-adjusted comparisons).

One can propose that these phospholipids are those converted, in priority, into TAG and PE. Indeed, TAG are known to accumulate in condition of nitrogen and/or phosphate limitation in most studied microorganisms (Lv et al., 2021; Maltsev et al., 2023; Santucci et al., 2019). In such conditions, membranous phospholipids (PLs) are usually converted into TAG even if de novo TAG biosynthesis can also occur. The conversion of PLs into TAGs starts by the cleavage off of the amino and phosphate groups, constituting the polar head of these phospholipids, by phospholipases C or D, yielding 1,2-DAG or phosphatidic acid (PA), respectively. The dephosphorylation of PA by specific phosphatases would also yield 1,2-DAG whose acylation results in the formation of TAG.

At last, glycylproline, ethanolamine and N-acetyl-D-mannosamine (Figure 4C) were more abundant in M1142 than in M1141. Glycylproline results from the condensation of glycine and proline that are, with serine, the amino acids used for RED biosynthesis. Its abundance in M1142 might be due to the absence of RED biosynthesis. Furthermore, our results suggest that a fraction of serine, used in M1141 to synthesize RED, was converted into ethanolamine in M1142 and enters in the composition of PE whose content was higher in M1142 (and M1148) than in M1141 (Figure 2).

At last, N-acetyl-D-mannosamine might result from the condensation of acetylCoA and mannosamine that possibly results from the degradation of PIMs that were found to be less abundant in M1142 (and M1148) than in M1141 (Figure 2).

To explain these unexpected results, we propose that in absence of RED biosynthesis the programmed cell death process (Tenconi et al., 2018) would be less efficient. This would result into N and P limitations responsible for the triggering of TAG biosynthesis (Lv et al., 2021; Maltsev et al., 2023; Santucci et al., 2019). Interestingly, the slower growth rate of M1142 compared with that of M1141 in ph3 and ph4 (Figure 1) and the previously reported precocious sporulation of a RED mutant (Tenconi et al., 2020) are consistent with the hypothesis that this strain is suffering from a nutritional limitation.

Comparative analysis of M1142 (Δ ACT, Δ RED) and M1148 (Δ ACT, Δ RED, Δ CDA)

The results shown in Figures 3 and 4 show an extremely contrasted abundance of most metabolites between M1148 and M1141 (Figure 3), whereas the abundance of these metabolites in M1142 is

intermediate between that of M1141 and M1148. The CDA cluster directs the biosynthesis of a peptidic antibiotic that requires 9 AA. These AA are Ser, Gly, hydrophenylGly stemming from glycolytic intermediates as well as Glu, methylGlu, Trp, Asp, Asn, and Thr stemming from intermediates of the TCA cycle. CDA is mainly transiently produced towards the “transition phase” (ph2) that is a short phase of growth arrest occurring between ph1 and ph3 (Granozzi et al., 1990; Puglia et al., 1995). CDA is an ionophore whose calcium dependent insertion into the plasma membrane leads to an increased permeability of the membrane to ions. We previously proposed that CDA might break the H⁺ gradient leading to a reduction of ATP synthesis (Virolle, 2020). Furthermore, M1148 contains 1.4-fold more TAG than M1142 and produces citric acid, whereas M1142 does not. TAG (Lv et al., 2021; Maltsev et al., 2023; Santucci et al., 2019) and citric acid (Anastassiadis et al., 2002; Kamzolova et al., 2022) biosynthesis are known to be triggered in condition of nitrogen and/or phosphate limitation in most studied microorganisms. These observations suggest that M1148 suffers more severe nitrogen and/or phosphate limitations than M1142. The possible causes of the specific metabolic features of M1148 will be addressed in Discussion section.

Besides its higher citric acid, TAG and PE content, M1148 has a higher content in glucose 6P, fructose 6P, isomaltose, glycerol-1P and glycerol as well as in galacturonic, lactic and citric acids than M1142 and a lower content in myo-inositol, xylose, D-mannitol and glycolic acid. ATP (Bang et al., 1977) and citric acid (Usenik & Legisa, 2010), are known to inhibit the activity of the phosphofructo kinase in other microorganisms, this inhibition might create a bottleneck responsible for the higher content in fructose-6P and glucose-6P as well as in galacturonic acid and isomaltose, metabolites derived from glucose, in M1148 than in M1142. The higher content in M1148 than in M1142 of glycerol-1P/glycerol (Figure 4A), in 1, 2 DAG and possibly in PA (in mixture with Ac-PIM2 and OL) (Figure 2) that is correlated with the lower content in FA and MAG in M1148 than in M1142 and the similar content of heptadecanoic, myristic and palmitoleic acids in these two strains (Figure 4B), is consistent with the 1.4-fold higher TAG content of M1148 compared with M1142 (Figure 2). An important consequence of the high storage of acetylCoA as TAG in M1148 is a reduction of the feeding and thus of the activity of the TCA cycle that forces M1148 towards a fermentative metabolism that is characterized by the generation of lactic acid from pyruvate catalysed by a NADH-dependent lactate dehydrogenase (Wang et al., 2014). Consistently, previous comparative study of M145 and M1146 (Δ CPK, Δ CDA, Δ RED and Δ ACT) demonstrated that M1146 produces 8-fold more pyruvate than M145, had a lower flux through the TCA

