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Disruption of the GDP-mannose synthesis pathway in *Streptomyces coelicolor* results in antibiotic hyper-susceptible phenotypes

Robert Howlett,1 Katri Anttonen,2 Nicholas Read1 and Margaret C. M. Smith1,2,*

**Abstract**

Actinomycete bacteria use polyprenol phosphate mannose as a lipid linked sugar donor for extra-xytoplasmic glycosyl transferases that transfer mannose to cell envelope polymers, including glycoproteins and glycolipids. We showed recently that strains of *Streptomyces coelicolor* with mutations in the gene *ppm1* encoding polyprenol phosphate mannose synthase were both resistant to phage φC31 and have greatly increased susceptibility to antibiotics that mostly act on cell wall biogenesis. Here we show that mutations in the genes encoding enzymes that act upstream of Ppm1 in the polyprenol phosphate mannose synthase pathway can also confer phage resistance and antibiotic hyper-susceptibility. GDP-mannose is a substrate for Ppm1 and is synthesised by GDP-mannose pyrophosphorylase (GMP; ManC) which uses GTP and mannose-1-phosphate as substrates. Phosphomannomutase (PMM; ManB) converts mannose-6-phosphate to mannose-1-phosphate. *S. coelicolor* strains with knocked down GMP activity or with a mutation in *sco3028* encoding PMM acquire phenotypes that resemble those of the *ppm1Δ* mutants i.e. φC31 resistant and susceptible to antibiotics. Differences in the phenotypes of the strains were observed, however. While the *ppm1Δ* strains have a small colony phenotype, the *sco3028::Tn5062* mutants had an extremely small colony phenotype indicative of an even greater growth defect. Moreover we were unable to generate a strain in which GMP activity encoded by *sco3039* and *sco4238* is completely knocked out, indicating that GMP is also an important enzyme for growth. Possibly GDP-mannose is at a metabolic branch point that supplies alternative nucleotide sugar donors.

**INTRODUCTION**

*Streptomyces* spp. are prolific producers of secondary metabolites, many with potent antibiotic activity. In nature *Streptomyces* spp. produce antibiotics either to inhibit competitors thus providing the producer with a growth advantage or as signalling molecules in microbial communities [1, 2]. Either way *Streptomyces* bacteria are constantly exposed to antibiotics produced by other soil microorganisms and consequently have evolved resistance mechanisms [3]. As such *Streptomyces* spp. are a model system to study how the mechanisms of antibiotic resistance evolve in an environmental organism.

We recently showed that strains of *S. coelicolor* lacking the ability to synthesise polyprenol phosphate mannose due to mutations in polyprenol phosphate synthase (*Ppm1*) were hyper-sensitive to multiple antibiotics (Howlett *et al.* [4]). We used RNA-seq and Raman spectroscopy to demonstrate that the strains had undergone changes to the membrane phospholipids, with possible subsequent changes to membrane functions. Polyprenol phosphate mannose synthase, Ppm1, transfers mannose from GDP-mannose to polyprenol phosphate (Fig. 1). Previously we demonstrated that the synthesis of polyprenol phosphate mannose was entirely dependent on membrane associated Ppm1 [5].

Polyprenol phosphate mannose is the mannose donor for extracytoplasmic glycosyl transferases. One of these is a protein mannosyl transferase (Pmt), which glycosylates periplasmic and membrane proteins in *Streptomyces* [5, 6]. Pmt defective strains also show increased antibiotic susceptibility compared to the parent strain, but to fewer antibiotics and to a lower level than the *ppm1Δ* mutants (Howlett *et al.* [4]). Loss of protein glycosylation is therefore likely to contribute
in part to the antibiotic hyper-susceptible phenotype of the ppm1 mutants. In addition both ppm1 strains and the pmt strains are resistant to the phage ϕC31, most likely through loss of the receptor, although the exact nature of the phage receptor is still unknown [6, 7].

