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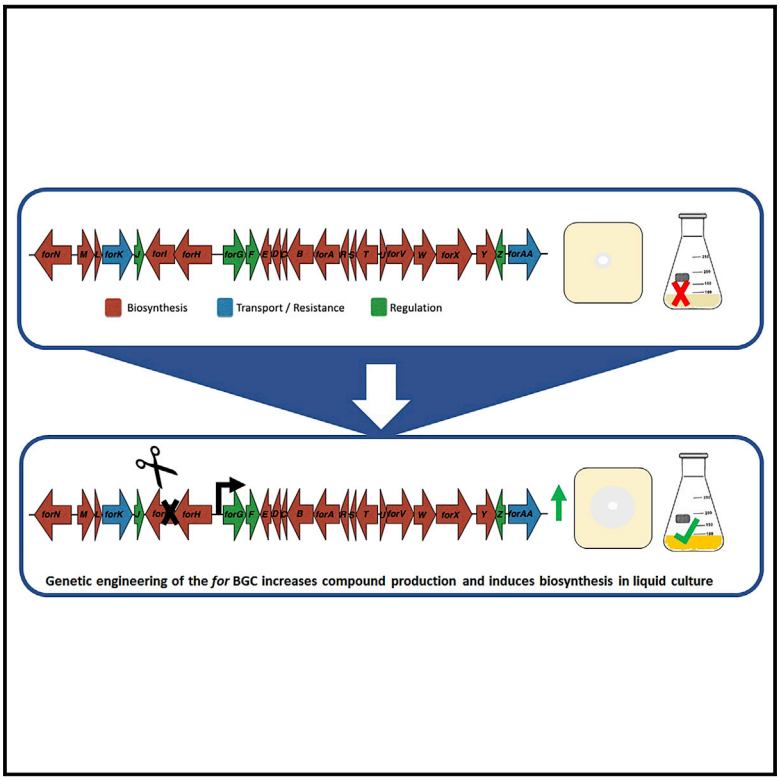
Cell Chemical Biology

Re-wiring the regulation of the formicamycin biosynthetic gene cluster to enable the development of promising antibacterial compounds

Graphical Abstract



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In Brief

Formicamycins are promising antibiotics with activity against drug-resistant pathogens. Here, Devine et al. show formicamycin biosynthesis is repressed by a MarR-family regulator and activated by a two-component system. Using CRISPR/Cas9 genetic engineering, Devine et al. have re-wired formicamycin biosynthesis to increase titers and enable further development of these promising compounds.

Highlights

- Formicamycin biosynthesis requires 24 genes expressed on nine transcripts
- Deleting the MarR regulator ForJ increases formicamycin biosynthesis
- De-repressing formicamycin biosynthesis induces production in liquid culture
- Re-wiring regulation and biosynthesis results in the production of new congeners

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Re-wiring the regulation of the formicamycin biosynthetic gene cluster to enable the development of promising antibacterial compounds

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SUMMARY

The formicamycins are promising antibiotics first identified in *Streptomyces formicae* KY5, which produces the compounds at low levels. Here, we show that by understanding the regulation of the *for* biosynthetic gene cluster (BGC), we can rewire the BGC to increase production levels. The *for* BGC consists of 24 genes expressed on nine transcripts. The MarR regulator ForJ represses expression of seven transcripts encoding the major biosynthetic genes as well as the ForGF two-component system that initiates biosynthesis. We show that overexpression of *forGF* in a Δ*forJ* background increases formicamycin production 10-fold compared with the wild-type. De-repression, by deleting *forJ*, also switches on biosynthesis in liquid culture and induces the production of additional, previously unreported formicamycin congeners. Furthermore, combining de-repression with mutations in biosynthetic genes leads to biosynthesis of additional bioactive precursors.

INTRODUCTION

Almost half of all known antibiotics are derived from the specialized metabolites of filamentous actinomycetes, particularly *Streptomyces* species, many of which were discovered during the golden age of antibiotic discovery that occurred between 1940 and 1960 (Kämpfer et al., 2014). Since this time, few new classes of antibiotics have been introduced into the clinic, and increasing problems of antimicrobial resistance pose a significant threat to modern medicine. Many synthetic antibiotics have failed to progress through clinical trials, so interest has returned to natural products (Hutchings et al., 2019). *Streptomyces* are primarily known as soil bacteria; however, their ability to produce antibiotics makes them competitive in a wide range of environments. By searching under-explored environments and ecosystems, new species can be isolated that may produce useful specialized metabolites (Devine et al., 2017). Furthermore, advances in genomic techniques has revealed that bacterial and fungal species encode many more biosynthetic gene clusters (BGCs) than previously thought, with only around 10% being expressed under laboratory conditions, meaning many more specialized metabolites remain to be discovered from their cryptic BGCs. Isolating bacteria from under-explored environmental niches and mining their genomes for novel BGCs is therefore a promising route toward antibiotic development (Genilloud, 2019). Following this hypothesis, we previously isolated a number of actinomycetes from the domatia of the African fungus-growing plant-ant *Tetraponera penzigi*, including *Streptomyces formicae* KY5 (Seipke et al., 2013). The genome of *S. formicae* encodes numerous specialized metabolite BGCs, including a type 2 polyketide synthase (PKS) BGC that is responsible for the biosynthesis of formicamycins (Holmes et al., 2018). These antibiotics are potent inhibitors of vancomycin-resistant enterococci (VRE) and methicillin-resistant *Staphylococcus aureus* (MRSA), with no resistance observed *in vitro* (Qin et al., 2017).

Previous work on the formicamycin (*for*) BGC has shown that the encoded biosynthetic pathway makes two distinct families of compounds in addition to the formicamycins. The fasamycins are biosynthetic precursors of the formicamycins that also exhibit antibacterial activity; they have been isolated from a number of actinomycete strains and given the alternative names accramycins, naphthacemycins, and streptovertimycins (Feng et al., 2012; Maglangit et al., 2019; Huo et al., 2020; Yang et al., 2020). In addition to the fasamycins, the formicapyridines are shunt metabolites produced when the cyclization stage of the biosynthetic pathway is derailed (Qin et al., 2017, 2019). Conversion of fasamycin precursors into formicamycins involves a unique two-step ring-expansion, ring-contraction pathway that proceeds via a Baeyer-Villigerase-derived lactone intermediate that undergoes a unique reduction Favorskii-like rearrangement (Qin et al., 2020) (Figure 1). In this work, we aimed to understand how *S. formicae* KY5 regulates the production of formicamycins with the view to refactoring the BGC to produce increased titers of these potentially valuable antibiotics. We show that the *for*

Gene	Annotation
forN	Acyl hydrolase
forM	Methyltransferase
forL	PKS cyclase
forK	Na^+/H^+ exchanger
forJ	MarR family transcriptional regulator
forI	ACC biotin carboxylase
forH	ACC carboxyl transferase
forG	Sensor histidine kinase
forF	LuxR family response regulator
forE	ACC biotin carboxy carrier protein
forD	PKS cyclase/dehydratase
forC	PKS ACP
forB	KS_β
forA	KS_α
forR	Putative PKS cyclase/chaperone
forS	PKS chaperone
forT	Methyltransferase
forU	Putative PKS cyclase/chaperone
forV	Halogenase
forW	Methyltransferase
forX	Monooxygenase
forY	Oxidoreductase
forZ	MarR family transcriptional regulator
forAA	Multidrug resistance protein

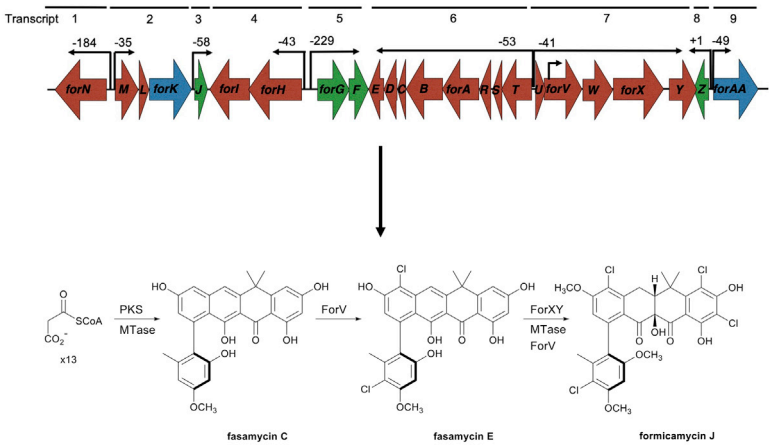


Figure 1. Formicamycin biosynthesis requires 24 genes expressed on nine transcripts

The minimal *for* BGC contains 24 genes required for formicamycin biosynthesis; red = biosynthetic genes, blue = transporters, green = regulatory genes. Cappable RNA sequencing identified 10 transcription start sites in the *for* BGC, nine of which are in intergenic regions that likely represent promoter regions for the biosynthetic genes. Formicamycin biosynthesis occurs by the formation of fasamycins through the action of the PKS and associated gene products, including methyltransferases (MTase) and a halogenase. ForX-catalyzed hydroxylation and ring expansion leads to a lactone intermediate that undergoes a reductive ring contraction catalyzed by the flavin-dependent oxidoreductase ForY to yield the formicamycin backbone.

BGC consists of 24 genes expressed on nine transcripts and is controlled by the combined actions of three cluster-situated regulators (CSRs). The MarR-family transcriptional regulator ForJ represses the expression of the majority of the biosynthetic genes, while the two-component system (TCS) ForGF is required to activate formicamycin biosynthesis. A third CSR, the MarR-family regulator ForZ, appears to autorepress its own expression and activate expression of the putative, divergent MFS transporter gene *forAA*. Deletion of the *forGF* operon abolished the production of fasamycins and formicamycins in the wild-type strain while deleting *forJ* increased formicamycin titers approximately 5-fold. Introducing a second copy of *forGF* into the Δ*forJ* mutant increased production of formicamycins to approximately 10 times the wild-type levels. De-repression of the BGC by deleting *forJ* also induced the production of fasamycins and formicamycins in liquid culture, including previously unreported congeners.