cycle and contained approximately 1.4-fold more TAG than M145 (Coze et al., 2013) and the specific metabolic features of a derivative of M1146 (M1152), were confirmed by another study (Sulheim et al., 2020).

Another consequence of the low activity of the TCA cycle in M1148 is its much lower content in all AA detected and in N-acetyl-D-mannosamine (Figure 4C) compared with M1142 as well as its lower level of oxidative stress. Indeed, trans-13-octadecenoic acid that was shown to have anti-oxidant properties (Furumoto et al., 2016) was at a lower level in M1148 than in M1142 (Figure 4B), whereas squalene, lauric acid were at a similar low level in both strains. Furthermore, since myo-inositol is a precursor of the anti-oxidant mycothiol (Newton et al., 2008) and xylose is stemming from the Pentose Phosphate Pathway (PPP) that generates NADPH, an important co-factor of thioredoxin reductases, enzymes involved in the resistance to oxidative stress (AIOkda & Van Raamsdonk, 2023), the lower content of these metabolites in M1148 than in M1142 might indicate a lower level of oxidative stress in M1148 than in M1142. At last, the conversion of pyruvate into lactic acid by a NADH-dependent lactate dehydrogenase in M1148 consumes NADH and generates NAD⁺ and thus constitutes an alternative route to the respiratory chain to re-oxidized reduced co-factors.

In contrast, N-acetyl-L-glutamic acid was at a higher level in M1148 than in M1142, whereas glycylproline, ethanolamine and GABA (derived from glutamate) were at a similar level in the two strains. N-acetyl-L-glutamic acid results from the condensation of Glu and acetylCoA, that are precursors of CDA biosynthesis, its higher abundance in M1148 than in M1142 might directly result from the high availability of these precursors linked to the deletion of the CDA cluster. As mentioned above glycylproline results from the condensation of glycine and proline that are, with serine, the amino acids used for RED biosynthesis. Serine, used in M1141 to synthesize RED, is most likely converted into ethanolamine in M1148 and M1142 and enters in the composition of PE that is at a similarly high level in both strains (Figure 2). In M1148, as in M1142, one notes the correlation between a high TAG and PE content and a low PI and PIMs content suggesting a link between the disappearance of these phospholipids and the higher content in TAG and PE as mentioned above.

Considering the high TAG content of M1148, we wished to test whether the two genes annotated as encoding putative phospholipase A2-like enzymes present in the CDA cluster had any impact on the TAG content of SC since some reports in the literature mentioned a positive role played by phospholipase A2-like enzymes in the formation of TAG-containing lipid droplets in eukaryotes (Guijas et al., 2014; Pucer et al., 2013). To

do so, the impact of the over-expression of these two genes on the TAG content of *S. coelicolor* M145 was assessed.

Impact of the over-expression of genes encoding putative phospholipases A2-like enzymes present the CDA cluster on the total lipid content and ACT production of *S. coelicolor* M145

To determine the impact of the over-expression of the putative phospholipases A2-like encoding genes (*sco3219* and *sco3222*) present in the CDA cluster on the lipidic content and ACT production of M145, the expression of these genes was put under the control of the strong *ermE* promoter via their cloning in the vector pOSV557 (Bibb et al., 1985). The plasmids pOSV557, pOSV557::*sco3219* and pOSV557::*sco3222* were introduced into SC and the resulting strains were grown on solid R2YE medium limited in Pi (1 mM). The lipidic content of these strains was assessed after, 48, 72 and 96 h of growth at 28°C, first using Fourier Transformed InfraRed Spectroscopy (FTIRS) (Deniset-Besseau et al., 2014; Millan-Oropeza, Rebois, et al., 2017). Data of Figure S2 indicated that the over-expression of *sco3222*, but not that of *sco3219*, had a strong positive impact on the total lipid content of SC.

