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Heterologous Production of Fosfomycin and Identification of the Minimal Biosynthetic Gene Cluster

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Summary

Fosfomycin is a clinically utilized, highly effective antibiotic, which is active against methicillin- and vancomycin-resistant pathogens. Here we report the cloning and characterization of a *complete* fosfomycin biosynthetic cluster from *Streptomyces fradiae* and heterologous production of fosfomycin in *S. lividans*. Sequence analysis coupled with gene deletion and disruption revealed that the minimal cluster consists of *fom1-4*, *fomA-D*. A LuxR-type activator that was apparently required for heterologous fosfomycin production was also discovered ~13 kb away from the cluster and was named *fomR*. The genes *fomE* and *fomF*, previously thought to be involved in fosfomycin biosynthesis, were shown not to be essential by gene disruption. This work provides new insights into fosfomycin biosynthesis and opens the door for fosfomycin overproduction and creation of new analogs via biomolecular pathway engineering.

Introduction

Microbial resistance to antibiotics is rapidly increasing, posing a real threat to human health and demanding treatments that are effective against resistant pathogens. Fosfomycin is a low molecular weight natural product produced by several species of *Pseudomonas* [1] and *Streptomyces* [2] that exhibits broad spectrum antimicrobial activity against gram-negative and gram-positive bacteria [3]. Fosfomycin was first identified nearly 40 years ago [2] and is an FDA approved drug that has become the first choice for treatment of certain types of infections [4–8]. Attractively, fosfomycin has been proven effective for the treatment of cephalosporin- and penicillin-resistant *Streptococcus pneumoniae* [9, 10] and is effective against methicillin-resistant [11] and vancomycin-resistant [12] strains of *Staphylococcus aureus*. Fosfomycin inactivates UDP-*N*-acetylglucosamine-3-*O*-enolpyruvyltransferase (MurA), an essential enzyme that catalyzes the first committed step in bacterial cell wall biosynthesis [13–16], by covalent alkylation of an active site cysteine [17, 18].

Antibiotics containing P-C bonds (phosphonates and phosphinates) as a class are virtually uncharacterized on the genetic and biochemical level. Although the biosynthetic pathway for fosfomycin production in *S. wedmorensis* has been proposed, heterologous production of fosfomycin has never been achieved, suggesting the possibility that additional required genes remain to be identified [19]. The enzymes encoded by *fom1-4* are generally thought to catalyze consecutive steps as displayed in Figure 1, where PEP is converted to phosphonopyruvate (PnPy) by PEP mutase [20], followed by a decarboxylation to form phosphonoacetaldehyde (PnAA). These first two steps are a common theme in the biosynthesis of most phosphonates [21, 22], where the decarboxylation reaction drives the unfavorable PEP mutase reaction forward [23–26]. PnAA is known to be a biosynthetic intermediate for several phosphonates such as bialaphos [23], fosfomycin [20], 2-aminoethylphosphonate (AEP) [27], and 2-hydroxyethylphosphonate (HEP) [28]. Both PEP mutase (Fom1) and PnPy decarboxylase (Fom2) activities have been mapped to the corresponding genes in the fosfomycin biosynthetic pathway of *S. wedmorensis* [20]. In the subsequent step of the pathway, PnAA is proposed to be attacked by a methyl anion derived from methylcobalamin (MeCbl) to form 2-hydroxypropylphosphonate (HPP) [19, 29]. This step is unusual, as it would involve an unprecedented use of methylcobalamin. Since the methyltransferase encoded by *fom3* has not been characterized in vitro due to difficulties with functional expression and purification, this proposed conversion is still purely speculative. In the final step, HPP is oxidized to form the epoxide [30, 31], completing the biosynthesis of fosfomycin. This unusual epoxidation step catalyzed by Fom4 has been characterized in detail [30, 32–38] with several different crystal structures reported for the enzyme [37, 38]. While the mechanistic details are still under debate, there is a consensus that this enzyme catalyzes the conversion of HPP to fosfomycin in a stereospecific manner using a mononuclear metal. Thus, several important features of fosfomycin biosynthesis have been discerned on the biochemical level.

On the other hand, little is known about the minimal required gene cluster for production, transcriptional control of the cluster, import/export of the antibiotic, and the unusual methyl transfer step, thus warranting further study. To date, heterologous production of the cluster from *S. wedmorensis* has not been achieved [19], and the vast majority of the *S. fradiae* fosfomycin cluster has not been characterized or sequenced; similarly, very little information is available about the cluster found in *Pseudomonas* species, other than limited information about the epoxidase and resistance protein [30, 39]. Therefore, we initially set out to isolate, sequence, and characterize the fosfomycin cluster from *S. fradiae* with the goal of solidifying the understanding of this biosynthetic pathway in terms of the open reading frames involved.

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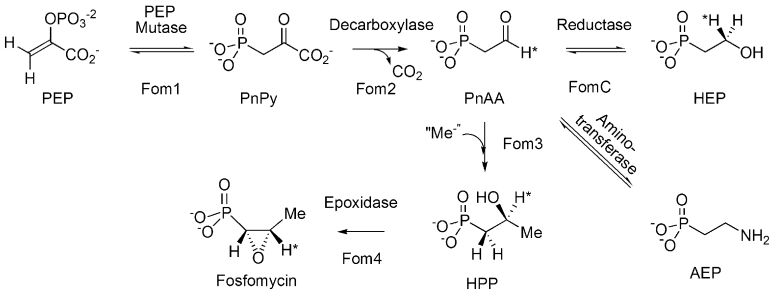

Figure 1. Previously Proposed Biosynthetic Pathway for Fosfomycin

PEP is converted to PnPy by Fom1 and PnPy is converted to PnAA by Fom2. PnAA is proposed to be converted to HPP by Fom3. Fosfomycin is the product of epoxidation of HPP by Fom4. Both HEP and AEP have been shown to complement production of pathways blocked at Fom2 [31].

Results and Discussion

Identifying the Fosfomycin Cluster from *S. fradiae*

To isolate the complete fosfomycin cluster from *Streptomyces fradiae,* a fosmid library of its genomic DNA was prepared. *S. fradiae* was cultivated and its genomic DNA was isolated in fragments of >200 kb followed by partial Sau3A1 digestion. Cloning of 30–50 kb fragments followed by lambda phage transduction yielded a library of 1×10^6 fosmid-containing clones of *E. coli* WM4489. A partial sequence of the methyltransferase gene (*fom3*) involved in fosfomycin biosynthesis in *S. fradiae* was obtained by PCR using primers designed for the same gene from *S. wedmorensis* (*fom3* NCBI accession number: BAA32490). A small fraction of the fosmid library (ca. 1500 clones) was screened by PCR for *fom3*. A total of six fosmids were identified to contain *fom3*, of which three were eliminated because the fosmid/insert-junction sequence contained an incomplete gene known to be involved in fosfomycin biosynthesis. The remaining three fosmids were introduced into *S. lividans* 66 via conjugal transfer and integrated into the ϕC31 site of the genome. The exconjugant integrants were verified by PCR and subsequently checked under a wide variety of conditions for fosfomycin production.