Polyprenol phosphate mannose is likely to be a mannose donor for other cell envelope macromolecules with one of these likely to be phosphoinositol mannosides (PIMs) [8, 9]. In other Actinobacteria including Mycobacterium and Corynebacterium spp. PIMs are precursors for the synthesis of lipoarabinomannan and lipomannan [10], but neither of these polymers has been reported in Streptomyces. Ppm1 is an essential enzyme in mycobacteria and a ppm1 strain of Corynebacterium is growth retarded indicating the central role polyprenol phosphate mannose has in both organisms [11, 12]. The protein O-glycosylation pathway is present in most Actinobacteria and Pmt in Mtb has been shown to be important for virulence [13, 14]. In Streptomyces coelicolor other putative glycosyl transferases are also likely to use polyprenol phosphate mannose as a sugar donor and some of these are described in Howlett et al. [4].

The role of polyprenol phosphate mannose in antibiotic resistance and the pathway leading to its synthesis is addressed further in this paper (Fig. 1). D-mannose is either taken up from the medium and converted by hexokinase to D-mannose-6-phosphate or the latter can be produced from D-fructose-6-phosphate by phosphomannomutase (ManA). Phosphomannomutase (ManB:PMM) then converts D-mannose-6-phosphate to D-mannose-1-phosphate which is a substrate for GDP-mannose pyrophosphorylase (ManC:GMP). In Corynebacterium glutamicum deletion of the manC homologue (NCgl0710) conferred retarded growth and loss of nearly all mannoglycans from the envelope [15]. This phenotype resembles that of the ppm1 mutant of C. glutamicum [11] and suggests that the ManB, ManC pathway is responsible for the synthesis of GDP-mannose. We hypothesised that strains containing blocks in the pathway leading to the synthesis of GDP-mannose ought to be phenotypically similar to the ppm1 strains as they too will be deficient in polyprenol phosphate mannose. Here we analysed the roles of three putative manC genes in the S. coelicolor genome and a manB gene. We show that both a GMP depleted strain and a strain lacking PMM do indeed have phenotypes reminiscent of the ppm1 mutants. The phenotype of the S. coelicolor manB strains constructed here varied from that reported previously for a manB strain [16, 17]. We conclude that GMP activity in S. coelicolor is provided by expression of two genes, sco3039 and sco4238. Moreover both GMP and PMM activities are part of the same metabolic pathway leading to the synthesis of polyprenol phosphate mannose and ultimately to glycoprotein biosynthesis in S. coelicolor.

**METHODS**

**DNA manipulations**

Chemically competent E. coli cells were prepared, stored and used in the transformation procedure as described previously [18]. Plasmid DNA extraction from E. coli was performed using a Spin Miniprep Kit following the protocol supplied by the manufacturer (QIAGEN). Cosmids were manipulated as described [19]. Restriction enzymes and T4 ligase were obtained from New England Biolabs (NEB) and used according to the manufacturer’s instruction. Phusion High-Fidelity DNA Polymerase (NEB) was employed for PCR amplification. Primers used in the present study are listed (Table S1, available in the online version of this article). In-fusion HD cloning kit (Clontech) was used according to the protocol supplied by the manufacturer. DNA sequencing (Sanger) was outsourced to Source Bioscience.

**Plasmid, cosmid and strain constructions**

A list of plasmids and cosmids used in this work is provided (Table 1). Plasmid pH1 was produced by cloning of the PCR amplified product from primers RH11 and RH12 and J1929 genomic DNA as template, into EcoRV digested vector pAV11b [20–22]. Plasmid pH12 was produced by

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**Fig. 1.** The GDP-mannose biosynthesis pathway in *Streptomyces coelicolor.*
cloning the PCR product from primers RH91 and RH92 and J1929 template into NdeI digested pIJ10257. Plasmids pRH11 and pRH12 were produced similar to pRH12 but using E.coli DH5a genomic DNA as template and primer pairs RH93/RH94, and RH140/RH141, respectively. Expression plasmids for sco3039 (pRH06) and sco4238 (pRH07) were produced through the ligation of XhoI and NdeI digested PCR products from primers pairs RH71/RH72.