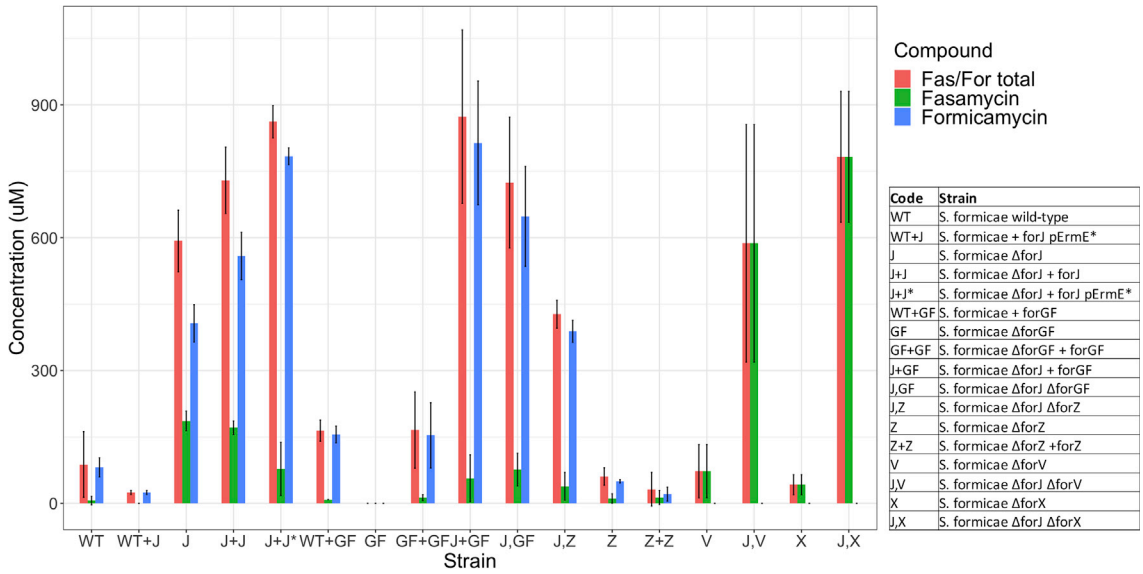
RESULTS

Formicamycin biosynthesis requires 24 genes expressed on nine transcripts

We previously showed that Cas9-mediated deletion of 46 kbp of DNA encompassing the *for* BGC in *S. formicae* abolished formicamycin biosynthesis. Production was restored in the *S. formicae Δfor* strain by introducing the phage-derived artificial chromosome (ePAC) pESAC13-215-G, which carries the entire *for* BGC plus 40–80 kbp of DNA on each side, thus proving this genomic region encodes biosynthesis of these molecules (Qin et al., 2017). Chromatin immunoprecipitation (ChIP) sequencing and cappable RNA sequencing cc (see below) suggested 24 genes form this cluster, and we confirmed this by deleting genes on each side of this 24-gene cluster contained on pESAC13-215-G and demonstrating their ability to induce formicamycin biosynthesis in the *S. formicae Δfor* strain. To elucidate the transcriptional organization of the *for* BGC we mapped the transcription start sites (TSSs) using cappable RNA sequencing (accession number E-MTAB-7975) and identified 10 TSSs, nine of which are in intergenic regions. We thus conclude that the 24 genes required for formicamycin biosynthesis and export are expressed as nine transcripts comprising *forN*, *forMLK*, *forJ*, *forHI*, *forGF*, *forTSRABCD*, *forUVWXY*, *forZ*, and *forAA*. The other TSS is located within the *forV* coding region and likely maintains the expression levels of *forWXY.* The *forX* and *forY* gene products are required for the unique ring conversion that changes a fasamycin precursor into a formicamycin (Qin et al., 2020) (Figure 1).

Formicamycin biosynthesis is repressed by the MarR-family regulator ForJ and activated by the TCS ForGF

The *for* BGC encodes three CSRs that were predicted to regulate the expression of the transcripts required for formicamycin biosynthesis and export. There are two putative MarR-family transcriptional regulators encoded by *forJ* and *forZ* and a TCS encoded by *forGF*. To determine the roles of these CSRs, we made CRISPR/Cas9-mediated deletions in each of their coding genes and measured formicamycin biosynthesis in the resulting mutants. Formicamycin and fasamycin biosynthesis were abolished in the Δ*forGF* mutant, whereas ectopic



Code	Strain
WT	S. formicae wild-type
WT+J	S. formicae + forJ pErmE*
J	S. formicae ΔforJ
J+J	S. formicae ΔforJ + forJ
J+J*	S. formicae ΔforJ + forJ pErmE*
WT+GF	S. formicae + forGF
GF	S. formicae ΔforGF
GF+GF	S. formicae ΔforGF + forGF
J+GF	S. formicae ΔforJ + forGF
J,GF	S. formicae ΔforJ ΔforGF
J,Z	S. formicae ΔforJ ΔforZ
Z	S. formicae ΔforZ
Z+Z	S. formicae ΔforZ +forZ
V	S. formicae ΔforV
J,V	S. formicae ΔforJ ΔforV
X	S. formicae ΔforX
J,X	S. formicae ΔforJ ΔforX

Figure 2. Manipulation of BGC-situated regulators affects formicamycin biosynthesis during solid culture

Deletion of *forJ* results in overproduction of formicamycins and accumulation of the fasamycin precursors. Deletion of *forGF* abolishes fasamycin and formicamycin production. Deletion of *forJ* combined with a second copy of *forGF* results in 10-fold higher formicamycin production than the wild-type strain. Deletion of *forJ* can also be combined with mutations in biosynthetic machinery to generate strains that accumulate precursors and intermediates. Deletion of *forZ* results in a reduction of formicamycin biosynthesis to around 60% of the wild-type strain on solid agar. Manipulation of *forJ* is enough to overcome any other regulatory mutation. Error bars represent SD across experimental replicates. Values are mean ± SD; Wild-type, n = 16; mutants, n = 3.

expression of an additional copy of *forGF* from the native promoter led to an almost doubling (1.9-fold increase) in formicamycin biosynthesis when grown in solid culture. The loss of production caused by deletion of *forGF* was rescued by ectopic expression of *forGF*. In contrast, formicamycin production increased 5-fold in the Δ*forJ* mutant compared with the wild-type, with the levels of fasamycin precursors increasing more than 28-fold. This suggests that the conversion of a fasamycin to a formicamycin is the limiting step in this biosynthetic pathway (the absolute titers of fasamycins are generally lower than those of the formicamycins). Overall, the combined production of fasamycin and formicamycin metabolites increased 6.7-fold in the Δ*forJ* mutant grown in solid culture. Introduction of *forJ* cloned downstream of either the putative *forJ* promoter or the constitutive *ermE** promoter failed to complement the *ΔforJ* mutant for reasons we cannot explain, but overexpression of *forJ* under control of the *ermE** promoter in the wild-type strain significantly reduced fasamycin and formicamycin biosynthesis. Deletion of *forZ* reduced formicamycin biosynthesis to approximately 60% of the wild-type levels (~70% of total metabolites). Taken together, these results show that formicamycin biosynthesis is activated by ForGF and repressed by ForJ, while ForZ may be involved in activation of formicamycin biosynthesis but is not absolutely required and most likely regulates the divergent transporter gene *forAA*. We then deleted both *forJ* and *forGF* in combination and this resulted in a mutant that over-produced formicamycins and accumulated fasamycins, even though loss of *forGF* was expected to result in biosynthesis being abolished. Furthermore, as noted above, the ectopic expression of a second copy of *forJ* under a native promoter is enough to almost abolish biosynthesis in the wild-type strain, even in the presence of the activating TCS. These combined data suggest that *forJ* sits at the top of the *for* BGC regulatory network as the effects of manipulating the other CSRs are overcome by its activity (Figure 2, Table 1).

To determine how these CSRs control the expression of the *for* biosynthetic genes, we generated 3x-FLAG-tagged fusion constructs to complement the deletion mutants and used ChIP sequencing to identify where these CSRs bind across the *S. formicae* chromosome (accession number E-MTAB-8006). The results show that ForF binds to a single site in the *for* BGC at the promoter region between the *forGF* operon and the divergent *forHI* operon, and therefore likely autoregulates and controls expression of *forHI*. ForZ binds to a single site between the divergent *forZ* and *forAA* genes and likely acts as a typical MarR-family regulator by controlling expression of itself and the MFS transporter gene *forAA*. ForJ, the apparent master CSR, binds to multiple locations across the BGC. There are binding sites between the divergent *forN* and *forMLK* operons, in the intergenic regions between the divergent *forHI* and *forGF* operons, and between the *forTSRABCDE* and *forUVWXY* operons that encode the majority of the core biosynthetic machinery. These data are consistent with the observation that ForJ represses the biosynthesis of fasamycins and formicamycins by repressing the transcription of most of the biosynthetic genes. ForJ also binds to its own coding region, presumably to autorepress via a roadblock mechanism, as well as binding within the coding region of *forE* at the end of the long *forTSRABCDE* transcript. We predict that its likely function here is to act as a roadblock to prevent the RNA polymerase transcribing the *forTSRABCDE* operon from running into RNA polymerase transcribing *forGF* since this transcript is required for activation of

Table 1. Fasamycin and formicamycin production by engineered *S. formicae* strains on solid soya flour mannitol agar and in liquid soya flour mannitol

Strain	Fasamycins titer (μM) Solid	Formicamycins titer (μM)	Combined titer (μM)	Fasamycins titer (μM) Liquid	Formicamycins titer (μM)	Combined titer (μM)
Wild-type	6.5 ± 9.6	81.3 ± 21.6	87.9 ± 74.2	0	0	0
Wild-type + *forJ*	0	24.8 ± 4.7	24.8 ± 4.7	0	0	0
Δ*forJ*	186.2 ± 22.2	406.5 ± 42.1	592.7 ± 69.4	6 ± 0.2	624.5 ± 29.4	630.5.4 ± 29.6
Δ*forJ* + *forJ*[a]	170.8 ± 15.4	558.8 ± 53.5	729.6 ± 74.8	2.7 ± 0.6	657.3 ± 40.7	660 ± 41.3
Δ*forJ* + *forJ*[b]	78.0 ± 60.2	784.0 ± 18.9	862.0 ± 36.6	210 ± 36.0	423.1 ± 72.4	633.1 ± 138.1
Wild-type + *forGF*	8.6 ± 0.02	155.7 ± 18.7	164.3 ± 23.7	Not tested	not tested	not tested
Δ*forGF*	0	0	0	not tested	not tested	not tested
Δ*forGF* + *forGF*	13.1 ± 6.7	153.7 ± 73.6	165.7 ± 86.1	not tested	not tested	not tested
Δ*forJ* + *forGF*	56.7 ± 52.9	814.2 ± 139.8	873.4 ± 195.9	275.4 ± 11.6	759.8 ± 193.8	1035.2 ± 212.6
Δ*forJ* Δ*forGF*	76.3 ± 36.8	648.1 ± 112.7	724.5 ± 147.8	242.9 ± 21.6	355.3 ± 82.1	598.1 ± 85.7
Δ*forJ* Δ*forZ*	38.6 ± 31.2	388.5 ± 24.8	427.1 ± 31.6	516.48 ± 297.4	409.1 ± 95.4	925.6 ± 341.2
Δ*forZ*	11.1 ± 10.6	49.8 ± 3.7	60.9 ± 19.7	not tested	not tested	not tested
Δ*forZ* + *forZ*	13.6 ± 15.8	21.0 ± 15.7	31.8 ± 38	not tested	not tested	not tested
Δ*forV*	72.8 ± 60.3	0	72.8 ± 60.3	1.3 ± 1.8	0	1.3 ± 1.8
Δ*forJ*Δ*forV*	587.5 ± 268.1	0	587.5 ± 268.1	79.4 ± 8.1	0	79.4 ± 8.1
Δ*forX*	42.3 ± 22.6	0	42.3 ± 22.6	2.54 ± 4.6	0	2.54 ± 4.6
Δ*forJ*Δ*forX*	782.6 ± 147.8	0	782.6 ± 147.8	274.3 ± 325.4	0	274.3 ± 325.4

Values are mean ± SD; wild-type, n = 16; mutants, n = 3.
[a]*forJ* under control of the native promoter.
[b]*forJ* under control of the *ermE** promoter.

the *for* BGC (Figure 3). Only three significant enrichments occurred outside the *for* BGC: ForF binds upstream of *KY5_0375*, which encodes a putative NLP/P60 family protein, while ForJ binds upstream of both *KY5_3182*, which encodes a putative MoxR-type ATPase, and *KY5_5812*, which encodes a hypothetical protein. The significance of these binding events is not known, and they were not considered further in this study.