Heterologous Fosfomycin Production

A bioassay based on inhibition zones of *E. coli* WM5923 grown on solid penicillin assay medium 2 was developed utilizing authentic fosfomycin. This assay was effective at concentrations of authentic fosfomycin as low as 1 μg/ml, and no inhibition zones were observed using wild-type (WT) *S. lividans* 66 under any of the production conditions tested. The three exconjugants containing different fosmids referred to as 39, 40, and 45 were tested for production of fosfomycin. Exconjugants harboring fosmids 39 and 40 never produced inhibition zones for any of the conditions tested. However, *S. lividans* 66 integrated with fosmid 45 (hereafter referred to as 45-*S. lividans*) reproducibly produced inhibition zones when grown on ISP2 or Hickey-Tresner [40] (HT) agar plates, with larger inhibition zones produced when grown on the latter. Additionally, lower production of fosfomycin was observed in liquid HT media. Previous reports have commented on extraction of fosfomycin from dried culture media using methanol [1]. Indeed, the methanol extractable material from dried liquid HT media used for 45-*S. lividans* growth gave reproducible inhibition zones, while similarly prepared samples from media used for WT-*S. lividans* 66 growth did not (Figure 2A). The material remaining after extraction for either strain did not produce inhibition zones. 45-*S. lividans* produced similar sized inhibition zones to *S. fradiae* when grown under similar conditions.

Fosfomycin specifically inhibits cell wall biosynthesis by forming a covalent adduct with C115 of UDP-*N*-acetylglucosamine enolpyruvyl transferase (MurA) [13, 17], which is responsible for the first committed step of peptidoglycan biosynthesis [41]. A single mutation of MurA, C115D, averts the formation of the covalent adduct, while the enzyme still retains some activity [18]. To verify that the produced antibiotic was fosfomycin, *E. coli* BL21 (DE3) harboring pET20-MurA-C115D (fosfomycin MIC ~20 mg/ml) was checked as a resistant strain. Neither authentic fosfomycin (100 μg/ml) nor the antibiotic produced by 45-*S. lividans* (500-fold concentrated extracted media) gave rise to inhibition zones with this fosfomycin-specific resistant strain (Figure 2B). Thus, the bioassay data are consistent with the heterologous production of fosfomycin.

Biochemical and Biophysical Identification of Fosfomycin

To further verify that 45-*S. lividans* was indeed producing fosfomycin, biochemical and physical identification were sought. Unmodified *murA* and UDP-*N*-acetylenolpyruvoylglucosamine reductase (*murB*) were cloned into overexpression vectors, overexpressed, and purified from *E. coli* as 6×His-tagged fusions. These enzymes were subsequently utilized in a coupled enzymatic assay, in which the product of MurA (UDP-*N*-acetylenolpyruvoylglucosamine) is reduced by NADPH-dependent MurB (Figure 2C), allowing convenient monitoring of consumed NADPH at 340 nm. MurA was incubated with MOPS buffer, fosfomycin, media extract from WT-*S. lividans* 66, or 45-*S. lividans* and then assayed in this coupled reaction. As displayed in Figure 2D, MurA activity was lost in the 45-*S. lividans* sample as well as the fosfomycin sample, while incubation with the WT-*S. lividans* sample only resulted in a 2-fold reduction in activity, likely as a result of the high salt concentration in the media extract. Furthermore, the covalent adduct of fosfomycin with MurA was analyzed by ESI-Q-TOF MS. MurA was incubated with buffer, WT-*S. lividans* extract, 45-*S. lividans* extract, or authentic fosfomycin in the presence of the substrate UDP-*N*-acetylglucosamine, which enhances the bioactivity of fosfomycin [15, 17, 41]. The resulting *m/z* values for the desalted samples are displayed in Table S1 (see the Supplemental Data available with this article online). MurA incubated with authentic fosfomycin or 45-*S. lividans* extract gave $[M + 138]^+$ *m/z* and $[M + 139]^+$ *m/z*

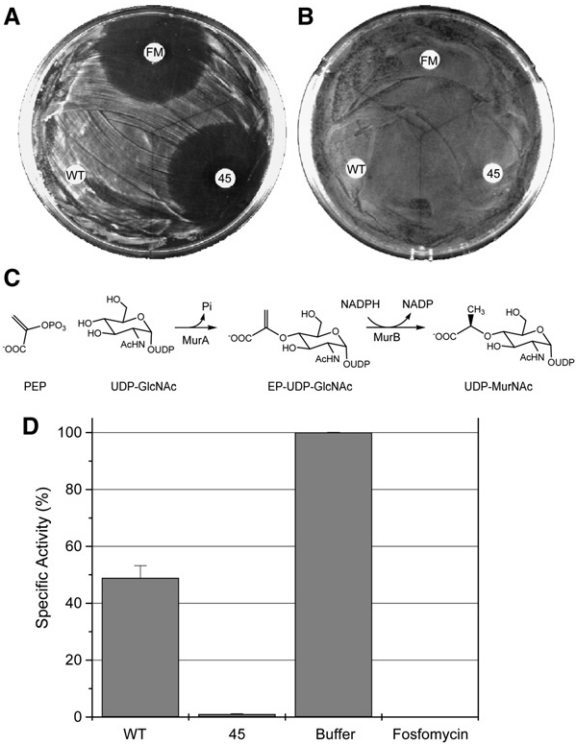

Figure 2. Biological Detection of Fosfomycin

(A) Growth inhibition assay of *E. coli* WM5923 with 10 μl 100 μM fosfomycin, 300-fold concentrated media extract from WT-*S. lividans* 66, or concentrated media extract from 45-*S. lividans*.

(B) The fosfomycin-resistant strain BL21 pET20-MurA-C115D did not produce inhibition zones for fosfomycin or the produced antibiotic.

(C) MurA was assayed indirectly by coupling its reaction to that of MurB, which can be analyzed at 340 nm due to the consumption of NADPH.

(D) MurA enzyme activity was measured after enzyme incubation with media extract from WT-*S. lividans*, 45-*S. lividans*, authentic fosfomycin, or buffer alone. Less than 1% of MurA activity remained for both authentic fosfomycin and the 45-*S. lividans* samples.

peaks, respectively, which corresponds well to addition of the molecular weight of fosfomycin (MW = 137). However, neither of these two peaks was observed for the WT-*S. lividans* sample. Instead, a peak at $[M + 160]^+ = m/z$ was observed, which was attributed to the formation of a covalent adduct with PEP (MW = 165) present in the concentrated media; MurA is known to form such an adduct [41]. Thus, the MurA activity assay and mass spectrometry data are consistent with production of fosfomycin.