Table 1. Bacteria, plasmids and cosmids

| Plasmid name | Description | References |
|--------------|-------------|------------|
| pAVIIb      | Integrating vector with tcp830 promoter and tetRiS cassette | [22] |
| pRH01       | sco4238 in pAVIIb | This study |
| pDT16       | sco1423 (ppm1) in pSET152 | [7] |
| pDT10       | sco3154 (pmt) in pSET152 | [6] |
| pET21a      | Overexpression vector containing HIS6-tag, T7 promoter | Merck Chemicals |
| pRH06       | sco3039 in pET21a | This study |
| pRH07       | sco4238 in pET21a | This study |
| pIJ10257    | Integrating vector with constitutive promoter ermEp* | [40] |
| pRH11       | epsG in pIJ10257 | This study |
| pRH12       | sco3028 in pIJ10257 | This study |
| pRH14       | pgm in pIJ10257 | This study |

| Cosmid       | Description | References |
|--------------|-------------|------------|
| St1A8A.1.B09 | SCO1388::Tn5062 at nt 572 | [28] |
| StD8A.2.D12  | SCO4238::Tn5062 at nt 69 | [28] |
| SE34.1.G05   | SCO3039::Tn5062 at nt 155 | [28] |
| SE34.1.B03   | SCO3028::Tn5062 at nt 590 | [28] |
| StD8A.2.D12  | StD8A.2.D12 with sco4238::Tn5062 | This study |
| St1A8A.1.B09 | St1A8A.1.B09 with sco1388::Tn5062 | This study |

| Streptomyces strain | Genotype | References |
|---------------------|----------|------------|
| M145                | Prototroph | [23] |
| J1929               | pglY mutant | [30] |
| DT3017              | ppm1E2183V mutant | [7] |
| DT1020              | ppm1H116D mutant | [7] |
| DT1029              | ppm1S661V mutant | [7] |
| DT1025              | pmt, frameshift from A121 | [6] |
| DT2008              | pmt       | [6] |
| SKA211              | sco3039::Tn5062 | This study |
| SKA311              | sco4238::Tn5062 | This study |
| RH501               | sco1388::Tn5062 | This study |
| RH25                | sco4238::Tn5062pprc | This study |
| RH221               | sco4238::Tn5062pprc, pRH01 | This study |
| RH2213              | sco4238::Tn5062pprc, sco3039::Tn5062, pRH01 | This study |
| RHB42               | sco3039::Tn5062 | This study |
| RHB4211             | sco3028::Tn5062, pRH11 | This study |
| RHB4212             | sco3028::Tn5062, pRH12 | This study |
| RHB4214             | sco3028::Tn5062, pRH14 | This study |

| E.coli strain       | Genotype | References |
|---------------------|----------|------------|
| ET12567(pUZ8002)   | dam- dcm- hsdS, RP4 transfer genes | [41] |
| BL21 (DE3)         | lon, ompT, gal, hsdS, ADE3 | [18] |
| DH5α               | F- Φ80lacZΔM15 Δ(lacZYA-argF) U169 recA1 endA1 hsdR17 (rk-, mk+) phoA supE44 Δ-thi-1 gyrA96 relA1 | [18] |
and RH73/RH74, respectively, and S. coelicolor J1929 template DNA, into Xhol and NdeI digested pET21a fusing both ORFs to an inframe C-terminal hexa-histidine tag. All constructs were confirmed as correct through Sanger sequencing performed by Source Bioscience.

The apramycin resistance markers within the Tn5062 transposon of Stt8A.2.D12 and Stt1A8A.1.B09 were replaced with spectinomycin and hygromycin markers, respectively, using the REDIRECT methodology [19]. Cosmids were introduced into S. coelicolor J1929 by conjugation and resistant exconjugants were selected according to the marker on Tn5062 (apramycin, spectinomycin or hygromycin resistance). Those that had undergone a double cross over recombination event were identified initially as they lost the marker (kanamycin resistance) on the cosmid vector backbone. Presence of the interrupted allele and loss of the wild-type allele was confirmed by PCR and Southern blotting.

**Phage sensitivity assays**

Plaque assays were performed as described [23]. Briefly Difco nutrient agar supplemented with 10 mM MgSO4 and 8 mM Ca (NO3)2 were inoculated with dilutions of C31 Δc25 (clear plaque) phage [24] and then overlayed with soft nutrient agar containing approximately 1 x 107 spores of the desired test strain. The streak plate assay was performed using square 10 cm plates containing Difco nutrient agar [10 mM MgSO4 and 8 mM Ca (NO3)2]. One half of the plate was inoculated with 100 µl of C31 Δc25 (approx 1 x 108 p.f.u. ml−1) and a single streak of the test spore preparation was inoculated across the plate beginning on the phage-free region. Plates were incubated at 30 °C.