To confirm this result, a comparative analysis of the lipid content of SC/pOSV557 and SC/pOSV557::*sco3222*, grown on solid R2YE medium containing either 1 mM or 5 mM phosphate (Pi), was carried out in LC/MS². The comparison of the lipid content of these strains grown in Pi limitation (1 mM, Figure 5A) or proficiency (5 mM, Figure 5B) revealed to that the TAG content was 3.37- and 1.4-fold higher in SC/pOSV557::*sco3222* than in SC/pOSV557, in Pi limitation and Pi proficiency, respectively. This indicated that Pi limitation triggers TAG biosynthesis in SC containing the plasmid pOSV557, whereas it was not the case in the native SC strain (Lejeune et al., 2021). This suggested

that the presence of a plasmid in this strain constitutes an energetic burden that aggravates the consequences of a limitation in Pi (Oftadeh & Hatzimanikatis, 2024) that usually triggers TAG biosynthesis in microorganisms (Wang et al., 2018; Wierzchowska et al., 2021; Wu et al., 2010). Interestingly, in Pi limitation, the strong increase of the TAG content was correlated with a strong reduction of ACT biosynthesis (Figure 5C) confirming the previously reported reverse correlation existing between the biosynthesis of TAG and that of ACT (Arabolaza et al., 2008; Esnault et al., 2017; Lejeune et al., 2021; Olukoshi & Packter, 1994) or of other bioactive specialized metabolites in various *Streptomyces* species (David et al., 2020; Dulermo et al., 2023).

Furthermore, the TAG, PE and PI content of both strains was higher in Pi proficiency than in Pi limitation. This was expected since the biosynthesis of these molecules requires ATP. At last, interestingly, PIMs were barely detectable in SC/pOSV557::*sco3222* compared with SC/pOSV557, at least in Pi proficiency. This suggested that the latter might be the substrate of the phospholipase SCO3222. The generation of PA then of DAG from PIMs by SCO3222 might stimulate the synthesis of TAG but also that of PE and PI. However, since the deletion of this gene in M1148 does not impair TAG nor PE and PI biosynthesis, this indicates that other phospholipases are able to fulfil SCO3222 function. The biological meaning of the presence of *sco3222* in the CDA cluster remains unclear even if we can speculate that if CDA does break the H⁺ gradient and thus impairs ATP generation, the storage of acetylCoA as TAG is another way to stop the activity of the TCA cycle and thus the generation of ATP.

DISCUSSION

The deletion of biosynthetic pathways directing the biosynthesis of peptidic and/or polyketide specialized metabolites led to an unanticipated consequences on the metabolism of *S. coelicolor* M145 (SC) that are summarized in Figure 6. The usual growth curves features

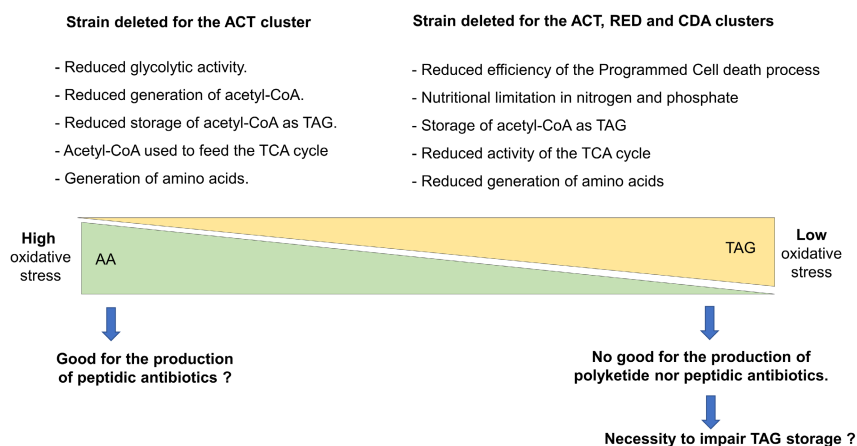


FIGURE 6 Schematic representation of the consequences of the deletion of specialized metabolic pathways on the metabolism of *S. coelicolor* M145 inferred from lipidomic and metabolomic analyses of the deleted strains.

of SC are maintained in the deleted strains but the huge differences in the level of most detected metabolites (glycolytic or over-flow metabolites, amino acids, lipids, etc.) in comparison with the original strain (Figures 2 and 3) highlighted the massive metabolic changes linked to the deletion of these specialized metabolites biosynthetic pathways.

Specialized metabolites are usually produced during the phases of growth slowdown or arrest linked to nutritional limitations and/or to other unidentified causes. In such condition, various metabolites (pyruvate, acetylCoA, amino acids, etc.) as well as co-factors (ATP, NAD(P)H, etc.) not used for growth would accumulate and generate potentially deleterious osmotic, redox or regulatory impacts. We thus propose that a function of the biosynthesis of specialized metabolites would be to consume these metabolites and co-factors to limit the potential deleterious effects of their accumulation. Consistently, a positive link between high osmolarity and the stimulation of antibiotic biosynthesis was previously reported (Bhowmick et al., 2023; Bishop et al., 2004; Godinez et al., 2015).