Finally, in order to directly observe the produced fosfomycin, 300-fold concentrated media extract was prepared from 45-*S. lividans*. This sample was analyzed by ^{31}P NMR spectroscopy since phosphonates give characteristic downfield peaks. The ^{31}P NMR signal for fosfomycin has been reported at 10.9 ppm [34], although the exact chemical shift of phosphonates is highly pH dependent. Figure 3A displays the ^{31}P NMR spectrum of the concentrated and extracted production media, which shows a peak at approximately the expected chemical shift for fosfomycin (11.9 ppm). To verify that fosfomycin was responsible for this peak, the sample was then spiked with 1 mM authentic fosfomycin, and the resulting spectrum (Figure 3A, inset) showed a relative increase in the peak area at 11.9 ppm, confirming fosfomycin production. The same sample was also analyzed by high-resolution Fourier-transform mass spectrometry (FTMS). Figure 3B displays the combined chromatographic and FTMS analysis of the concentrated and extracted production media, which shows peaks at the correct retention time (15.2 min) and relative molecular weight value (137.0008 Da) that match those with authentic fosfomycin, with 0.15 min and 0.96 ppm errors, respectively. Since there are no other known empirical formulas that match the exact mass within 8 ppm and the MS/MS fragmentation matches fosfomycin (data not shown), these data provide clear evidence for heterologous fosfomycin production. The whole fosmid 45 was subsequently sequenced using primers that annealed to a randomly inserted transposon. The final insert sequence was 31.8 kb and was comprised of 278 overlapping sequence reads averaging ~800 bp.

Analysis of the Gene Cluster

Figure 4 displays the completely sequenced insert with putative open reading frames (Orfs). To determine which portion of the ~32 kb insert encoded genes required for biosynthesis of fosfomycin, deletions of varying size were made from each end of the insert to determine the boundaries of the ability to confer antibiotic production. We thus identified an approximately 23 kb region that is sufficient to direct the production of fosfomycin within *S. lividans* (Figure 4). This region contains 21 putative Orfs, 10 of which were previously identified in *S. wedmorensis* (*fomA-F*, *fom1-4*) [19]. The central cluster from Fom3 to FomC was identical in orientation to that of *S. wedmorensis*, and the average predicted protein homology was 90% (Table 1).

Table 1 lists both the *S. wedmorensis* and next closest homolog to Orfs *fom1-4* and *fomA-F* from the nonredundant protein database as determined by a BLAST search [42]. Possible functions of the Orfs were assigned based on the functions of similar proteins identified by the BLAST searches as well as conserved domain searches. The genes *fom1* and *fom2* closely resembled the homologous genes from *S. wedmorensis* with 98% and 95% deduced protein homology, respectively, which have been studied in *S. wedmorensis* by chemical complementation of loss of function mutations, enzymatic assays, and gene manipulation [19, 20]. In addition to the PEP mutase domains, both *fom1* genes have a domain with homology to cytidylyl transferases of unknown function. The next closest homolog to *fom1* is the PEP mutase from *Bacteroides fragilis* (61% homology), a known producer of AEP [25]. The next closest homolog to *fom2* is a putative PnPy decarboxylase from *Treponema denticola* (63% homology). Therefore, the bioinformatics studies with *fom1* and *fom2* from *S. fradiae* are consistent with the proposed function of the homologous enzymes in *S. wedmorensis* (Figure 1).

A BLAST search of the deduced protein sequence of Fom3 showed that it had 95% sequence identity to Fom3 from *S. wedmorensis*. These proteins have two conserved domains. The N-terminal domain was identified as a B_{12}-like binding domain, and the C-terminal domain shows consensus to the radical-SAM protein

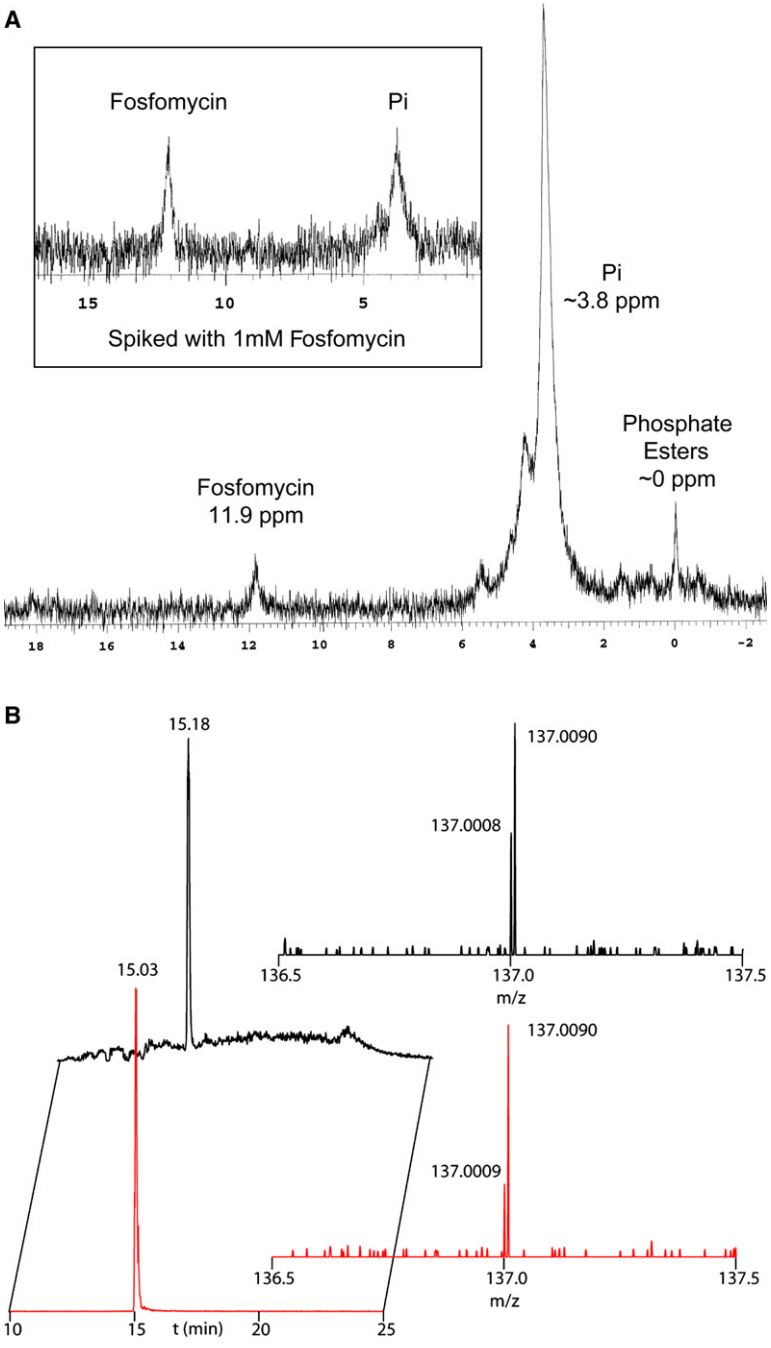

Figure 3. ^{31}P NMR and MS Detection of Fosfomycin in Production Media

(A) Culture media from 45-*S. lividans* was extracted with methanol, concentrated, and redissolved in D_2O. The ^{31}P NMR spectrum displays a peak with a chemical shift of 11.9 ppm. The inset displays the same sample when spiked with 1 mM fosfomycin, in which this peak is increased in relative intensity, suggesting the peak at 11.9 ppm is fosfomycin.