**Protein expression**

An overnight culture of E. coli BL21DE3 (pRH07) in LB containing ampicillin was grown at 37 °C and used to inoculate 2YT, which was grown to OD 0.6. IPTG (0.15 mM) was then added to induce expression and the culture was further incubated (22 °C for 22 h). The bacteria were harvested by centrifugation and resuspended in binding buffer (30 ml; 20 mM Tris HCl pH 7.4, 0.5M NaCl, 30 mM imidazole) and sonicated. The cell lysate was cleared by centrifugation (4 °C, 5 min, 10 000 g) to remove unlysed cells and debris and then the supernatant was loaded onto a HitTrap Ni2+ affinity column (AKTA Purifier). After washing with 2 column volumes of binding buffer the bound protein was eluted with a gradient of increasing imidazole concentration using the elution buffer (20 mM Tris HCl pH 7.4, 0.5M NaCl, 500 mM imidazole). Pooled fractions were then loaded onto a desalting column to remove imidazole and eluted in 20 mM Tris HCl pH 7.4, 0.5M NaCl. The protein was concentrated using Vivaspin (GE healthcare) spin columns to approximately 10 mg ml−1. Glycerol was added to a final concentration of 50 % and aliquots were stored at −80 °C. Protein concentration was assayed using the BioRad protein assay solution and is based on the Bradford assay [25].

**GDP mannose pyrophosphorylase assays**

Activity was measured by monitoring the release of pyrophosphate using the EnzChek pyrophosphate assay kit (ThermoFisher). Briefly, the kit includes a pyrophosphatase that catalyses the conversion of the pyrophosphate released from the GMP activity to two equivalents of phosphate, which is then used as a substrate in a reaction with 2-amino-6-mercaptop-7-methylpurine ribonucleoside (MESG) and purine nucleoside phosphorylase (PNP) to release ribose 1-phosphate and 2-amino-6-mercapto-7-methyl-purine. The latter compound was detected spectrophotometrically by absorbance at 360 nm. Assays were performed according to the manufacturer’s instructions except that they were scaled down to enable use of a 96 well plate reader (200 µl assay volume per well). GMP activity rates were obtained using different nucleotides (1 mM ATP, GTP, CTP or dTTP) and sugars, (1 mM mannose-1-phosphate or mannose-6-phosphate) as substrates. Initial rates were calculated and plotted against substrate concentration using SIGMAplot.

**RESULTS**

**Identification of putative GDP-mannose pyrophosphorylases**

GDP-mannose, a substrate for Ppm1, is synthesised by GDP-mannose pyrophosphorylase (GMP) encoded by manC (Fig. 1). BLAST searches of the Streptomyces coelicolor genome with the characterised Corynebacterium glutamicum GMP/ManC (encoded by NCgl0710) and Mycobacterium tuberculosis GMP/ManC (encoded by Rv3264c) identified SCO1388, SCO3039 and SCO4238 as putative GMP candidates (Fig. 2) [15]. The nucleotidyl transferase domains of all three Streptomyces GMP/ManC candidates contain the GXGXRxK signature motif of phosphorylases, and variations on the F(V) motif characteristic of the GMP active site (Fig. 2) [26, 27]. SCO3039 and SCO1388 have protein domains in addition to the nucleotidyl transferase domain; SCO1388 in particular appears to be a bifunctional enzyme with both GMP and phosphomannomutase (ManB) activity (Fig. 2).

**SCO3039 and SCO4238 have overlapping functions**

The S. coelicolor genes sco1388, sco3039 and sco4238 were disrupted by allelic exchange with cosmids containing Tn5062 in the gene of interest to produce single insertion mutants RH501 (sco1388::Tn5062), SKA211 (sco3039::Tn5062) and SKA311 (sco4238::Tn5062). The cosmids were obtained from the transposon insertion cosmid library [28] (Table 1). Validated mutants were tested for C31 resistance as a potential indicator for a loss of GMP/ManC activity due to a lack of protein O-glycosylation [6, 7], but all of the mutants were still sensitive to phage infection. A mild increase in blue pigment production was seen in the mutant strains SKA311 (sco4238::Tn5062) and SKA211 (sco3039::Tn5062) when grown on supplemented minimal media (SMM) (not shown).