To determine the roles of ForGF, ForJ, and ForZ in regulating *for* BGC expression, we compared the mRNA levels of all *for* transcripts in the Δ*forJ*, Δ*forGF*, and Δ*forZ* mutants with those in the wild-type strain, with the exception of *forN* as no suitable primers for qRT-PCR could be found within this relatively short transcript (Figure 4). The results show that levels of all transcripts encoding core biosynthetic machinery were higher in the Δ*forJ* mutant compared with the wild-type strain, consistent with the hypothesis that binding of ForJ represses the expression of these transcripts. Since the *forJ* transcript is missing from the Δ*forJ* mutant, we made a transcriptional fusion between the *forJ* promoter and *gusA* (encoding β-glucuronidase [GUS]) and found that GUS activity was 2-fold higher in Δ*forJ* relative to the wild-type, suggesting ForJ is autorepressed (Table S1). In contrast, levels of the core biosynthetic transcripts were greatly reduced in the Δ*forGF* mutant compared with the wild-type strain, which is consistent with the fact that this mutant does not make fasamycins or formicamycins. Activity levels of the *forG* and *forH* promoters was also reduced in the Δ*forGF* mutant, suggesting ForGF auto-activates. Interestingly, levels of the *forJ* transcript were slightly increased in the *forGF* mutant, suggesting levels of repression from *forJ* are higher in the absence of activation by *forGF*. In the Δ*forZ* mutant, levels of the *forAA* transcript were decreased more than 3-fold, suggesting that ForZ is required to activate the production of this putative transporter. Levels of some of the transcripts encoding biosynthetic machinery were also reduced in the *forZ* mutant compared with the wild-type strain, suggesting that *forZ* may play an indirect role in activating formicamycin biosynthesis (Figure 4). Interestingly, the activity of the *forZ* promoter was increased in the Δ*forZ* mutant, suggesting that ForZ may be an example of a dual activator-repressor MarR regulator that activates expression of the divergent transporter while repressing its own transcription. Together these results show that ForJ represses the majority of the core biosynthetic genes by binding to multiple regions across the *for* BGC. We also propose that ForGF is required to activate the divergent *forGF* and *forHI* promoters. The *forHI* genes encode subunits of the acetyl-CoA carboxylase, which converts acetyl-CoA into malonyl-CoA, the substrate of the *for* PKS, and are essential for the initiation of formicamycin biosynthesis. We hypothesize that expression of the remaining biosynthetic genes is repressed by ForJ in order to ensure biosynthesis only begins when sufficient levels of malonyl-CoA for formicamycin biosynthesis have been achieved. The results also indicate that ForZ auto-represses while activating the transporter gene *forAA* to ensure that compounds do not accumulate intracellularly once biosynthesis has been initiated.

BGC de-repression results in the production of additional formicamycins and induces biosynthesis in liquid culture

Wild-type *S. formicae* does not produce fasamycins or formicamycins during liquid culture and production levels on solid

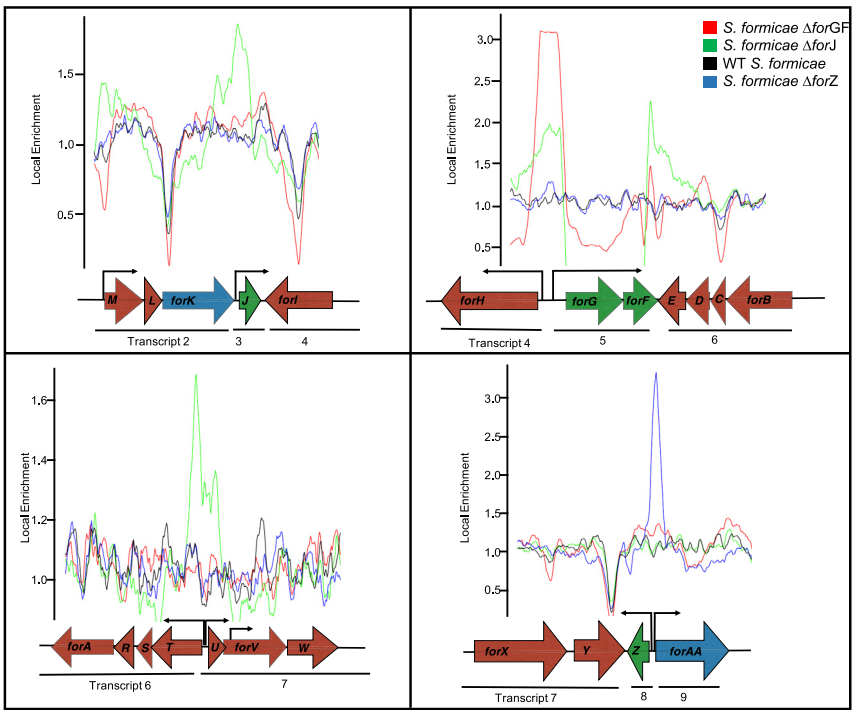


Figure 3. The *for* CSRs bind to multiple promoter regions within the *for* BGC

ForJ binds to multiple locations across the *for* BGC to regulate the expression of the majority of the genes required for formicamycin biosynthesis. ForGF binds to a single promoter within the *for* BGC to regulate itself and the divergent *forHI* transcript. ForZ binds a single promoter between itself and the divergent transporter gene *forAA* and is not predicted to directly regulate biosynthesis of the formicamycins.

agar are low. This limits the scope for further investigation of the antibiotic potential of these compounds since they cannot easily be purified on a large scale. As noted, the ForGF TCS is essential for biosynthesis in the wild-type, while deletion of *forJ* leads to 5-fold higher production of formicamycins (6.7-fold increase in combined fasamycin and formicamycins) on solid medium. We therefore expressed a second copy of the *forGF* operon in the *ΔforJ* strain and found the resulting strain makes 10-fold higher levels of formicamycins on solid culture compared with the wild-type (Figure 2, Table 1). Further analysis of the *ΔforJ* and *ΔforJ* + *forFG* strains gave the surprising result that deletion of *forJ* induced production of the formicamycins in liquid medium. The Δ*forJ* mutant produced 1.5-fold more formicamycins when grown in liquid culture compared with on solid culture, although with significantly reduced levels of accumulated fasamycins; this equates to approximately the same overall production level of both sets of metabolites combined, and to an overall 7.1-fold increase in total productivity versus the wild-type strain grown on solid culture. The Δ*forJ* + *forGF* mutant was even more impressive, producing 1.6-fold the total levels of metabolites compared with the Δ*forJ* mutant in liquid culture and 11.8-fold more total metabolites (9.3-fold more formicamycins) than the wild-type strain grown in solid culture. By enabling production of the formicamycins during liquid culture, these mutants will facilitate fermenter-scale production to accelerate their further investigation. Deletion of *forJ* also induced the production of two additional formicamycin congeners (formicamycins S and R), each carrying five chlorine atoms (Figure 5) (Devine et al., 2020). Previously we had observed a maximum of four chlorination events for any congener. Both molecules exhibited potent antibacterial activity against both methicillin-sensitive *S. aureus* (MSSA) and MRSA (Table 2).

Combining mutations in regulatory and biosynthetic genes results in the accumulation of additional fasamycin congeners

Our observations suggested that combining de-repression (deletion of *forJ*) with deletion of key biosynthetic genes would lead to strains that accumulate elevated levels of pathway intermediates and, potentially, more congeners. We previously showed that the halogenase ForV performs a gatekeeper function, with chlorination of fasamycin intermediates controlling the ability of downstream enzymes to utilize these molecules as substrates and their conversion into the formicamycin skeleton (Qin et al., 2017). We thus constructed an *S. formicae* Δ*forJ*Δ*forV* strain and found that it accumulates fasamycin C exclusively at a titer similar to that of the combined fasamycins and formicamycins produced by the Δ*forJ* strain in solid culture; this corresponds to a titer 90-fold higher than the total fasamycins produced by the wild-type strain (Table 1, Figure 2). This provides a powerful route for selectively producing non-halogenated fasamycin congeners, and the lack of formicamycin biosynthesis by this strain is consistent with the proposed gatekeeper function of ForV.

In a similar vein, we next made a strain lacking *forJ* and *forX*, which encodes the flavin-dependent monooxygenase ForX, the enzyme responsible for the first ring-expansion step involved in converting fasamycin precursors into formicamycins (Qin et al., 2020). The resulting *ΔforJΔforX* strain did not make formicamycins but instead accumulated chlorinated fasamycin congeners to approximately 120 times the level of the wild-type strain (8.9-fold increase in total metabolites) when grown in solid culture (Table 1, Figure 2). Moreover, the congener profile of this strain changed considerably and six additional fasamycin congeners (L-Q) were isolated (Figure 5) (Devine et al., 2020). These include molecules carrying up to four chlorine atoms, whereas a maximum of two had previously been observed for fasamycins isolated from the wild-type strain. These congeners all displayed potent antibacterial activity against both MSSA and MRSA (Table 2).

The structures of these fasamycin and formicamycin congeners were assigned using high-resolution liquid chromatography-mass spectrometry and 2D NMR based on our published data (Qin et al., 2017). The substituent variations in chlorination and *O*-methylations were determined from the 2D heteronuclear

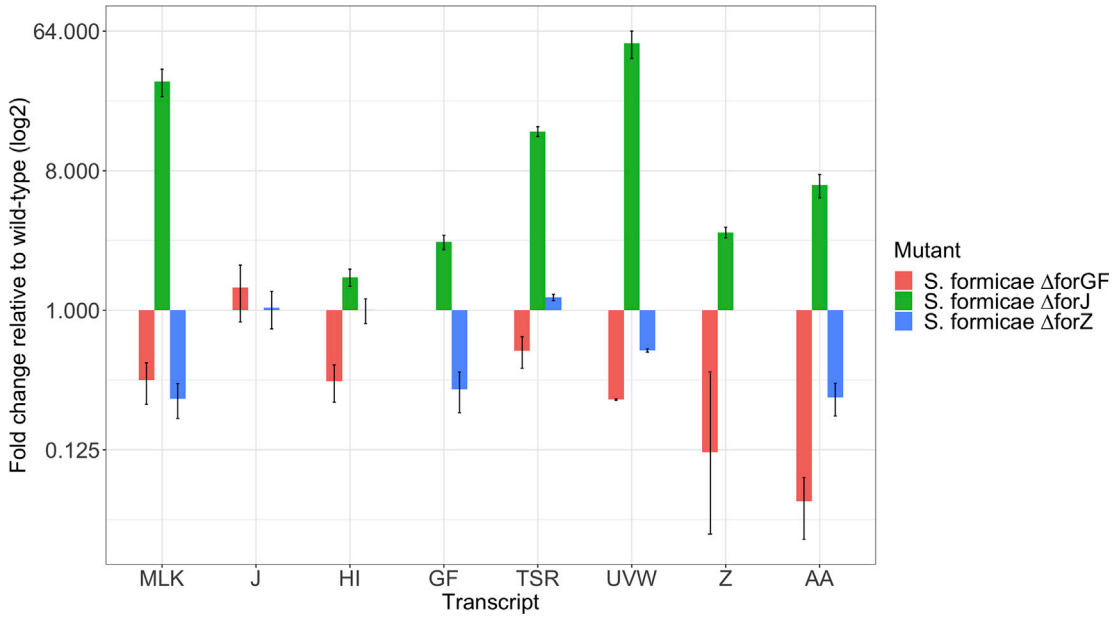


Figure 4. Deletion of *forJ* results in increased transcription of *for* genes and deletion of *forGF* decreases expression

In accordance with observed titers, deletion of *forJ* results in an increase in the expression of all other *for* cluster transcripts. Deletion of *forGF* results in a decrease in expression of all biosynthetic transcripts but an increase in the expression of the repressor gene *forJ*, thereby inhibiting biosynthesis. In the *forZ* deletion mutant, expression of the resistance transporter *forAA* is decreased, suggesting *forZ* is required to activate its transcription. There is a small decrease in the levels of other transcripts in the *forZ* mutant, suggesting this regulator may also indirectly influence expression of these genes without binding to their promoters. Error bars represent SD across three biological and two technical experimental replicates.

single quantum coherence spectroscopy (HSQC) and ^{1}H-^{1}H correlation spectroscopy (NOESY) data. Supplementary chemistry data are available here: https://doi.org/10.6084/m9.figshare.13222070.v1.