(B) Overlay of chromatograms from LC/FTMS in SRM mode from the standard (red, bottom) and the concentrated supernatant (black, top) and the corresponding high-resolution mass spectra of fosfomycin from both runs (insets at right).

family that utilizes *S*-adenosylmethionine (SAM) to catalyze reactions involving organic free radicals [43]. Similar domain structure is found for the methyltransferases involved in clorobiocin biosynthesis [44] (CloN6), bialaphos biosynthesis (PhpK) [45], fortimycin biosynthesis [46], and several unknown proteins proposed to be methyltransferases. Therefore, the role of Fom3 as a methyltransferase is likely. The deduced protein sequence of Fom4 from *S. fradiae* has 94% identity to the epoxidase Fom4 from *S. wedmorensis* (Table 1). Interestingly, both enzymes have helix-turn-helix motifs that are typical of DNA binding enzymes. However, the epoxidation activity of this enzyme is unquestioned as it has been unambiguously demonstrated [19, 31, 33, 34, 47]. The next closest and the only other known enzyme with significant homology is the epoxidase involved in fosfomycin production in *Pseudomonas syringae* [30]. Therefore, all the previously proposed biosynthetic enzymes in the *S. wedmorensis* pathway (*fom1-4*) are present in *S. fradiae*.

FomA and FomB from *S. fradiae* have high homology to the corresponding sequences from *S. wedmorensis.* FomA shares homology with the amino acid kinase family, which includes kinases that phosphorylate a variety of amino acid substrates, as well as uridylate kinase and carbamate kinase. The known resistance gene *fosC* from *Pseudomonas syringae* is also homologous to *fomA* [39]. FomB on the other hand has no recognized

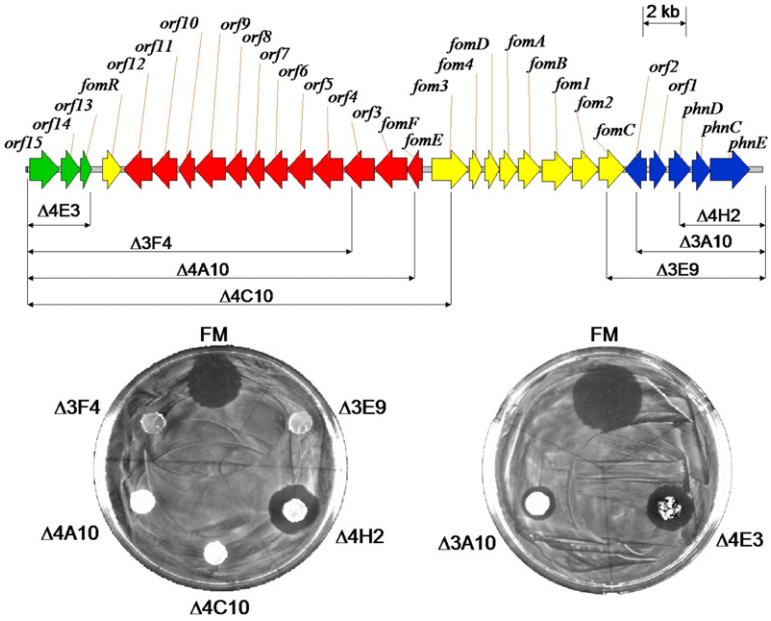

Figure 4. The Complete Fosfomycin Biosynthetic Cluster

Open reading frame analysis found 28 Orfs. Deletion analysis was performed to determine the minimal cluster. The smallest deletions that no longer resulted in fosfomycin production were Δ3F4 and Δ3E9. The largest deletions that allowed continued fosfomycin production were Δ4E3 and Δ3A10.

conserved domains or significant homology to any other proteins; only weak homology to ATP binding proteins can be found. The biochemical action of both FomA and FomB from *S. wedmorensis* as phosphotransferases that inactivate fosfomycin has been demonstrated [48, 49].

Four genes (*fomC-F*) that have not previously been assigned a function were present in both the *S. wedmorensis* and *S. fradiae* clusters. The genes *fomE* and *fomF* have homology to aspartate racemase and threonyl-tRNA synthetase, respectively, and as such are not suspected to be involved in fosfomycin biosynthesis. These two genes do have relatively high homology between *S. fradiae* and *S. wedmorensis*. The genes *fomC* and *fomD*, however, are found in the central cluster and appear to be transcriptionally coupled to other genes in this cluster (Figure 4). The FomC protein sequence from *S. fradiae* had much lower homology (58%) to the *fomC* gene product of *S. wedmorensis* than other respective gene products in the two clusters. However, the first ~230 amino acids have very high homology (92%) and both sequences have conserved domains similar to the Fe(II)-dependent alcohol dehydrogenase (ADH) family. The *S. fradiae* FomC sequence is 120 amino acids longer than that of *S. wedmorensis*. Curiously, the previously reported *S. wedmorensis* FomC [19] appears to be truncated with respect to other members of the Fe(II)-dependent ADH family, which are typically between 350 and 400 amino acids in length [50–54], while FomC for *S. fradiae* is of the expected size (382 amino acids). Comparisons of the DNA sequence shows that over the entire length of the *fomC* gene from *S. fradiae*, 92% of the nucleotides are conserved in *S. wedmorensis*, suggesting that there is a missense mutation or sequencing error in the *S. wedmorensis* gene, resulting in the different C-terminal protein sequences and relatively low overall homology. Several proteins that have moderate homology (35%–25%) to FomC have been studied in detail such as Fe^{2+}-dependent ADH from *Thermotoga maritime* [55] and 1,2-propanediol oxidoreductase from *E. coli* [51]. PhpC from the bialaphos pathway of *Streptomyces viridochromogenes* is another enzyme that shares 31% homology [56]. Interestingly, this is the only other sequenced

Table 1. Open Reading Frame Analysis of the Fosfomycin Gene Cluster

Predicted ORF (Amino Acids)	*S. wedmorensis* Homolog	Identity	BLAST Homolog	Identity
FomC (382 aa)	FomC (262 aa)	58%	CAB45024 putative alcohol dehydrogenase (*Amycolatopsis orientalis*, 377 aa)	35%
Fom2 (384 aa)	Fom2 (384 aa)	95%	NP_972020 putative phosphonopyruvate decarboxylase (*Treponema denticola*, 378 aa)	63%
Fom1 (435 aa)	Fom1 (435 aa)	98%	BAD49328 putative phosphoenolpyruvate phosphomutase (*Bacteroides fragilis*, 435 aa)	61%
FomB (330 aa)	FomB (330 aa)	96%	Q08381 molybdenum import ATP-binding protein ModC (*Rhodobacter capsulatus*, 363 aa)	34%
FomA (266 aa)	FomA(266 aa)	95%	YP_562175 aspartate glutamate uridylate kinase (*Shewanella denitrificans*, 279 aa)	25%
FomD (207 aa)	FomD (209 aa)	91%	CAC05886 hypothetical protein (*Streptomyces coelicolor*, 236 aa)	32%
Fom4 (198 aa)	Fom4 (198 aa)	94%	BAA94418 epoxidase (*Pseudomonas syringae*, 190 aa)	35%
Fom3 (534 aa)	Fom3 (543 aa)	95%	ABE54454 radical sam protein (*Shewanella denitrificans*, 562 aa)	29%

The closest homologs in the *S. wedmonensis* pathway are shown along with the next closest BLAST hit and accession code.