Double mutants were created to assess whether there is redundancy in gene function between sco3039, sco4238 and
The cosmid StD8A.2.D12\textsuperscript{spec} (sco4238::Tn5062\textsuperscript{spec}) was introduced into J1929 by conjugation to create the spectinomycin resistant sco4238 insertion mutant, RH25. The cosmid StE34.1.G05 (sco3039::Tn5062) was then introduced into RH25 by conjugation, selecting for apramycin resistance. Only eight exconjugants from several hundred that were screened had the spectinomycin-resistant, apramycin-resistant, kanamycin-sensitive phenotype indicative of a sco4238::Tn5062\textsuperscript{spec}, sco3039::Tn5062 double mutant. However subsequent analysis by polymerase chain reaction (PCR) to amplify the genomic region containing sco3039 showed that this gene was uninterrupted in all eight candidate double mutant strains and mutations must have occurred elsewhere to confer resistance to apramycin. Thus we were unable to create a simple double mutant containing Tn5062\textsuperscript{spec} insertions in both sco3039 and sco4238, suggesting that these genes share an important function for growth. Multiple sco4238::Tn5062\textsuperscript{spec}, sco1388::Tn5062 and sco3039::Tn5062, sco1388::Tn5062\textsuperscript{hyg} double mutant strains were produced and confirmed through kanamycin sensitivity. The phenotypes of these strains were no different from the individual sco4238::Tn5062 and sco3039::Tn5062 mutants, SKA311 and SKA211, respectively. The product of sco1388 therefore probably contributes little to the total GMP activity in S. coelicolor.

We were able to create a strain containing both sco4238::Tn5062\textsuperscript{spec} and sco3039::Tn5062 insertions in the presence of a conditionally expressed sco4238. Plasmid pRH01, encoding sco4238 under the control of the anhydrotetracycline (ATC) inducible promoter, tcp830\textsuperscript{[21]}, was introduced into RH25 to create strain RH221 (sco4238::Tn5062\textsuperscript{spec}, tcp830-sco4238, hyg). Conjugation of StE34.1.G05 (sco3039::Tn5062) into RH221 in the presence of ATC resulted in multiple spec\textsuperscript{R}, apra\textsuperscript{R}, hyg\textsuperscript{R}, kan\textsuperscript{S} exconjugants (RH2213) that were subsequently confirmed as sco4238::Tn5062\textsuperscript{spec}, sco3039::Tn5062 double mutants through PCR. Surprisingly RH2213 could grow in the absence of ATC, an observation that was at odds with our inability to isolate the transposon double mutants in the absence of pRH01. Colony sizes of the RH2213 strains in the absence of ATC were indistinguishable from the wild-type parent strain, J1929, but a significant increase in blue pigments were observed compared to the single mutants RH25 (sco4238::Tn5062\textsuperscript{spec}) and SKA211 (sco3039::Tn5062) (Fig. 2b). The tcp830 promoter has been shown by others to

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**Fig. 2.** GDP-mannose pyrophosphorylases in Streptomyces coelicolor (a). Domain structures for S. coelicolor genes with putative GDP-mannose phosphorylase activity. (b). Pigment overproduction in sco4238, sco3039 double mutants. Strain RH2213 (sco4238::Tn5062\textsuperscript{spec}, sco3039::Tn5062, pRH01 encoding inducible sco4238) overproduced blue pigment on supplemented minimal medium solid (SMMS) agar in the absence of anhydrotetracycline (ATC) but not in the presence of 0.5 µg ml\textsuperscript{-1} ATC.
be incompletely turned off in the absence of ATC and we propose that this is also the case in our experiments [20]. It seems likely that RH2213 grown in the absence of ATC has a depleted level of GMP compared to the parent strain and compared to RH2213 grown in the presence of ATC.

Strains depleted in the putative GMPs SCO3039 and SCO4238 are hyper-susceptible to antibiotics and partially resistant to \( \varphi \text{C31} \)

Ppm1 uses GDP-mannose as a substrate and we therefore hypothesised that inability to synthesise GDP-mannose, for example through GMP depletion, should result in a similar phenotype to those strains deficient in Ppm1. RH2213 isolates (sco4239 :: Tn5062\(^{spec}\), sco3039 :: Tn5062, pRH01 encoding inducible sco4238) were still able to support \( \varphi \text{C31} \) plaque formation but displayed resistance to \( \varphi \text{C31} \) on a streak assay in the absence of ATC (Fig. 3a).