DISCUSSION

Using targeted gene deletions and cappable RNA sequencing, we have shown that 24 genes are required for formicamycin biosynthesis and export. These are expressed as nine transcriptional units and are under the control of three CSRs: ForJ and ForZ are MarR-type regulators, while ForGF is a TCS. The latter is responsible for activating expression of these transcripts by binding to a single divergent promoter in the *for* BGC and is essential for formicamycin biosynthesis in the wild-type strain but not in a Δ*forJ* deletion mutant. TCSs are one of the major ways in which bacteria sense changes in their environment, and the large genomes of *Streptomyces* species generally encode for high numbers of TCSs, which enable them to survive in dynamic and sometimes extreme environments (Hutchings et al., 2004). Due to the availability of genome sequencing data and protocols for genetic manipulation, the majority of characterized TCSs are from model organisms such as *Streptomyces coelicolor* and *Streptomyces venezuelae* (Rodríguez et al., 2013). Of the TCSs studied in these model species, several have been shown to control secondary metabolism directly, while the majority coordinate secondary metabolism and morphological development either via global regulation (e.g., PhoPR, MtrA/B) or by interacting with CSRs (e.g., AfsQ1) (McLean et al., 2019). In contrast, ForGF is a rare example of a cluster-situated TCS that specifically activates transcription of the *for* biosynthetic genes. Another example of a cluster-situated TCS is *cinKR*, which, when deleted, abolishes biosynthesis of the lanthipeptide antibiotic cinnamycin much like deletion of *forGF* in *S. formicae* (O'Rourke et al., 2017). However, it should be noted that CinKR is responsible for the activation of a resistance transporter that is absolutely required for cinnamycin biosynthesis, rather than directly activating biosynthetic genes as ForGF appears to. Cluster-situated TCSs that activate biosynthesis in this way represent a promising target for overexpression to activate BGCs that may be cryptic or expressed at low levels, like the *for* BGC. Indeed, ectopic expression of an extra copy of *forGF* resulted in a significant increase (2-fold) in the level of formicamycin production compared with the wild-type strain in solid culture.

MarR-family regulators such as ForJ usually repress transcription of their target genes by binding to DNA sequences within promoter regions (Grove, 2017). We have shown that ForJ is the major *for* cluster repressor and deletion of *forJ* leads to increased production of multiple pathway products and the induction of biosynthesis during liquid culture of *S. formicae*. Deletion of CSRs is a known method of inducing biosynthesis from cryptic BGCs (Aigle and Corre, 2012); however, to our knowledge, MarR regulators that repress entire biosynthetic pathways in this way are relatively rare. MarR regulators typically bind to intergenic regions to autoregulate their own gene expression and the divergently transcribed gene (Wilkinson and Grove, 2006). Often, the genes under their control are involved in the control of export of secondary metabolites (Perera and Grove, 2010). ForZ appears to function in this way: through binding to the *forZ-forAA* intergenic region it autoregulates and controls

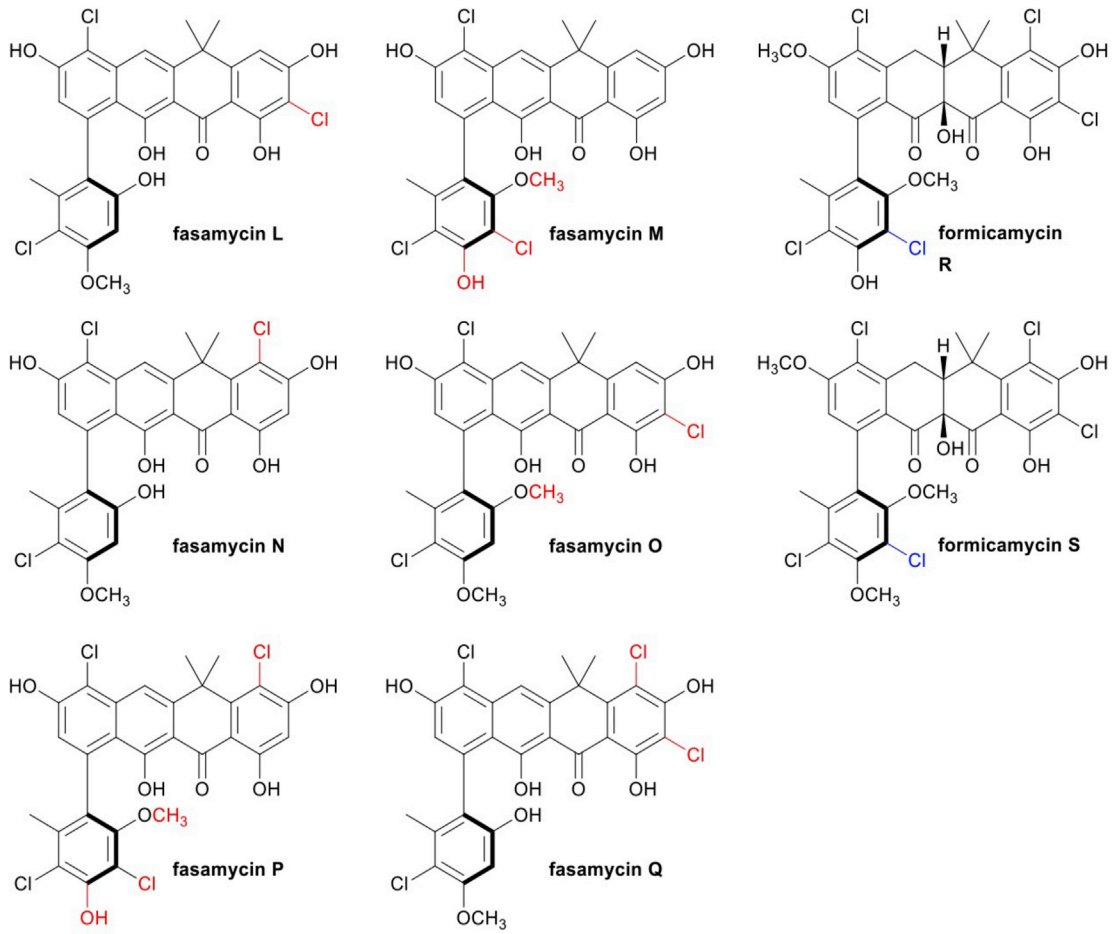


Figure 5. Fasamycin and formicamycin congeners isolated from de-repressed *for* cluster mutants

Deletion of *forJ* results in accumulation of all previously identified congeners from the *for* BGC in addition to the production of two additional formicamycin congeners (R and S), which exhibit a unique C4 chlorination (highlighted in blue). De-repressing the *for* BGC by deleting *forJ* in the *S. formicae ΔforX* mutant results in the production of six additional fasamycin congeners (L to Q), which have different chlorination and methylation patterns (highlighted in red) compared with fasamycins C–E produced by the wild-type and *S. formicae ΔforX* strains. All these congeners displayed potent antibacterial activity against MRSA.

expression of the divergent *forAA* gene, encoding a putative resistance transporter. Interestingly, ForZ appears to activate transcription of *forAA* rather than repressing it, which is unusual, although not unique. ForZ is not required for formicamycin biosynthesis and ChIP sequencing data show that it specifically binds to only a single site within the *for* BGC. However, our data indicate that ForZ may indirectly activate the expression of some *for* BGC transcripts to increase formicamycin biosynthesis.

Based on these data, we propose a model in which ForG senses an (unknown) environmental change and phosphorylates ForF, which then activates expression of *forHI*, which encode subunits of the acetyl-CoA carboxylase that converts acetyl-CoA to the polyketide precursor malonyl-CoA. MarR-family regulators are also known to bind small molecule ligands, and often these ligands are products of the pathways within which they are encoded (Perera and Grove, 2010). We therefore hypothesize that production of malonyl-CoA above a certain threshold level either directly or indirectly induces ForJ and leads to de-repression of the *for* BGC. This results in the expression of the seven transcripts under the regulation of ForJ, which contain the biosynthetic machinery required for formicamycin biosynthesis. To prevent these compounds accumulating intracellularly, we suggest that ForZ activates transcription of *forAA* to export formicamycins. Where MarR regulators have been shown to regulate an efflux pump, they generally bind and respond to the molecule required for export (Grove, 2013). An example is OtrR, encoded within the oxytetracycline BGC of *Streptomyces rimosus*, which controls the expression of the divergent transporter gene *otrB* in response to the presence of oxytetracycline and biosynthetic pathway intermediates (Pickens and Tang, 2010; Mak et al., 2014). It is possible that ForZ is sensitive to fasamycin and/or formicamycin levels and activates expression of *forAA* to prevent toxic accumulation while ensuring expression of other *for* transcripts remains low until this resistance mechanism is activated. This will be investigated in future studies.

The formicamycins represent promising candidates for investigation as a new structural class of antibiotics due to their potent bioactivity against drug-resistant pathogens and their high barrier to the development of resistance (Qin et al., 2017). Until now, further investigation into the formicamycins has been hindered

Table 2. Minimal inhibitory concentration of fasamycin and formicamycin congeners against *S. aureus* ATCC BAA-1717 (MRSA) and ATCC 6538P (MSSA)

	Minimum inhibitory concentration (μg/mL)										
	Fasamycin								Formicamycin		
	C	E	L	M	N	O	P	Q	J	R	S
MRSA	16	4	2	4	2	2	4	4	4	2	2
MSSA	16	4	2	4	4	4	2	4	4	2	2

by their low production levels in *S. formicae* and the fact they were only produced in solid culture. In this work, we show that de-repression of the *for* BGC results in not only increased titers but also the induction of biosynthesis during liquid culture. This is significant because the majority of industrial antibiotic production is performed in liquid cultures (Manteca and Yagüe, 2018). Furthermore, using our knowledge of *for* regulation and biosynthesis, we created a series of targeted mutants by combining mutations in genes encoding the biosynthetic machinery with pathway de-repression. This led to *S. formicae* strains that produce high titers of specific metabolites in both solid and liquid culture. This work will greatly accelerate further investigations into these exciting molecules and demonstrates the importance of studying both the biosynthesis and regulation of BGCs encoding specialized metabolites with antibiotic potential.

SIGNIFICANCE

Antimicrobial resistance poses a major threat to public health, therefore the discovery and development of new antibiotics is vital. The formicamycins are promising antibiotics with potent activity against drug-resistant pathogens like MRSA. Further development of the formicamycins has so far been limited by the fact that they are only produced in low levels during solid culture. In this study, CRISPR/Cas9 genome editing was used to rewire formicamycin biosynthesis in the native host to increase titers and induce production during liquid culture. As well as leading to the discovery of additional formicamycin congeners and biosynthetic intermediates, this work will accelerate further investigations into these clinically relevant molecules and demonstrates the power of synthetic biology for refactoring antibiotic biosynthesis.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.chembiol.2020.12.011.