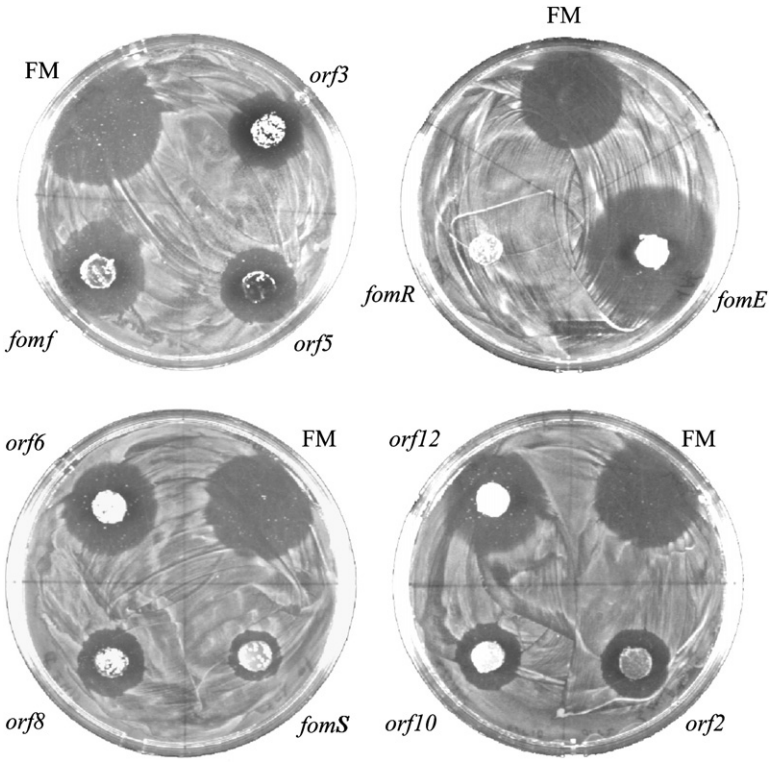

Figure 5. Gene Disruption Analysis

The minimal gene cluster was identified using a selectable transposon inserted in the genes of interest. Gene clusters with disruptions of *fomE*, *fomF*, *fomR*, *orf1*, *orf2*, *orf3*, *orf5*, *orf6*, *orf8*, *orf10*, and *orf12* were each integrated into *S. lividans* and checked for bioactivity.

pathway in which HEP is a known intermediate [57]. It has been proposed in several studies that HEP and PnAA are interconverted by a dehydrogenase (Figure 1) [19, 29]. It is likely that FomC is the enzyme responsible for this interconversion.

The only remaining gene without assigned function is *fomD*, for which the deduced protein sequence has 91% homology to the originally published sequence from *S. wedmorensis* (NCBI accession number: BAA32492) and 73.2% identity to a sequence reported later (S60209). The closest homologs of *fomD* are both listed as hypothetical proteins from *S. coelicolor* and *S. avermitilis* with 32% and 34% homology, respectively. Interestingly, the homologs from both related Streptomycetes are located in the genome downstream of class I and class II fumarase enzymes. Class I fumarases typically contain 4Fe4S clusters that can be oxidized under oxidative conditions to form a 3Fe4S cluster [58, 59] in much the same way as many members of the radical SAM protein family [43, 60, 61]. Therefore, it can be speculated that *fomD* might be related to Fom3 or Fom4 activity, perhaps as a reductase, reactivating protein, or maturation protein, since both Fom3 and Fom4 are metalloproteins that catalyze redox chemistry.

Minimal Cluster Analysis

In addition to the previously identified genes, several other genes in the gene cluster appeared to be important. Deletion of the two genes (designated *orf1* and *orf2)* downstream of the aforementioned *fom* genes resulted in noticeably lower production of fosfomycin, but they were not required for fosfomycin biosynthesis (Figure 4). Deletion of a larger DNA fragment (Δ3E9) resulted in loss of fosfomycin production, but a smaller deletion (Δ4H2) resulted in a typical level of fosfomycin production (Figure 4). These data strongly suggest that FomC is required for fosfomycin production. To determine the relative importance of *orf1* and *orf2*, disrupting insertions into both *orf1* and *orf2* were obtained from the transposon sequencing protocol and were reintroduced into *S. lividans* by conjugal transfer, integrated into the chromosome, and checked by bioassay for fosfomycin production. As displayed in Figure 5, disruption of *orf2* did not affect fosfomycin production, whereas disruption of *orf1* resulted in reduced fosfomycin production similar to that observed for the *orf1* and *orf2* deletion. A BLAST search was performed on these two genes, with the results presented in Table 2. Interestingly, *orf1* appears to be a DNA regulating element, which may partially regulate fosfomycin production, whereas *orf2* is not obviously involved.

Deletion analysis also revealed that an upstream fragment encoding eleven additional open reading frames was required for antimicrobial activity (Figure 4). Ten of these Orfs (designated *orf3-12)* seemed to comprise a biosynthetic cluster along with *fomE-F*. A BLAST search using these genes as query sequences produced the best hits listed in Table 2. No clear biological function can be assigned based on homology to the *fomE-F* and *orf3-12* gene cluster, although these genes seem unlikely to be involved directly in fosfomycin biosynthesis. The final gene product in this upstream DNA fragment has homology to LuxR-type helix-turn-helix activators (Table 2) including homology (30% sequence identity) with the activator in the bialaphos pathway (*PhpR*), one of the few other completely sequenced phosphonate antibiotic pathways [56, 62]. This gene is therefore proposed to be an activator of the fosfomycin cluster and is termed *fomR* (Figure 4). It is interesting to note that for *fomE*, *fomF*, *orf7*, *orf9*, and *orf12*, no