We then tested the putative GMP deficient strains for their susceptibilities to antibiotics, notably those to which the ppm1 and pmt mutants were particularly sensitive. S. coelicolor strains SKA211 and RH25 containing Tn5062 insertions in sco3039 and sco4238, respectively, had the same antibiotic resistances as the parent strain J1929. However RH2213, with depleted levels of GMP in the absence of ATC, was highly susceptible to antibiotics, strongly resembling the phenotypes of the ppm1 mutants (Fig. 3b). The phenotypes of the GMP depleted mutants indicate that sco3039 and sco4238 provide the majority of the GMP activity in S. coelicolor.

Fig. 3. *Streptomyces coelicolor* strains depleted in GDP mannose pyrophosphorylase activity are partially resistant to \( \varphi \text{C31} \) and are hyper-susceptible to some antibiotics (a). Spores of the indicated *S. coelicolor* strains were streaked from an area free from \( \varphi \text{C31} \) to an area inoculated with \( 1 \times 10^7 \) p.f.u. \( \varphi \text{C31} \) on Difco nutrient agar plates with or without the supplementation of \( 0.5 \mu\text{g ml}^{-1} \) anhydrotetracycline (ATC). RH2213 (sco4239 :: Tn5062\(^{spec}\), sco3039 :: Tn5062, pRH01 encoding inducible sco4238) showed conditional phage resistance growing only in the absence of ATC. For comparison the phage resistant phenotype of the ppm1\(^{-}\) mutant (DT3017), the parent strain (J1929) and the strains with single mutations in the manC candidate genes, sco3039 and sco4238, (SKA211 and RH25, respectively). (b). RH2213 showed increased susceptibility to antibiotics in the absence of ATC but not in the presence of ATC. This phenotype is comparable to the antibiotic hyper-susceptible phenotype of the ppm1\(^{-}\) mutant (DT3017). Results show the mean diameter of the disc diffusion inhibition zones from at least 3 replicates. Antibiotics were all used at 4 µg/disc with the exception of ampicillin that was used at 40 µg/disc.
sco4238 encodes a highly specific GDP-mannose pyrophosphorylase activity

To confirm the phenotypes mentioned above were due to a depletion of GMP activity in RH2213, sco4238 and sco3039 were overexpressed in E. coli in order to assay GMP activity on purified proteins. Overproduced SCO4238 showed high GMP activity (Fig. 4). The enzyme was highly specific for GTP and D-mannose-1-phosphate substrates, with no or very low rates achieved with CTP, ATP and dTTP (not shown). Approximately 50% activity was observed with D-mannose-6-phosphate and GTP, with the Hill coefficient showing a loss of the cooperativity seen with D-mannose-1-phosphate. In Mycobacterium tuberculosis the essential enzyme, RmlA, catalyses the synthesis of dTDP-glucose, an intermediate in dTDP-rhamnose biosynthesis required for the integrity of the cell wall [29]. Given the apparent essentiality of GMP in S. coelicolor we tested whether SCO4238 had activity on glucose-1-phosphate in combination with any nucleotide, including dTTP but no activity was detected. Attempts to obtain soluble, active SCO3039 from several overexpression constructs in E. coli failed (not shown).

Strains with a mutation in the manB gene, sco3028 are also phenotypically similar to the ppm1 mutants

Previous work has shown that SCO3028 is a dual functioning enzyme capable of phosphomannomutase (PMM, mannose-6-phosphate to mannose-1-phosphate) and phosphoglucomutase (PGM, glucose-6-phosphate to glucose-1-phosphate) activity [17]. The authors constructed a manB deletion mutant, ΔmanB, which had increased actinorhodin production and had lost chloramphenicol resistance but displayed apparently similar growth to the parent strain, M145 [16, 17]. Both phenotypes of the ΔmanB strain were complemented when the wild-type E. coli manB was introduced whilst the S. coelicolor pgm gene (sco7443) failed to complement. Thus PMM activity was shown to be solely responsible for an increase in chloramphenicol sensitivity and actinorhodin production in S. coelicolor M145. If SCO3028 is the sole PMM enzyme in S. coelicolor we would expect a similar phenotype in the sco3028 mutant as we see for the GMP depleted strains. However, Yang et al. did not detect increased susceptibility of their ΔmanB strain to vancomycin, bacitracin or ampicillin [17].