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AUTHOR CONTRIBUTIONS

R.D., H.M., Z.Q., C.A., K.N., B.W., and M.I.H. designed the research. R.D., H.M., K.N., B.W., and M.I.H. wrote the paper and all authors commented. R.D., H.M., K.N., and C.A. performed the molecular genetics experiments, H.M. and Z.Q. performed the chemical experiments. G.C. conducted the bioinformatics analysis.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial strains		
Streptomcyes formicae wild-type	Lab stock	N/A
Streptomcyes formicae mutants	This work	For details, see Table S3
Staphylococcus aureus (MSSA)	ATCC	ATCC 6538P
Staphylococcus aureus (MRSA)	ATCC	ATCC BAA-1717
Chemicals, peptides, and recombinant proteins		
PCRBIO Taq DNA Polymerase Mix, Red	PCR Biosystems	PB10.13-02
Q5 High-fidelity DNA polymerase	NEB	M0491S
Gibson assembly master mix	NEB	E2611S
ANTI-FLAG M2 Affinity Gel	Sigma	A2220
Luna Universal qPCR Master Mix	NEB	M3003S
Critical commercial assays		
Plasmid prep mini kit	Qiagen	12123
Gel purification kit (QIAquick)	Qiagen	28506
RNAeasy mini kit	Qiagen	74104
LunaScript RT SuperMix	NEB	ES010S
Deposited data		
Cappable RNA-Seq data	This work	E-MTAB-7975
ChIP-Seq data	This work	E-MTAB-8006
Oligonucleotides		
For details of oligonucleotides generated and used, see Table S5	This work	N/A
Recombinant DNA		
pCRISPomyces-2 for CRISPR/Cas deletions	(Cobb, Wang and Zhao, 2015)	N/A
pMF96 for GUS assay	(Feeney et al., 2017)	N/A
pMS82 for integration of DNA into *S. formicae*	(Gregory, Till and Smith, 2003)	N/A
pESAC-13 215-G ePAC containing the formicamycin BGC	BioS&T and (Qin et al., 2017)	N/A
For details of other plasmids used or generated in this study, see Table S4	This work	N/A
Software and algorithms		
Integrated Genome Browser	(Nowlan, Norris and Loraine, 2016)	https://www.bioviz.org/download.html
Bowtie2	(Langmead and Salzberg, 2012)	http://bowtie-bio.sourceforge.net/bowtie2/index.shtml

RESOURCE AVAILABILITY

Lead contact

Further requests for information should be addressed to Matthew I. Hutchings at matt.hutchings@jic.ac.uk.

Materials availability

Requests for materials should be made via the lead contact.

Data and code availability

Sequencing data are available at EMBL-EBI (accession numbers E-MTAB-7975 and E-MTAB-8006). Other data and code are available on request via the lead contact.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

For details of strains used and generated in this study see Table S3**.** Generally, *Streptomyces* strains were grown at 30°C and other organisms at 37°C, with shaking at 200 rpm for liquid cultures, unless otherwise stated (Table S3). *Streptomyces* spores were harvested from confluent lawns streaked from single colonies using a sterile cotton bud and stored in 1.5 ml 20% glycerol at -80°C. Glycerol stocks were made by resuspending overnight culture in 50:50 LB and glycerol (final concentration 20%). All plasmids and ePACs used in this study are described in Table S4 and all primers in Table S5. Standard DNA sequencing was conducted by Eurofins Genomics using the Mix2Seq kit (Ebersberg, Germany).

METHOD DETAILS

Chemicals and reagents were laboratory standard grade or above and purchased from Sigma Aldrich (UK) or Thermo Fisher Scientific (UK) unless otherwise stated. All media and solutions were made using deionised water (dH_2O) except where stated otherwise (Table S2).

Standard molecular techniques

Genomic DNA and ePACs were isolated by resuspending 1 ml overnight culture in 100 μl solution I (50 mM Tris/HCl, pH 8; 10 mM EDTA). Alkaline lysis was performed by adding 200 μl solution II (200 mM NaOH; 1% SDS) followed by 150 μl solution III (3M potassium acetate, pH 5.5). The supernatant was extracted in 400 μl phenol:chloroform:isoamyl alcohol and the upper phase mixed with 600 μl 2-propanol and incubated on ice for precipitation of the DNA. The DNA was pelleted and washed with 200 μl 70% ethanol, air dried and resuspended in sterile dH_2O. Plasmid DNA was prepared using the QIAprep Spin Miniprep kit (QIAGEN) according to the manufacturer's instructions. All DNA samples were quantified using the Nanodrop 2000 UV-Vis Spectrophotometer and the Qubit assay using the Qubit® fluorimeter 2.0.

PCRs were conducted using either the PCRBIO Taq DNA Polymerase (PCR Biosystems) for diagnostic reactions or the Q5 High-fidelity DNA polymerase for amplification of fragments required for cloning. Both were used according to the manufacturer's recommendations with a final concentration of 100 nM primers. PCR products were analysed using gel electrophoresis using 1% agarose gels in TBE buffer (90 mM Tris HCl, 90 mM Boric Acid, 2 mM EDTA) with 2 μg/ml ethidium bromide and visualised by UV-light. When required, bands of interest were excised from the gel and the DNA recovered using the QiaQuick Gel Extraction Kit (QIAGEN) according to the manufacturer's instructions.

For golden gate assembly, 100 ng purified backbone was incubated with 0.3 μl insert, 2 μl T4 ligase buffer (NEB), 1 μl T4 ligase (NEB) and 1 μl of the relevant restriction enzyme in a total volume of 20 μl made up in dH_2O. Reactions were incubated under the following conditions: 10 cycles of 10 minutes at 37°C and 10 minutes at 16°C, followed by 5 minutes at 50°C and 20 minutes at 65°C. Plasmids were digested in 50-100 μl total volumes with restriction enzymes and their appropriate buffer (ether Roche or NEB) in accordance with the manufacturers recommendations (typically 1 μg of DNA was digested with 1 unit of enzyme for 1 hour at 37°C). Restriction enzymes were heat inactivated at 65°C for 10 minutes and 2 μl shrimp alkaline phosphatase was added to single-enzyme digestions to prevent re-ligation. Ligations were carried out using T4 DNA Ligase according to the manufacturer's recommendations with a standard ratio of 1:3 plasmid:insert. Multiple DNA fragments were assembled into digested vector backbones using Gibson assembly by designing overlaps of between 18 and 24 nucleotides. Gel extracted DNA fragments were incubated in a ratio of 1:3 of plasmid to insert (1:5 for inserts smaller than 300 nucleotides) in the presence of Gibson Assembly master mix (NEB) at 50°C for 1 hour.

Plasmids were transformed into *E. coli* using heat shock (30 seconds at 42°C) for chemically competent cells or electroporation in a BioRad electroporator (200 Ω, 25 μF and 2.5 kV). ePACs were moved into *E. coli* using tri-parental mating by incubating 20 μl of each strain in the centre of an LB agar plate at 37°C and then re-streaking the spot onto selective agar for the desired plasmid combination. Plasmids were conjugated into *S. formicae* via the non-methylating *E. coli* ET12567/pUZ8002 as described previously using between 10 and 200 μl of spores depending on the application (more spores for conjugation of pCRISPomyces-2) (MacNeil et al., 1992; Keiser et al., 2000; Gust et al., 2004).

To analyse protein content, whole cell lysates were incubated at 100°C for 10 minutes in 50 μl SDS loading buffer (950 μl Bio-Rad® Laemmli buffer, 50 μl β-mercaptoethanol) and analysed by gel electrophoresis on a standard resolving gel of 16% (w/v) acrylamide: Bis-Acrylamide 37.5:1 (Fisher BioReagents). To confirm expression of tagged proteins, proteins were analysed by western blot by transferring to a nitrocellulose membrane (Pall Corporation) in a Trans-Blot transfer cell (10 V, 1 hour). After blocking in 5% (w/v) fat-free skimmed milk powder in TBST (50 mM Tris Cl pH 7.5, 150 mM NaCl, 1% Tween) overnight at room temperature, 20 ml HRP-conjugated anti-FLAG antibody diluted 1 in 20000 in TBST was added to the membrane for 1 hour. The membrane was washed 3 times for 10 minutes in TBST before developing in a 50:50 mix of 100 mM Tris pH 8.5, 100 μl luminal, 45 μl coumaric acid and 100 mM Tris pH 8.5, 6 μl 30% hydrogen peroxide for imaging using the ECL setting on a SYNGENE G:Box.

Editing pESAC-13 to define the borders of the *for* BGC

Targeted mutagenesis of pESAC-13 was conducted by ReDirect as described previously (Gust et al., 2004) except that the *oriT* was removed from the apramycin cassette to avoid undesired recombination events with the ePAC. The truncated cassette was PCR amplified, gel purified and electroporated into *E. coli* BW25113 pIJ1790 transformed (by tri-parental mating) with pESAC-13

215-G (containing the *for* BGC, supplied by BioS&T, Quebec(Qin et al., 2017)) using the transfer plasmid pR9604 (Piffaretti, Arini and Frey, 1988; Datsenko and Wanner, 2000). Primer overhangs targeting the edges of the formicamycin cluster were designed so that genes at either the left-hand or right-hand edge were deleted. The PCR-confirmed, edited ePACs were isolated and conjugated into *S. formicae Δfor* and the resulting mutants were grown under formicamycin producing conditions (as above). The metabolites were extracted as described below and then analysed by chromatography over a Phenomenex Gemini reversed-phase column (C18, 100 Å, 150×2.1 mm) using an Agilent 1100 series HPLC system and eluting with the following gradient method: 0–2 min 50% B; 2–16 min 100% B; 16–18 min 100% B; 18–18.1 min 100–50% B; 18.1–20 min 50% B; flowrate 1 mL min^{-1}; injection volume 10 μL; mobile phase A: water+0.1% formic acid; mobile phase B: methanol. UV absorbance was monitored at 250, 285, 360 and 415 nm.

Cappable RNA sequencing

Samples for RNA-sequencing were crushed in liquid nitrogen using a sterile pestle and mortar on dry ice and resuspended by vortexing in 1 mL RLT Buffer (Qiagen) supplemented with β-mercaptoethanol (10 μl in every 1 ml buffer). Following lysis in a QIA-shredder column (Qiagen) the sample was extracted in 700 μl acidic phenol-chloroform and the upper phase mixed with 0.5 volumes 96% ethanol. RNA was purified using the RNeasy Mini spin column (Qiagen) according to the manufacturers protocol, including on column DNase treatment. Following elution, the Turbo-DNase kit was used according to the manufacturers protocol and a further Qiagen RNeasey mini clean-up was conducted before samples were flash frozen in liquid nitrogen for storage at -80°C. Once quantified by Nanodrop and gel electrophoresis, RNA was sent to Vertis Biotechnologie (Freising, Germany) for sequencing and analysed by capillary electrophoresis to map transcription start sites.

Generating *S. formicae* mutants

Gene deletions were made using the pCRISPomyces-2 system as described previously (Cobb, Wang and Zhao, 2015; Qin et al., 2017, 2019). Approximately 20 bp protospacers were designed so that the last 15 nucleotides, including the PAM (NGG) were unique in the genome. These were annealed and assembled into the sgRNA using a Golden Gate reaction with BbsI and pCRISPomyces-2. The resulting plasmid was then digested with XbaI to allow for the insertion of the repair template (usually approximately 1 Kb from either side of the deletion) using Gibson assembly. Once confirmed by PCR and sequencing, the final vector was conjugated into *S. formicae* via the non-methylating *E. coli* ET12567/pUZ8002. The deletion was confirmed using PCR to amplify the region across the repair template and into the surrounding genomic DNA. Once confirmed, loss of the editing plasmid was encouraged by repeated re-streaking on plates lacking the antibiotic selection and incubating at 37°C.

Complementation of gene deletions were achieved by fusing the gene to either a native promoter in pMS82(Gregory, Till and Smith, 2003) by Gibson assembly or by ligating the digested gene product downstream of the constitutive *ermE** promoter in pIJ10257(Hong et al., 2005) and transferring the plasmid into the relevant mutant by conjugation.