Table 2. Open Reading Frame Analysis of Neighboring Genes

Predicted ORF (Amino Acid)	BLAST Homolog	Identity
FomE (228 aa)	ZP_00997194 putative aspartate racemase (*Janibacter sp.*, 235 aa)	35%
FomF (469 aa)	AAS09418 threonyl-tRNA synthetase (*Lactobacillus johnsonii*, 643 aa)	25%
Orf1 (308 aa)	CAB45044 putative transcriptional regulator (*Amycolatopsis orientalis*, 335 aa)	32%
Orf2 (244 aa)	ZP_00599669 putative dienelactone hydrolase (*Rubrobacter xylanophilus*, 220 aa)	31%
Orf3 (439 aa)	AAN85500 antibiotic transport protein (*Streptomyces atroolivaceus*, 432 aa)	25%
Orf4 (440 aa)	CAA60474 OrfE (*Streptomyces hygroscopicus*, 465 aa)	33%
Orf5 (366 aa)	YP_288556 succinyl-diaminopimelate desuccinylase (*Thermobifida fusca*, 354 aa)	48%
Orf6 (333 aa)	YP_236144 HMG-CoA lyase-like (*Pseudomonas syringae*, 342 aa)	36%
Orf7 (255 aa)	AAP05735 putative coenzyme PQQ synthesis protein c (*Chlamydophila caviae*, 242 aa)	25%
Orf8 (312 aa)	YP_012407 branched-chain amino acid aminotransferase (*Desulfovibrio vulgaris*, 309 aa)	35%
Orf9 (449 aa)	ABB08584 hypothetical protein (*Burkholderia sp.*, 462 aa)	22%
Orf10 (225 aa)	ZP_00945807 demethylmenaquinone methyltransferase (*Ralstonia solanacearum*, 221 aa)	50%
Orf11 (363 aa)	ZP_00800093 mandelate racemase (*Alkaliphilus metalliredigenes*, 363 aa)	43%
Orf12 (251aa)	ZP_00863202 hypothetical protein (*Bradyrhizobium sp.*, 244 aa)	26%
Orf13 (165 aa)	CAD85407 conserved hypothetical protein (*Nitrosomonas europaea*, 151 aa)	42%
Orf14 (260 aa)	BAB39459 bioH pimeloyl-CoA synthesis (*Kurthia sp.*, 267 aa)	35%
Orf15 (426 aa)	BAC71944 putative membrane protein (*Streptomyces avermitilis*, 489 aa)	31%
FomR (277 aa)	BAA09943 erythropoiesis stimulating activity protein (*Streptomyces thermoviolaceus*, 334 aa)	32%
PhnD (322 aa)	YP_481256 phosphonate-binding periplasmic protein (*Frankia sp.*, 275 aa)	54%
PhnC (265 aa)	YP_481257 phosphonate ABC transporter PhnC, ATP-binding (*Frankia sp.*, 253 aa)	56%
PhnE (574 aa)	ZP_00380826 ABC-type phosphonate permease (*Brevibacterium linens*, 559 aa)	48%

The NCBI accession code is given for homologous genes along with the organism and number of amino acids.

homologs are found in the two completely sequenced *Streptomyces* (*avermertilis* and *coelicolor*) or any other partially sequenced Streptomycete (excluding *wedmorensis*). At present it is unclear whether FomD is required since no disrupting insertions were obtained. Therefore, future studies will focus on using a different method to knockout this gene.

To test the hypothesis that FomR is a regulator of fosfomycin production and to determine if any of the other genes (*orf3-12* or *fomE,F*) were required, gene disrupted clones were created. Note that it was apparent from the deletion analysis that some gene(s) were required between *orf13* and *orf3* (Figure 4). Disrupting insertions into *fomE*, *fomF, orf3*, *orf5*, *orf6*, *orf8*, *orf10*, *orf12*, and *fomR* were obtained and reintroduced into *S. lividans*, and checked by bioassay for fosfomycin production. Only gene disruption of *fomR* resulted in a loss of fosfomycin production (Figure 5), while the remaining eight disruptions did not. This result clearly confirms that *fomE,F* and *orf3*, *orf5*, *orf6*, *orf8*, *orf10*, *orf12* are not essential for fosfomycin biosynthesis. Taking the deletion analysis in account, the most straightforward interpretation of this result is that *fomR* is involved in transcriptional regulation since its deletion (Figure 4) or disruption results in loss of fosfomycin production, it is not part of the central cluster, and it is homologous to DNA regulators. Although we cannot rule out that *orf4*, *orf7*, *orf9*, and *orf11* are essential, we believe this to be unlikely as these genes are clustered (Figure 4, red) with genes that are not essential according to the gene disruption studies. Therefore, we propose that the minimal gene cluster is comprised of nine Orfs including *fom3* to *fomC*, and a required regulator, *fomR* (Figure 4, yellow).

Although not required for heterologous production in *S. lividans* based on deletion analysis, it is interesting to note that genes for an ABC family transporter system are downstream of the *fom* genes (Figure 4) and share high homology with phosphonate uptake transporters (*phnDCE*) (Table 2). These Orfs were therefore labeled as *phnD*, *C*, and *E*. This transporter could be involved in scavenging intermediates or degradation products, or could be an exporter. If in fact these three genes constitute an active phosphonate transporter, to the best of our knowledge, it would be the first ABC family transporter for phosphonates discovered in a gram-positive bacterium.

Significance

To date, only three complete phosphonate biosynthetic pathways (as demonstrated by heterologous production) have been uncovered: AEP [27], bialaphos [56], and FR900098 (A. Eliot and W.W.M., unpublished data). We describe here the recombinant production of fosfomycin. The gene cluster was successfully isolated from *S. fradiae* and integrated into the chromosome of *S. lividans* 66 under the control of the natural regulatory elements. Production was verified by a variety of biochemical and physical means including ^{31}P NMR spectroscopy, high-resolution FTMS, and the observation of an enzyme-fosfomycin covalent adduct on the target enzyme by ESI-Q-TOF MS. Heterologous production indicates that the ~32 kb region of *S. fradiae* genomic DNA contains all of the genes required for biosynthesis of this compound.

Since this study successfully demonstrated heterologous production of fosfomycin, it provided the opportunity to study the biosynthetic pathway in new ways. The minimal cluster was identified by gene deletion analysis to be located in an ~23 kb region of *S. fradiae* genomic DNA containing 21 putative Orfs. Gene disruption analysis coupled with bioinformatics studies narrowed down the number of Orfs involved to 13 total Orfs including what we believe to be the minimal gene cluster *fom1-4*, *fomA-D*, and *fomR*. Thus, two

genes previously thought to be involved in biosynthesis *fomE* and *fomF*, were found not to be required and a proposed transcriptional activator (*fomR)* was discovered. Interestingly, a probable ABC family phosphonate transporter was identified and is the first recognized in gram-positive bacteria.

With the heterologous producer in hand, more detailed genetic studies of *fomC* and *fomD* will be carried out to determine their role in fosfomycin biosynthesis. Additionally, because we have shown that *fom3* and *fomC* can be functionally expressed in *S. lividans*, future studies will focus on the heterologous overexpression and characterization of these enzymes using this host. Moreover, heterologous production of fosfomycin gives knowledge of the genes required for biosynthesis and opens the door for strain and pathway engineering on the molecular level, which could in the future yield high-level overproduction strains in an amenable host such as *E. coli* or the production of novel fosfomycin variants by introducing new or mutated genes.