In order to assess the phenotype of an sco3028 mutant in our C31 sensitive strain S. coelicolor J1929, a pglY derivative of M145 [30], the cosmid Ste34.1.B03 (sco3028::Tn5062) was introduced into J1929 by conjugation. Exconjugants that had undergone a double crossover (RHB42 strains, validated by PCR) were isolated at low frequency and had an extreme small (XS) colony phenotype, even smaller than the colony size seen in the ppm1 mutant DT3017 (Fig. 5). The XS colony phenotype in RHB42 could be fully restored to wild-type through complementation with S. coelicolor sco3028 (manB), and Escherichia coli manB (cpsG) as observed in strains RHB4212 and RHB4211, respectively. RHB42 containing Escherichia coli pgm, encoding phosphoglucomutase, was capable of partially restoring colony size (RHB4214), suggesting it is the loss of both PMM and PGM activity that had resulted in the XS colony phenotype in RHB42.
RHB42 was highly resistant to ϕC31 infection in a plaque assay, resembling phage resistance in the pmt and ppm1 mutants (Fig. 5a). RHB42 was also highly susceptible to a number of cell wall acting antibiotics, as well as the RNA polymerase targeting antibiotic, rifampicin (Fig. 5b). Phage sensitivity and antibiotic resistance were restored to wild-
type in RHB4212 (sco3028<sup>Δ</sup>) and RHB4211 (cpsG<sup>Δ</sup>) but not in RHB4214 (pgm<sup>Δ</sup>). No difference in chloramphenicol resistance between RHB42 and J1929 was observed. These phenotypes are consistent with SCO3028 being the primary PMM in <i>S. coelicolor</i> and in the same metabolic pathway that synthesises polyrenol phosphate mannose.

An increase in pigment production was recorded in RHB42, similar to that seen previously following sco3028 deletion [17] and that seen in the GMP depleted strain, RH2213 (Fig. 5c). The production of blue pigment was reduced to wild-type level in RHB4212 (sco3028<sup>Δ</sup>) and RHB4211 (cpsG<sup>Δ</sup>) but not RHB4214 (pgm<sup>Δ</sup>) (Fig. 5). To further validate our observations (as they differ from those of Yang et al. [16, 17]), we created two more sets of sco3028:: Tn5062 mutants: First we used a different Tn5062 insertion in J1929 using cosmid, STE34.2.D03, generating strain JD182 and second we generated derivatives of M145 containing the Tn5062 insertions from both STE34.1.B03 and STE34.2.D03 to generate strains MD202 and MB92, respectively. All three strains had an identical phenotype to RHB42 (Figs S1 and S2).

**DISCUSSION**

Mannose is a component of cell envelope polymers including mannolipids, phosphoinositol mannosides (PIMs) and glycoproteins in many bacteria [10, 31–33]. Extracytoplasmic glycosyl transferases use polyrenol phosphate mannose as the lipid linked sugar donor in the biosynthesis of mannose containing polymers [5, 34]. The synthesis of polyrenol phosphate mannose by Ppm1 is therefore an important activity and pppm1 mutants are considerably less fit than the parent strains [11, 12]. In the case of <i>S. coelicolor</i>, pppm1<sup>Δ</sup> mutants have a small colony growth phenotype and are hyper-susceptible to multiple antibiotics, most of which inhibit cell wall biogenesis suggesting that these mutants are pleiotropically deficient in membrane and/or periplasmic function (Howlett et al., [4]). Mutants lacking Ppm1 or Pmt are also resistant to phage infection and we have proposed that ϕC31 uses a glycoprotein(s) as its receptor [6, 7]. We show here that depletion of enzymes in the mannose metabolism pathway prior to Ppm1 display a phenotype that resembles that of the pppm1<sup>Δ</sup> mutants. We conclude that synthesis of polyrenol phosphate mannose and its subsequent role as a mannose donor in the periplasm is required for a wild-type antibiotic resistant phenotype.