ChIP-sequencing

For sampling, spores were inoculated onto cellophane discs and grown for 2, 3 or 4 days. These time points were chosen as day 5 is the earliest formicamycins have been observed in the culture extract and expression of regulator genes would be predicted to happen before biosynthesis. Day 2 was the earliest point that enough biomass could be harvested. Expression of the genes of interest was also confirmed using RT-PCR using the OneStep RT-PCR Kit (Qiagen) before the experiment was conducted. At sampling, discs were removed and the mycelium submerged in 10 ml 1% (v/v) formaldehyde for 20 minutes, followed by 10 ml 0.5 M glycine for 5 minutes. After washing the discs in 25 ml ice-cold PBS (pH 7.4), the samples were frozen at -80°C for storage and a small aliquot retained for western blot analysis to confirm expression of the tagged proteins in each sample.

For chromatin immunoprecipitation, cell pellets were resuspended in 2 ml lysis buffer (10 mM Tris-HCl pH 8.0, 50 mM NaCl, 10 mg/ml lysozyme, EDTA-free protease inhibitor) and incubated at 37°C for 30 minutes. To fragment the DNA, 1 ml IP buffer (100 mM Tris-HCl pH 8.0, 500 mM NaCl, 1% v/v Triton-X, EDTA-free protease inhibitor) was added and samples sonicated 20 times at 50 Hz for 10 seconds per cycle, ensuring cool-down on ice for at least 2 minutes between pulses. A small sample of this crude lysate was extracted with phenol:chloroform, treated with RNaseA and analysed by gel electrophoresis to confirm DNA fragments were within the desired size range for sequencing. The remaining crude lydate was cleared by centrifugation and incubated with 40 μl prepared Anti-FLAG M2 beads (Sigma-Aldridge) with rotation at 4°C overnight. To elute the bound DNA, the samples were then incubated in 100 μl elution buffer (50 mM Tris-HCl pH 8.0, 10 mM EDTA, 15 SDS) overnight at 65°C. Another 50 μl elution buffer was then added and the samples incubated for a further 5 minutes at 65°C before DNA from the total 150 μl eluate was purified by adding proteinase K and incubating at 55°C for 1.5 hours. DNA was extracted in 150 μl phenol-chloroform and purified on a QIAquick column (Qiagen) and eluted in 50 μl EB buffer (10 mM Tris-HCl pH 8.5) for quantification and sequencing by Genewiz (Leipzig, Germany) using the Illumina HiSeq platform. Data analysis was conducted as described previously (Bush et al., 2016; Munnoch et al., 2016; Som et al., 2017). Briefly, reads were aligned to the genome of *S. formicae* and Samtools v1.8 were used to sort and index BAM files produced by Bowtie2 and compute the depth of reads at every nucleotide position. Perl scripts were used to calculate local enrichment in 30 nucleotide windows along the entire genome. Assuming normal distribution, adjusted p-values were calculated and regions $p < 0.00005$ were selected and visualised in Integrated Genome Browser.

qRT-PCR

RNA samples were confirmed to be DNA-free by conducting test PCRs on the 16S rRNA gene and converted to cDNA using the LunaScript RT SuperMix Kit (NEB) according to the manufacturer's instructions. Primers for each transcript were optimised using serial dilutions of ePAC template DNA and checked for specificity by melt-curve analysis and gel electrophoresis. Reactions were run in biological triplicate and technical duplicate using the Luna Universal qPCR Master Mix according to the manufacturer's guidelines in a total volume of 20 μl with approximately 100 ng template cDNA and a final concentration of 0.25 μM of each primer. ΔC_T values were normalised to the 16S rRNA gene.

GUS assay

Strains were generated using pMF96 plasmids containing the *gusA* vector under to control of each relevant promoter and a pMF23 plasmid as a negative control (Feeney et al., 2017) (Table S3). GUS plasmids were conjugated into to *S. formicae* WT, *ΔforJ*, *ΔforGF* and *ΔforZ* strains via ET12567/pUZ8002. Three biological replicates of each new GUS strain were then confirmed by PCR.

GUS strains were grown on SFM agar with a cellophane disk for 4 days before mycelium was harvested by scraping with a sterile metal spatula and resuspended in 1 mL dilution buffer (50 mM phosphate buffer, 0.1 % Triton X-100 [v/v], 5mM DTT). Samples were sonicated in 3 rounds of 8-10 seconds at 80 Hz before centrifugation at 13,000 rpm, 4°C for 10 minutes. A total of 95 μL of each sample supernatant was added to a 96 well plate and freeze-thawed at -80°C and 37°C, respectively. As strains were grown on agar plates rather than in liquid culture, the protein concentration of lysates were quantified by measuring absorbance at 280 nm rather than measuring cell density at 600 nm. The hydrolysis of PNPG by β-glucuronidase produces galactose and chromophoric 4-nitrophenol (PNP) with a peak absorbance at 420 nm. The intensity of this peak is dependent upon the quantity of enzyme present which correlates with transcription at the inserted promoter. PNPG was added to each well (final concentration 3 μg/mL) to initiate the reaction. Samples were loaded into a plate reader at 37°C for optimum enzyme activity and quantified at 415 nm (due to some interference with fasamycin peak absorbance at 419 nm) and 550 nm every 5 minutes for 40 minutes, inclusive. Softmax® Pro7 used to extract raw data which was used calculate Miller units/ mg protein (1 unit = 1000*[Abs415–{1.75*Abs550}]/{t*v*Abs280}]). Each promoter's transcriptional activity was tested in both biological and technical triplicate and mean Miller Units/mg protein calculated. All statistical analysis was performed using IBM SPSS™ Statistics 23.

Fasamycin and formicamycin congener analysis

Solid culture

S. formicae WT ($n = 16$) and mutant strains ($n = 3$) were grown on soya flour mannitol (SFM) agar at 30°C for 10 days. Equal size agar plugs (1 cm^3) were taken in triplicate from each plate and shaken with ethyl acetate (1 mL) for 1 hour before being centrifuged at 3100 rpm for 5 minutes. Ethyl acetate (300 μl) was transferred to a clean tube and solvent was removed under reduced pressure. The resulting extract was dissolved in methanol (200 μL) before being analysed by HPLC (Agilent 1290 UHPLC). To verify peak identity a representative set of samples were analysed by LCMS (Shimadzu IT-ToF LCMS platform). Chromatography was undertaken for both HPLC and LCMS analysis using the following method: Phenomenex Gemini NX C18 column (150 × 4.6 mm); mobile phase A: water + 0.1% formic acid; mobile phase B: methanol. Elution gradient: 0–2 min, 50% B; 2–16 min, 50–100% B; 16–18 min, 100% B; 18–18.1 min, 100–50% B; 18.1–20 min, 50% B; flow rate 1 mL min^{-1}; injection volume 10 μL.

Liquid culture

S. formicae WT or mutant strains ($n = 3$) were grown in liquid TSB (10 mL) for 2 days. 100 μL of 2-day liquid culture was aliquoted into 10 mL of SFM liquid media into sterile 50 mL falcon tubes with sterile bungs. Strains were incubated at 30°C with shaking at 250 rpm. After 10 days, an aliquot (1 mL) of culture was removed from each sample in triplicate and shaken with ethyl acetate (1 mL) for 1 hour and then centrifuged at 3100 rpm for 5 minutes. Ethyl acetate extract (300 μl) was transferred to a clean tube and solvent was removed under reduced pressure. The resulting extract was dissolved in methanol (200 μl) before being analysed by HPLC and LCMS as described before.

Titre determination

Titres of fasamycin and formicamycins were determined by comparing peak areas from the above HPLC analysis to those of standard calibration curves and correcting for the concentration that occurred during the extraction process. Calibration curves were determined using standard solutions of fasamycin E (10, 20, 50, 80 and 200 μM) and formicamycin I (10, 20, 50, 100, 200 and 400 μM) (Figure S1). The content of fasamycin E and formicamycin I were determined by UV absorption at 418 nm and 285 nm respectively. Each standard solution was measured three times.

Scale up fermentation of *S. formicae* Δ*forJ* and Δ*forJX*

A seed culture was prepared by inoculating the fresh mycelium from an MS agar plate into TSB liquid medium (50 mL) and incubating for 24 h at 30°C with shaking at 250 rpm. The seed culture was then used to inoculate TSB (3 L; 6 × 2.5 L flasks, 1:500 inoculation). The cultures were incubated for seven days under the same condition as above after which the whole culture broth was extracted twice with equal amount of ethyl acetate. The organic phase was separated, and the solvent removed by evaporation under reduced pressure. The resulting material was dissolved in methanol and first analysed by LCMS and then subjected to semi-prep purification. In this way six additional fasamycin congeners (fasamycins L to Q) were isolated from the Δ*forJX* mutant, and two additional formicamycin congeners (formicamycins R and S) from the Δ*forJ* mutant. The structures of these compounds were determined using HRLCMS and 2D NMR as described in the Supplementary section.

Analytical LCMS method

For LCMS following analytical LCMS method was used: Phenomenex Kinetex C18 column (100 Å~ 2.1 mm, 100 Å); mobile phase A: water +0.1% formic acid; mobile phase B: acetonitrile. Elution gradient: 0–1 min, 20% B; 1–8 min, 20–100% B; 8–11 min, 100% B; 11–11.1 min, 100–20% B; 11.1–12 min, 20% B; flow rate 0.6 mL min^{-1}; injection volume 10 μL.

Semi-prep HPLC method for isolation of formicamycins R & S

Chromatography was achieved over a Phenomenex Gemini-NX semi-prep reversed-phase column (C18, 110 Å, 150 Å~ 10 mm) using an Agilent 1100 series HPLC system and eluting with the following gradient method: 0–2 min 60% B; 2–18 min 60–100% B; 18–23 min 100% B; 23–23.1 min 100–60% B; 23.1–26 min 60% B; flowrate 3.5 mL min^{-1}; injection volume 20 μL; mobile phase A: water +0.1% formic acid; mobile phase B: acetonitrile. The UV absorbance was monitored at 250 and 285 nm.

Semi-prep HPLC method for isolation of fasamycins L to Q

Chromatography was achieved over a Phenomenex Gemini-NX semi-prep reversed-phase column (C18, 110 Å, 150 Å~ 10 mm) using an Agilent 1100 series HPLC system and eluting with the following gradient method: 0–2 min 70% B; 2–18 min 70–100% B; 18–20 min 100% B; 20–20.1 min 100–70% B; 20.1–24 min 70% B; flowrate 3.5 mL min^{-1}; injection volume 100 μL; mobile phase A: water + 0.1% formic acid; mobile phase B: acetonitrile. UV absorbance was monitored at 250, 285, 360 & 415 nm.

Bioassays

Resazurin assays were performed to determine minimum inhibitory concentrations. A stock solution of compound was prepared in DMSO and diluted in LB or TSB media to give a concentration range of 256 μg/ml to 1 μg/ml containing 5% DMSO. Positive control (PC) for preparations in LB and TSB was apramycin at 50 μg/ml. Negative control (NC) and media control (MC) contained media (LB or TSB) and DMSO at 5%. Methods were followed as detailed in Heine *et al* (Heine et al., 2018). In brief, cultures were grown to confluence overnight and then diluted 1/100 in fresh media and grown to 0.4 OD600nm, diluted to match a 0.5 McFarlands standard and further diluted 1/100. 5 μl of culture was then aliquoted into each well of the 96 well plate excluding the MC well. Plates were grown at 37°C before being inoculated with 5 μl resazurin dye (6.75 mg / ml, Sigma Aldrich). Colorimetric results were observed 4 hours after inoculation. MSSA (ATCC 6538P) and MRSA (ATCC BAA-1717) indicator strains were obtained from the American Type Culture Collection.