Experimental Procedures

Materials

Streptomyces fradiae and *S. lividans* 66 were obtained from the Agricultural Research Service Culture Collection (Peoria, IL), NRRL-3417 and NRRL-B-16148, respectively. Plasmids pJK050, pAE4, pAE5, pAE19, pCP20 *E. coli* strains WM4489, WM5923, and WM3608 were developed by the Metcalf group (A. Eliot and W.W. Metcalf, submitted). Complete sequences of plasmids and details of strain and plasmid construction can be obtained by request from the authors. Fosfomycin disodium salt was obtained from Fluka. Kanamycin, chloramphenicol, nalidixic acid, IPTG, proteinase K, lysozyme and 2-aminoethylphosphonic acid were obtained from Sigma-Aldrich (St. Louis, MO). ISP2, ISP4, YM, agar, beef extract, yeast extract, malt extract, and other reagents required for cell culture were obtained from Difco (Franklin Lakes, NJ). BP clonase was obtained from Invitrogen (Carlsbad, CA). MuA Transposase and Phusion DNA polymerase were from Finnzymes (Espoo, Finland). Buffer G and MaxPlax lambda particles from Epicentre (Madison, WI). Micro biospin gel filtration columns were obtained from Bio-Rad (Hercules, CA).

Identification of the *fom3* Gene in *S. fradiae*

A single colony of *S. fradiae* grown on ISP medium 2 agar plates was homogenized in 100 μl DMSO and 1 μl was used as template for PCR. The PCR contained Phusion DNA polymerase and its corresponding buffer and 500 nmol of the primers Fom3F1 (5′-TAC GAT CAC ATA TGA CGA TCG GTT CTC TGG-3′) and Fom3R1 (5′-TAC GAT CAA AGC TTT CAG TAC TGG TTT GCG TTC C-3′). These primers were designed based on the *fom3* sequence from *S. wedmorensis*. The PCR product was sequenced using the same primer set at the University of Illinois Biotechnology Center. The sequence had high similarity to *fom3* from *S. wedmorensis* and thus the following screening primers were designed in regions of homology: FomScreenF (5′-GGC AGC TCG GGC TTG ATC TGG-3′) and FomScreenR (5′-CAC GAC CTT CCG CGA TGT ACG TAC C-3′). These primers were checked for their efficiency to amplify the *fom3* fragment from *S. wedmorensis* and *S. fradiae*.

Construction of a Library of Genomic DNA of *S. fradiae*

Genomic DNA was prepared from *S. fradiae* grown in 700 ml glucose yeast malt (GYM) broth [40] for 72 hr at 30°C in a 2 liter baffled flask containing approximately fifty 3 mm glass beads. The cells were harvested and homogenized with a sterile ground glass tissue homogenizer and pelleted by centrifugation (2000 × g for 10 min), washed with 10 ml TE25S buffer (25 mM Tris-HCl, 25 mM EDTA, 0.3 M sucrose [pH 8]), and resuspended in 10 ml TE25S buffer, and lysozyme was added to 2 mg/ml. After incubation at 37°C for 20 min, the protoplasted cells were treated with 0.5 mg/ml proteinase K and incubated at 50°C for 30 min, and sodium dodecyl sulfate was added to 0.5% (w/v) final concentration. After 20 min at 50°C, the lysate was washed with one volume of a 25:24:1 mixture of buffer saturated phenol, chloroform, and isoamyl alcohol. After centrifugation at 22,500 × g for 45 min, the aqueous layer was removed, washed with one volume of a 24:1 mixture of chloroform and isoamyl alcohol, and centrifuged a second time. Genomic DNA was precipitated from the aqueous layer by addition of 0.1 vol (0.7 ml) of 5 M NaCl and 0.7 vol (5.5 ml) of isopropanol. The precipitated DNA was spooled onto a glass rod, washed three times with 70% EtOH and once with 100% EtOH, dried, and resuspended in 1.5 ml of TE buffer (pH 8.0). The genomic DNA was analyzed by field inversion gel electrophoresis (FIGE) and determined to be primarily DNA fragments of >200 kb.

The prepared genomic DNA was partially digested with Sau3A1 by performing serial dilutions of the enzyme in the DNA. The fraction with the highest yield of ~20–60 kb fragments as determined by FIGE was then treated with shrimp alkaline phosphatase (SAP). The fosmid vector was prepared by sequential NheI digestion, SAP treatment, and BamHI digestion. The SAP-treated genomic DNA fraction was ligated with prepared pJK050 overnight at 16°C, followed by ethanol precipitation and packaging into lambda phage using the MaxPlax packaging extract according to the manufacturer's instructions. *E. coli* WM4489 cells were transfected with the packaged library and plated on LB + 12 μg/ml chloramphenicol (Cm) agar plates. WM4489 is an *E. coli* DH10B derivative that contains the *trfA33* gene under the control of a rhamnose-inducible promoter. Individual colonies (~1500) were picked into standard 96-well plates with each well containing a single 3 mm glass bead (to aid mixing) and 200 μl LB + 12 μg/ml Cm and grown overnight with shaking at 37°C.

Library Screening

Cell culture (10 μl) from 48 clones was pooled, boiled in water (100 μl), and used as a template for PCR reactions containing 500 nmol of primers FomScreenF and FomScreenR and *Taq* DNA polymerase in 1× PCR premix G, with the annealing temperature of 55°C, extension at 72°C for 40 s, and melting temperature at 95°C—30 cycles in total. Subsequent row and column pooling allowed positive clones to be identified. Fosmid DNA was isolated from single positive clones grown overnight in 5 ml LB + 12 μg/ml Cm + 0.2% rhamnose using a Qiagen Miniprep kit. In order to add the functions necessary for transfer and integration into the genomic DNA of *S. lividans*, the purified fosmids were individually recombined in vitro with similarly purified pAE4 using a BP clonase kit according to the manufacturer's instructions. The reaction mixtures were used to transform *E. coli* WM3608 [56], and successfully recombined plasmids were selected on LB + 12 μg/ml Cm + 50 μg/ml apramycin (Apr) agar plates. These transformants were then used as donors for conjugal transfer of the fosmids to *S. lividans* 66 following the "high-throughput" protocol described previously with the exception that the entire *E. coli*/*S. lividans* mixture was spotted on R2 no-sucrose media in 2.5 μl aliquots [63]. After 16–20 hr at 30°C, plates were flooded with 2 ml of a mixture of 1 mg/ml each nalidixic acid (Nal) and Apr and incubated at 30°C for an additional 5–7 days, at which point *S. lividans* exconjugants were picked and restreaked on ISP2 + 50 μg/ml Nal + 30 μg/ml Apr + 12 μg/ml Cm and allowed to grow for several days, at which time integration was confirmed by PCR using the screening primers for Fom3.