Although the overall phenotypes of the GDP-mannose pyrophosphorylase (GMP) deficient and the manB mutant strains resembled the pppm1<sup>Δ</sup> strain there were some minor differences. We had difficulty in generating a GMP deficient strain. <i>S. coelicolor</i> has three candidate genes that could express GMP activity and we could only obtain a double sco3039<sup>Δ</sup>, sco4238<sup>Δ</sup> mutant if sco4238 was expressed conditionally using the anhydrotetracycline-inducible tcp830 promoter. While this is not absolute proof that the GMP activity is essential in <i>S. coelicolor</i>, it would seem that some low level of activity, possibly that provided by the leakiness of the repressed tcp830 promoter reading into an integrated copy of sco4238, is required for the simultaneous interruption of both sco3039 and sco4238 by Tn5062. Similarly the insertion in sco3028 (manB) was obtained at very low frequency and the colonies were extremely small, indicative of a requirement for both phosphomannomutase (PMM) and phosphoglucomutase (PGM) activities encoded by this gene. If GMP activity is essential then we would also expect PMM activity to be essential, but there may be sufficient PMM activity from other closely related enzymes (such as other PGM paralogues; sco7443 or sco4916 a possible alternative phosphomannomutase) to allow growth. The more severe phenotypes of the GMP depletion mutant and the manB mutant also suggest that GDP-mannose could be located at a metabolic branch point i.e. GDP-mannose is required for polyrenol phosphate mannose synthesis but also perhaps for modification into other nucleotide sugars. For example, the <i>S. coelicolor</i> genome encodes a GDP-mannose dehydrogenase (SCO0382) that is predicted to make GDP-mannuronate, one of the building blocks in the synthesis of alginites in <i>Pseudomonas</i>. Sco382 lies within an operon sco381 to sco386 that has features of an extracellular polysaccharide biosynthesis gene cluster including a polyrenol dependent glycosyl transferase and various other membrane proteins.

Enzyme assays with purified sco4238 showed it to be a monofunctional GMP (ManC) with a slim substrate tolerance similar to Rv3264 (previously miss-annotated as rmlA) of <i>Mycobacterium</i> [29, 35] and in contrast to the more promiscuous ManC enzymes of <i>E. coli</i> and <i>P. furiosus</i> [36, 37]. In <i>Mycobacterium</i> and in <i>Corynebacterium</i> the ManC enzymes (Rv3264 and NCG0710, respectively) provide essential supplies of GDP-mannose for phosphatidyl inositol mannoside (PIM) biosynthesis and lipoglycans [15].

The phenotype described here for RHB42 (sco3028::Tn5062, manB<sup>Δ</sup>) has differences and similarities to a ΔmanB strain of <i>S. coelicolor</i> M145 that was described previously [16, 17]. A notable difference is the sensitivity to antibiotics of RHB42 as Yang et al. did not detect an increase in susceptibility of their ΔmanB strain to vancomycin, bacitracin or ampicillin [17]. Whilst we cannot explain these differences in phenotypes, both RHB42 and the ΔmanB of Yang et al. have increased pigment production. Pigment production is also greatly increased in the ManC deficient strains. The increase in pigment production could be indicative of the activation of several stress pathways or, as discussed by Yang et al., could be due to the increase in carbon flux through glycolysis as the pathway to GDP-mannose is blocked. The pppm1<sup>Δ</sup> strain DT3017 has a mild pigment overproduction phenotype (data not shown). Neither Yang et al., or Rajesh et al. could test phage sensitivity in their ΔmanB strain as they used a Pgl<sup>Δ</sup> strain of <i>S. coelicolor</i>, which confers ϕC31 resistance.

Mannose is used in other <i>Streptomyces</i> spp in the biosynthesis of antibiotics e.g. mannopeptimycins and amphotericin [38, 39]. ManB and ManC activities are required in <i>S. nodosus</i> for the glycosylation of amphotericin [39]. The identification of
the *manC* genes and the construction of the *manC* deficient strain could be useful in heterologous expression and combinatorial biosynthesis of several antibiotic pathways in *S. coelicolor*.

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**Conflicts of interest**

The authors declare that there are no conflicts of interest.

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