Furthermore, the bio-activity of these metabolites might also play important roles in the physiology of the producing bacteria. For instance CDA and ACT were both proposed to have a negative impact on ATP generation (Virolle, 2020) via different processes, dissipation of H⁺ gradient and capture of electrons of the respiratory chain (Esnault et al., 2017; Virolle, 2020), respectively. These metabolites may thus contribute to a reduction of anabolism and thus to growth slowdown. In contrast, RED was shown to be involved in the “Programmed Cell Death” process that leads to that the release in the growth medium of the cellular content of dying mycelial filaments that constitute nitrogen and phosphate sources to support growth of the surviving mycelia (Abbas & Edwards, 1990; Tenconi et al., 2018).

Unexpectedly, the concomitant deletion of the ACT, RED and CDA clusters in M1148 led to a higher TAG content than in M1142 and to the biosynthesis of citric acid that does not occur in M1142. Since the biosynthesis of these molecules is known to be triggered in condition of N and/or P limitations (Anastassiadis et al., 2002; Kamzolova et al., 2022; Lv et al., 2021; Maltsev et al., 2023; Santucci et al., 2019), this suggests that M1148 suffers from more severe N and P limitations than M1142. To explain these unexpected observations, we proposed two hypotheses. First, since CDA and ACT were both proposed to have a negative impact on ATP generation via different process (Esnault et al., 2017; Virolle, 2020). The deletion of these two clusters in M1148 is predicted to have a positive impact on ATP generation resulting into growth stimulation. Growth stimulation would aggravate N and P limitations leading to a further

stimulation of TAG biosynthesis. Alternatively, since CDA is produced towards the transition phase that is a phase of growth arrest, CDA might be involved, as RED, in a process leading to the lysis of a fraction of the population. The concomitant absence of these two molecules in M1148, might result into a further reduction of the efficiency of the PCD process compared with M1142, resulting into more severe nitrogen and phosphate limitations in M1148 than in M1142 leading to further stimulation of TAG biosynthesis. In the framework of this hypothesis, the observed high biomass yield of M1148 compared with M1142 might not be due to growth “per se” but to the weight of accumulated TAGs. These two hypotheses are not mutually exclusive and both might contribute to the high biomass yield of M1148.

At last, our unexpected data question the efficiency of these strains as chassis strains. Indeed, the strains deleted for the ACT and CPK polyketide biosynthetic clusters, did not led, as anticipated, to a high TAG content but was correlated with a high content in AA (Figures 3 and 4C). Such metabolic features are expected to be beneficial for the biosynthesis of peptidic specialized metabolites. In contrast, the strains deleted for the RED and CDA peptidic biosynthetic clusters, were characterized by high TAG content and AA paucity. The accumulation of TAG in these strains would limit acetylCoA availability to feed the TCA cycle and thus limit AA biosynthesis. One would predict that the metabolic features of the RED and CDA deleted strains would be detrimental for the biosynthesis of both peptidic and polyketide specialized metabolites. The introduction of heterologous polyketide or peptidic biosynthetic pathways in these deleted strains may avoid or limit the establishment of metabolic features described in this paper and allow the efficient production of these heterologous polyketide and peptidic antibiotics. Indeed peptidic antibiotics such as congoicin, chloramphenicol, caprazamycin (Flinspach et al., 2010; Gomez-Escribano & Bibb, 2011) as well as some small polyketide antibiotics such as spinosad, germicidin and flaviolin (Li et al., 2022; Thanapipatsiri et al., 2015) were reported to be efficiently produced in M1146 (Δ ACT, Δ RED, Δ CPK, Δ CDA). However, to improve further the efficiency of these strains as chassis strains, genetic engineering strategies aimed at reducing the TAG content of these strains via inhibition of synthesis or activation of degradation of these storage lipids, should be beneficial for enhanced production of heterologous polyketide and/or peptidic antibiotics as already reported by some authors (Li et al., 2022; Wang et al., 2020).

AUTHOR CONTRIBUTIONS

Clara Lejeune: Data curation; formal analysis; investigation; methodology; visualization; writing – review and

editing. **Sonia Abreu:** Data curation; formal analysis; investigation; methodology; validation; visualization. **Florence Guérard:** Data curation; formal analysis; investigation; methodology; validation; visualization. **Ahmed Askora:** Funding acquisition; investigation; methodology. **Michelle David:** Investigation; methodology. **Pierre Chaminade:** Funding acquisition; methodology; project administration; resources; supervision. **Bertrand Gakière:** Data curation; methodology; supervision; validation; writing – review and editing. **Marie-Joelle Virolle:** Conceptualization; formal analysis; funding acquisition; investigation; methodology; project administration; supervision; validation; visualization; writing – original draft; writing – review and editing.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data are available in the manuscript.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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