QUANTIFICATION AND STATISTICAL ANALYSIS

In general, data were analysed in Excel and figures plotted in R using ggplot2. Details of replicates and data analysis for each experiment can be found in the figure legends.

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Supplemental information

Re-wiring the regulation of the formicamycin biosynthetic gene cluster to enable the development of promising antibacterial compounds

Rebecca Devine, Hannah P. McDonald, Zhiwei Qin, Corinne J. Arnold, Katie Noble, Govind Chandra, Barrie Wilkinson, and Matthew I. Hutchings

Supporting information

GUS assay results from *for* BGC promoters

Table S1: The activities of *for* gene cluster promoters were measured by fusing the promoters upstream of the β-glucoronidase reporter gene in pMF96. Activity of β-glucoronidase was measured by hydrolysis of PNPG using absorbance at 420 nm as described in **Method Details: GUS assay**.

Strain Name	Mean Miller Units/ mg protein ± Standard Error
S. formicae WT pMF96	0.37 ± 1.10
S. formicae WT pMF23	91.75 ± 21.75
S. formicae ΔforJ pMF96	0.00 ± 1.67
S. formicae ΔforJ pMF23	86.98 ± 18.81
S. formicae ΔforGF pMF96	2.01 ± 2.49
S. formicae ΔforGF pMF23	100.83 ± 12.22
S. formicae ΔforZ pMF96	0.37 ± 0.56
S. formicae ΔforZ pMF23	94.44 ± 16.37
S. formicae WT p*forJ*	32.82 ± 12.83
S. formicae ΔforJ p*forJ*	84.85 ± 10.05
S. formicae WT p*forG*	50.56 ± 14.34
S. formicae ΔforGF p*forG*	26.68 ± 8.11
S. formicae WT p*forH*	33.62 ± 12.52
S. formicae ΔforGF p*forH*	11.86 ± 5.07
S. formicae WT p*forZ*	0.38 ± 1.65
S. formicae ΔforZ p*forZ*	11.41 ± 7.68

Table S2: Media used in this study, related to **Method Details**.

Media	**Recipe (per litre)**	**Water**	**pH**
SFM	20 g soy flour 20 g mannitol 20 g agar	Tap	
MYM	4 g maltose 4 g yeast extract 10 g malt extract +/- 18 g agar	50:50 Tap:deionised	7.3
LB	10 g tryptone 5 g yeast extract 10 g NaCl (omitted when selecting with Hygromycin) +/- 20 g agar	Deionised	7.5
2YT	16 g tryptone 10 g yeast extract 5 g NaCl	Deionised	7.0

Table S3: Bacterial strains used and generated in this study, related to **Experimental Model and subject details.**

Strain	Description/Genotype	Plasmid	Resistance	Reference/Source
E. coli Top10	F– *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lacZ*ΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara leu*) 7697 *gal*U *gal*K *rps*L (StrR) *end*A1 *nup*G			Invitrogen™
E. coli BW25113	λ⁻, Δ(*araD-araB*)*567,* Δ*lacZ4787*(*::rrnB-4*), *lacIp-4000*(*lacIQ*), *rpoS369*(*Am*), *rph-1,* Δ(*rhaD-rhaB*)*568, hsdR514*	pIJ790	Cml^R	(Datsenko and Wanner, 2000)
E. coli ET12567	*dam⁻ dcm⁻ hsdS⁻*	pUZ8002	Cml^R/Tet^R	(MacNeil *et al.*, 1992)
S. formicae wild-type				Lab stock
MSSA	ATCC 6538P			American Type Culture Collection
MRSA	ATCC BAA-1717			American Type Culture Collection
S. formicae ΔforJ	*forJ* deletion strain			This work
S. formicae ΔforGF	*forGF* deletion strain			This work
S. formicae ΔforZ	*forZ* deletion strain			This work
S. formicae ΔforJ: ΦBT1 forJ pforM	*forJ* complementation under forM promoter	pRD030	Hyg^R	This work
S. formicae ΔforJ: ΦBT1 forJ	*forJ* complementation under forJ promoter	pRD063	Hyg^R	This work
S. formicae ΔforJ: ΦBT1 pErmE forJ*	*forJ* complementation under ErmE* promoter	pRD06	Hyg^R	This work
S. formicae ΔforGF: ΦBT1 forGF	*forGF* complementation under native promoter	pRD031	Hyg^R	This work
S. formicae Δfor: ΦC31 *for 1-4 aac(3)IV*	Whole *for* cluster deletion complemented with pESAC-13 215-G with genes 1-4 (as annotated by AntiSMASH) replaced with apramycin gene	pRD037	Apr^R	This work
S. formicae Δfor: ΦC31 *for 1-7 aac(3)IV*	Whole *for* cluster deletion complemented with pESAC-13 215-G with genes 1-7 replaced with apramycin gene	pRD038	Apr^R	This work
S. formicae Δfor: ΦC31 *for 32-43 aac(3)IV*	Whole *for* cluster deletion complemented with pESAC-13 215-G with genes 32-43 replaced with apramycin gene	pRD039	Apr^R	This work

S. formicae Δfor: ΦC31 *for 36-43 aac(3)IV*	Whole *for* cluster deletion complemented with pESAC-13 215-G with genes 36-43 replaced with apramycin gene	pRD040	Apr^R	This work
S. formicae ΔforJ: ΦBT1 forJ 3x Flag	*forJ* deletion mutant complemented in-trans with 3x flag-tagged *forJ* for ChIP	pRD034	Hyg^R	This work
S. formicae ΔforGF: ΦBT1 forGF 3x Flag	*forGF* deletion mutant complemented in-trans with 3x flag-tagged *forGF* for ChIP	pRD035	Hyg^R	This work
S. formicae ΔforZ: ΦBT1 forZ 3x Flag	*forZ* deletion mutant complemented in-trans with 3x flag-tagged *forZ* for ChIP	pRD036	Hyg^R	This work
S. formicae ΔforV	*forV* deletion strain			(Qin *et al.*, 2017)
S. formicae ΔforX	*forX* deletion strain			(Qin *et al.*, 2020)
S. formicae ΔforY	*forY* deletion strain			(Qin *et al.*, 2020)
S. formicae ΔforS	*forS* deletion strain			(Qin *et al.*, 2019)
S. formicae ΔforJ, ΔforV	*forV* deletion strain with *forJ* deletion			This work
S. formicae ΔforJ, ΔforX	*forX* deletion strain with *forJ* deletion			This work
S. formicae ΔforJ, ΔforY	*forY* deletion strain with *forJ* deletion			This work
S. formicae ΔforJ, ΔforS	*forS* deletion strain with *forJ* deletion			This work
S. formicae: pMF96	Wildtype strain with GUS but no promoter controlling expression (negative control)	pMF96	Hyg^R	This work
S. formicae: pMF23	Wildtype strain with GUS but no promoter controlling expression (negative control)	pMF23	Apr^R	This work
S. formicae ΔforJ: pMF96	*forJ* deletion strain with GUS but no promoter controlling expression (negative control)	pMF96	Hyg^R	This work
S. formicae ΔforJ: pMF23	*forJ* deletion e strain with GUS but no promoter controlling expression (negative control)	pMF23	Apr^R	This work
S. formicae ΔforGF: pMF96	*forGF* deletion strain with GUS but no promoter controlling expression (negative control)	pMF96	Hyg^R	This work
S. formicae ΔforGF: pMF23	*forGF* deletion e strain with GUS but no promoter controlling expression (negative control)	pMF23	Apr^R	This work
S. formicae ΔforZ: pMF96	*forZ* deletion strain with GUS but no promoter	pMF96	Hyg^R	This work

	controlling expression (negative control)			
S. formicae ΔforZ: pMF23	*forZ* deletion e strain with GUS but no promoter controlling expression (negative control)	pMF23	Apr^R	This work
S. formicae ΔforJ: ΦBT1 GUS pforJ	*forJ* deletion strain with GUS expressed under *pforJ*	pRD062	Hyg^R	This work
S. formicae: ΦBT1 GUS pforJ	Wildtype strain with GUS expressed under *pforJ*	pRD062	Hyg^R	This work
S. formicae: ΦBT1 GUS pforG	WIldtype strain with GUS expressed under *pforG*	pRD054	Hyg^R	This work
S. formicae ΔforGF: ΦBT1 GUS pforG	*forGF* deletion strain with GUS expressed under *pforG*	pRD054	Hyg^R	This work
S. formicae: ΦBT1 GUS pforH	Wildtype strain with GUS expressed under *pforH*	pRD055	Hyg^R	This work
S. formicae ΔforGF: ΦBT1 GUS pforH	*forGF* deletion strain with GUS expressed under *pforH*	pRD055	Hyg^R	This work
S. formicaa: ΦBT1 GUS pforZ	Wildtype strain with GUS expressed under *pforZ*	pRD058	Hyg^R	This work
S. formicae ΔforZ: ΦBT1 GUS pforZ	*forZ* deletion strain with GUS expressed under *pforZ*	pRD058	Hyg^R	This work

Table S4: ePACs and plasmids used or generated in this study, related to **Experimental Model and subject details.**

Plasmid	Description	Resistance	Reference
pUZ8002	RK2 derivative with a mutation in *oriT*	Kan^R	(Keiser *et al.*, 2000)
pMS82	*ori,* pUC18, *hyg, oriT,* RK2, int ΦBT1	Hyg^R	(Gregory, Till and Smith, 2003)
pIJ773	*aac(3)IV oriT bla*	Apr^R	(Gust *et al.*, 2004)
pR9604	pUB307 derivative	$Carb^R$	(Piffaretti, Arini and Frey, 1988)
pESAC-13 215-G	*aph*II, *tsr*	Kan^R/Tsr^R	BioS&T and (Qin *et al.*, 2017)
pCRISPomyces-2	Apr^R, *oriT,* $rep^{pSG5(ts)}$, ori^{ColE1}, *sSpcas9,* synthetic guide RNA cassette	Apr^R	(Cobb, Wang and Zhao, 2015)
pIJ10257	*oriT*, ΦBT1 *attB-int*, *ermEp**, pMS81 backbone	Hyg^R	(Hong *et al.*, 2005)
pMF96	ΦBT1 *attB-int*, uidA CDS, GUS plasmid	Hyg^R	(Feeney *et al.*, 2017)
pIJ10740 (pMF23)	ΦC31 *attB-int, ermEp**	Apr^R	(Feeney *et al.*, 2017)
pRD026	pCRISPomyces-2 f*orJ* flanking DNA and gRNA	Apr^R	This work
pRD027	pCRISPomyces-2 f*orGF* flanking DNA and gRNA	Apr^R	This work
pRD028	pCRISPomyces-2 *ForZ* flanking DNA and gRNA	Apr^R	This work

pRD030	pMS82 *pforM* forJ	Hyg^R	This work
pRD031	pMS82 *pforG* forGF	Hyg^R	This work
pRD032	pMS82 *pforZ* forZ	Hyg^R	This work
pRD034	pMS82 *pforM* f*orJ 3x Flag*	Hyg^R	This work
pRD035	pMS82 *pforG* f*orGF 3x Flag*	Hyg^R	This work
pRD036	pMS82 *pforZ* *forZ 3x Flag*	Hyg^R	This work
pRD037	*pESAC-13* 215-G *1-4 aac(3)IV oriT*	Kan^R/Tsr^R	This work
pRD038	*pESAC-13* 215-G *1-7 aac(3)IV oriT*	Kan^R/Tsr^R	This work
pRD039	*pESAC-13* 215-G *32-43 aac(3)IV oriT*	Kan^R/Tsr^R	This work
pRD040	*pESAC-13* 215-G *36-43 aac(3)IV oriT*	Kan^R/Tsr^R	This work
pRD054	pMF96 pforGF GUS	Hyg^R	This work
pRD055	pMF96 pforH GUS	Hyg^R	This work
pRD058	pMF96 pforZ GUS	Hyg^R	This work
pRD062	pMF96 pforJ GUS	Hyg^R	This work

pRD063	pMS82 *pforJ* forJ	Hyg^R	This work
pRD064	pIJ10257 forJ	Hyg^R	This work

Table S5: Primers designed and used for this study (5'-3'), related to **Experimental Model and subject details.** Capital bases indicate overhangs, restriction sites etc.

Primer name	Description	Sequence
pCRISP Test F	Test XbaI site pCRISP2	aggctagtccgttatcaacttgaaa
pCRISP Test R	Test XbaI site pCRISP2	tcgccacctctgacttgagcgtcga
Spacer test	Test BbsI site of pCRISP2	atacggctgccagataaggc
ForJ For1	Repair template *forJ* KO, left flank	gctcggttgccgccgggcgtttttaTCTAGAggtgtgcgcgaagaacggcc
ForJ Rev 1	Repair template *forJ* KO, left flank	GCTGCTGCGACCAGGCGAGCTCGCcactgacgcggtcgttcccg
ForJ For 2	Repair template *forJ* KO, right flank	GCGAGCTCGCCTGGTCGCAGCAGCtgacgtgcttcgagaccgcc
ForJ Rev 2	Repair template *forJ* KO, right flank	gcaacgcggccttttacggttcctggccTCTAGAcctcttcatgttcctggtgggcc
ForJ gRNA For	sgRNA *forJ* deletion	ACGCtgccgacaccttctccatga
ForJ gRNA Rev	sgRNA *forJ* deletion	AAACtcatggagaaggtgtcggca
ForJ KO Test 1F	Test *forJ* deletion in genome	cctcttcggtgagcgcttcgagg
ForJ KO Test 2R	Test *forJ* deletion in genome	cctgttggacttcgcgcaggc
ForJ KO Test 2F	Test *forJ* deletion in genome	gtacgccaggaggacgtgcg
ForJ KO Test 1R	Test *forJ* deletion in genome	gccgacgcggcacttctatcc
ForZ For1	Repair template *forZ* KO, left flank	gctcggttgccgccgggcgtttttaTCTAGAcgaacaggccgacgctgaacag
ForZ Rev 1	Repair template *forZ* KO, left flank	GCTGCTGCGACCAGGCGAGCTCGCcatggcttgaagtccagcacgtcc
ForZ For 2	Repair template *forZ* KO, right flank	GCGAGCTCGCCTGGTCGCAGCAGCtcatccgtacctggcagctcgtcg
ForZ Rev 2	Repair template *forZ* KO, right flank	gcaacgcggccttttacggttcctggccTCTAGAccgaggcggacggatcgcgtcc
ForZ gRNA For	sgRNA *forZ* deletion	ACGCgtcggcggtcaactcgactg
ForZ gRNA Rev	sgRNA *forZ* deletion	AAACcagtcgagttgaccgccgac
ForZ KO Test 1F	Test *forZ* deletion in genome	gccggtgccgaaccggacgc
ForZ KO Test 2R	Test *forZ* deletion in genome	cgcacgccgccacgacgagc
ForZ KO Test 2F	Test *forZ* deletion in genome	cgcacgccgccacgacgagc
ForZ KO Test 1R	Test *forZ* deletion in genome	cgcacgccgccacgacgagc
ForGF For1	Repair template *forGF* KO, left flank	gctcggttgccgccgggcgtttttaTCTAGAggagccggtcttggccatctgc
FoGF Rev 1	Repair template *forGF* KO, left flank	GCTGCTGCGACCAGGCGAGCTCGCggcagcctcgttcacagcagc
ForGF For 2	Repair template *forGF* KO, right flank	GCGAGCTCGCCTGGTCGCAGCAGCtgaggctcaggcgggttcgatgg
ForGF Rev 2	Repair template *forGF* KO, right flank	gcaacgcggccttttacggttcctggccTCTAGAcgagatcgtcatccacgcgcc
ForGF gRNA For	sgRNA *forGF* deletion	ACGCtggcgaagatgttgcgcaga

ForGF gRNA Rev	sgRNA *forGF* deletion	AAACtctgcgcaacatcttcgcca
ForGF KO Test 1F	Test *forGF* deletion in genome	gcagttcctggacgatgcgc
ForGF KO Test 1R	Test *forGF* deletion in genome	cgagggtctggagaacgcgc
ForGF KO Test 2F	Test *forGF* deletion in genome	cgtcggcaccttctaccaccg
ForGF KO Test 2R	Test *forGF* deletion in genome	gcctgcgtgattcatcggctg
pMS82 f*orJ pforM* F1	*forJ* complementationunder *pforM*	gccgagaaccTAGGATCCAAGCTTgatgccggtgagcagggcgag
pMS82 *forJ pforM* R1	*forJ* complementationunder *pforM*	ggcgccgtggtcgtggtcataccggctcccatcggttgctg
pMS82 *forJ pforM F2*	*forJ* complementationunder *pforM*	cagcaaccgatgggagccggtatgaccacgaccacggcgcc
pMS82 *forJ pforM R2*	*forJ* complementation under *pforM*	CTGGTACCATGCATAGATCTAAGCTTgcggaggcggaccgtgcctag
pMS82 *forZ* F	*forZ* complementation	gccgagaaccTAGGATCCAAGCTTccggtcaccacccattggag
pMS82 *forZ* R	*forZ* complementation	CTGGTACCATGCATAGATCTAAGCTTaggagttgtgcgccctcgc
pMS82 *forGF F*	*forGF* complementatio	gccgagaaccTAGGATCCAAGCTTcgtgtaccccctgtgcacg
pMS82 *forGF* R	*forGF* complementatio	CTGGTACCATGCATAGATCTAAGCTTccgctgctcgccatcgaac
pMS82 TEST F	Test HindIII site pMS82	gcaacagtgccgttgatcgtgctatg
pMS82 TEST R	Test HindIII site pMS82	gccagtggtatttatgtcaacaccgcc
ForJ-3xFLAG R	*forJ 3x*Flag for ChIP	gcctgaaccgcctccaccgtgccccgcgggcacctg
ForJ-3xFLAG F	*forJ 3x*Flag for ChIP	caggtgcccgcggggcacggtggaggcggttcaggc
FLAG-pMS82 R	3*x*Flag in pMS82	CTGGTACCATGCATAGATCTAAGCTTtcaCTTGTCGTCATCGTCCTTG
pMS82 ForF prom F	*forGF 3x*Flag for ChIP	gccgagaaccTAGGATCCAAGCTTcgtgtaccccctgtgcacg
ForF-prom R	*forGF 3x*Flag for ChIP	ggtcaccacggtctgcatagcagcctccccggttcg
ForGF F	*forGF 3x*Flag for ChIP	cgaaccggggaggctgctatgcagaccgtggtgacc
ForF-3xFLAG R	*forGF 3x*Flag for ChIP	gcctgaaccgcctccaccgccccggtcgccctgcg
FLAG-pMS82 F	3*x*Flag in pMS82	cgcagggcgaccggggcggtggaggcggttcaggc
ForZ-3xFLAG R	*forZ 3x*Flag for ChIP	gcctgaaccgcctccaccccgctcgcacgccgccacg
ForZ-3xFLAG F	*forZ 3x*Flag for ChIP	cgtggcggcgtgcgagcggggtggaggcggttcaggc
ForJ Test F	To check expression by RT-PCR	gcaaggcggcgcagagcg
ForJ Test R	To check expression by RT-PCR	gccgacaccttctccatgagg
ForZ Test F	To check expression by RT-PCR	gaaccggacgcagccgcag
ForZ Test R	To check expression by RT-PCR	cctcgacgcgtgccacgag
ForF Test F	To check expression by RT-PCR	cctcgacgcgtgccacgag
ForF Test R	To check expression by RT-PCR	gcgaccagggtcatgacctcg
pMF96 HindIII Test For	Test HindIII site of pMF96	gctcaatcaatcaccggatcc
pMF96 HindIII Test Rev	Test HindIII site of pMF96	catgtccgtacctccgttg
16S r RNA For	qPCR reference gene	cgggtctgcagtcgatacgg

16S r RNA Rev	qPCR reference gene	gctttcgctcctcagcgtcag
MLK For	qPCR expression	ctgatcttcggtgccttcctgtcc
MLK Rev	qPCR expression	cggcgagcagtccgaggtc
J For	qPCR expression	ccgaccgtgcggaaactcg
J Rev	qPCR expression	gtccggatccacatgccgc
HI For	qPCR expression	ccttcgagttcgtcgtggacg
HI Rev	qPCR expression	gctgctcggcgaccagatc
GF For	qPCR expression	gctccaccactacgaacagcg
GF Rev	qPCR expression	ggagcgagtcctcgatcacg
TSRABCDE For	qPCR expression	cgacaccatcgacaccgcc
TSRABCDE Rev	qPCR expression	cgttccactccacgaccacc
UVWXY For	qPCR expression	gcagcttctccaggagttcc
UVWXY Rev	qPCR expression	gccaagaagatcctcgacagg
Z For	qPCR expression	ctcatccggctcgtcacgc
Z Rev	qPCR expression	cagatggcggttggcgagc
AA For	qPCR expression	gaccggaggaacgcctgg
AA Rev	qPCR expression	cggtgtcgaggtccttgctc
*pforH-G*F F For	*pforG* in pMF96	AAAAAcatatggcgctgctcacggtcatcg
*pforH-G*F Rev	*pforG* in pMF96	AAAAActcgaggcagcctcgttcacagcag
pforZ-AA F For	*pforZ* in pMF96	AAAAAcatatggaatccctgacgcgccgcg
pforZ-AA F Rev	*pforZ* in pMF96	AAAAActcgaggacgatggtggtgtcgagcac
forJ pMF96 360 bp prom for	*pforJ* in pMF96	caattaatctagaggatccatatgcctgttcgtcgcggtggc
forJ pMF96 360 bp prom rev	*pforJ* in pMF96	gtacctccgttgctcgactcgaggttcgctcacctctgctgtgacg
pforGF-H For	*pforH* in pMF96	AAAAAcatatggcagcctcgttcacagcagc
pforGF-H Rev	*pforH* in pMF96	AAAAActcgaggcgctgctcacggtcatcg
pMS82 *forJ pforJ* F1	*forJ* complementationunder *pforJ*	gccgagaaccTAGGATCCAAGCTTcctgttcgtcgcggtggc
pMS82 *forJ pforJ* R1	*forJ* complementationunder *pforJ*	ggcgccgtggtcgtggtcatgttcgctcacctctgctgtgacg
pMS82 *forJ pforJ* F2	*forJ* complementationunder *pforJ*	cgtcacagcagaggtgagcgaacatgaccacgaccacggcgcc
pMS82 *forJ pforJ* R2	*forJ* complementationunder *pforJ*	CTGGTACCATGCATAGATCTAAGCTTgcggaggcggaccgtgcctag
pIJ10257 *forJ* F	*forJ* complementationunder *pErmE**	gtctagaacaggaggccccatatgatgaccacgaccacggcgc
pIJ10257 *forJ R*	*forJ* complementationunder *pErmE**	catgagaacctaggatccaagcttggaacgaccgcgtcagtgcc

Figure S1. Calibration curve for Fasamycin E and Formicamycin I, related to **Figure 2, Table 1 and Method Details: Titre Determination.**