Fosmid Junction Sequencing

Each of the fosmid constructs that yielded positive PCR products for *fom3* were sequenced from their junction points with the fosmid vector pJK050 using primers that anneal to the T7 promoter on one side of the vector or T3 promoter on the other side of the vector. The sequences were analyzed using BLASTX for any open reading frames that might help identify a fosmid that did not contain the complete cluster.

Production of Fosfomycin by *S. lividans*

Each of the *S. lividans* exconjugants was plated on HT agar plates [40] or inoculated into HT liquid media and incubated at 30°C.

Fosfomycin is known to be extractable by methanol [1]; therefore, in the case of liquid media, samples were concentrated to dryness under vacuum and extracted with methanol several times. The methanol extract was filtered and dried under vacuum resulting in a fine powder that was dissolved in water to effectively concentrate the samples between 10- and 500-fold. Several other production media in solid and liquid form were tested including HT + phosphate, ISP2, ISP2 + phosphate, S media, ISP4, and mannitol soy media [40]. Of these, only ISP2 also allowed production of fosfomycin, however, at a significantly lower level.

Bioauthentication

E. coli WM5923 has the three component *E. coli* phosphonate transporter *phnCDE* integrated into the chromosome under an IPTG inducible promoter resulting in a MIC value of ~1 μg/ml fosfomycin in the presence of IPTG (W.W.M., unpublished data). *E. coli* BL21 pET20-MurA C115D has a MIC value >20 mg/ml for fosfomycin in the presence of IPTG, due to MurA C115D being catalytically competent but unable to form a covalent adduct with fosfomycin [18]. An overnight culture of either WM5923 or BL21 pET20-*murA* C115D was diluted 10-fold in water, and 100 μl was spread on the surface of a Penicillin Assay Medium 2 plate with 25 μl 0.5 M IPTG. The plate was allowed to dry and then plugs of solid production medium or filter discs saturated with methanol extracted liquid medium from the *S. lividans* exconjugants were placed on the surface. After overnight incubation at 30°C, the plates were analyzed for inhibition zones.

Cloning and Purification of *E. coli* MurA and MurB

The *E. coli murA* gene was amplified from *E. coli* BL21 (DE3) genomic DNA using PCR with NdeI-MurA-forward (5′-CGA CAG TCC ATA TGG ATA AAT TTC GTG TTC-3′) and XhoI-MurA-reverse (5′-ATT TCA ATC TCG AGT TCG CCT TTC ACA CGC TC-3′) primers, and the resulting PCR product was digested with NdeI and XhoI and ligated with similarly prepared pET20b. The *murB* gene was amplified using HindIII-MurB-forward (5′-GAC GAT CGA AGC TTG CAT GAA CCA CTC CTT AAA ACC-3′) and XhoI-MurB-reverse (5′-CAG CAT GAC TCG AGT GAA ATT GTC TCC ACT GC-3′) primers, digested with HindIII and XhoI, and ligated into similarly prepared pET28a. These two constructs were sequenced at the University of Illinois Biotechnology Center and utilized to transform BL21 (DE3). A *murA* mutant C115A was created using a QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) following the manufacturer's instructions.

Expression of either *murA* or *murB* gene was induced by addition of IPTG to a final concentration of 0.2 mM in a 1 liter culture grown in LB broth + 50 μg/ml kanamycin (Km) at 37°C to an OD_{600} of 0.6. The culture was incubated at 20°C for an additional 16 hr, at which point the cells were harvested and lysed by addition of lysozyme, a freeze-thaw cycle, and sonication. The lysate was cleared by centrifugation, and the enzyme was purified by metal affinity chromatography using Ni-NTA agarose according to the manufacturer's instructions. The purified enzymes were concentrated and stored in 20 mM HEPES, 15% glycerol, 500 mM NaCl (pH 7.5) at a concentration of 30 mg/ml for MurA and 10 mg/ml for MurB. MurA was desalted directly before use by gel filtration using micro biospin columns.

Enzyme Assay for Fosfomycin Detection

Activity of MurA was measured indirectly utilizing MurB, by observing the decrease of absorbance at 340 nm resulting from oxidation of NADPH during the reaction (Figure 2A). All substrate and enzyme solutions were prepared in 50 mM MOPS (pH 7.5). An assay mix containing MurB (100 μg/ml), NADPH (100 μM), and PEP (150 μM) was prepared. An enzyme solution was prepared containing MurA (70 μg/ml) and UDP-*N*-acetylglucosamine (3.5 mM). The enzyme solution (15 μl) was incubated with various concentrations of authentic fosfomycin (15 μl), or 100-fold concentrated media extract from either suspected fosfomycin-producing *S. lividans* exconjugants or WT-*S. lividans* 66. After 10 min incubation at 25°C, the 30 μl MurA mixture was added to 500 μl of the assay mix and the decrease in absorbance at 340 nm was monitored by Varian CARY Bio-100 UV-Vis spectrophotometry (Palo Alto, CA). The background rate of NADPH oxidation was recorded without addition of MurA and substracted from all readings. Multiple readings were averaged and normalized to the addition of uninhibited MurA enzyme mixed only with buffer.

^{31}P NMR Detection of Fosfomycin

HT culture supernatant was extracted as described above and the extracted material was redissolved in D_2O to an effective concentration of 300-fold. ^{31}P NMR spectra were recorded on a Varian Unity 500 spectrometer with a 5 mm QUAD probe (Nalorac Cryogenics, Martinez, CA) in the Nuclear Magnetic Resonance Laboratory at the University of Illinois at Urbana-Champaign. Spectra were externally referenced to an 85% phosphoric acid standard set at 0 ppm. A total of 10,000 acquisitions were averaged and in order to verify that the observed peak was fosfomycin, 1 mM fosfomycin was added to the sample and an additional 100 acquisitions were averaged.

Fourier-Transform Mass Spectrometric Detection of Fosfomycin

Authentic fosfomycin was diluted with 1:1 methanol/water and infused into a 7 Tesla LTQ-FT mass spectrometer (Thermo Finnigan, San Jose, CA) at a flow rate of 3 μl/min in negative ion mode. The instrument was tuned to acquire high-resolution FTMS data of intact ions and lower-resolution ion trap data for MS/MS fragmentation. The standard was then diluted 10-fold in acetonitrile for on-line LC/MS using hydrophilic interaction chromatography (PolyLC, Columbia, MD) in negative ion mode with three repeating events: an intact low-resolution ion trap scan, an intact high-resolution FT scan, and a selected reaction monitoring (SRM) scan using three breakdown product ions of fosfomycin at *m/z* 63, 79, and 81. Fosfomycin eluted at 15 min during a gradient of 5% 100 mM ammonium acetate (pH 4)/95% acetonitrile to 80% 100 mM ammonium acetate (pH 4)/20% acetonitrile over 20 min. The same LC/MS run was used for the concentrated culture supernatant (10 μl), first diluted 10-fold with water before being diluted 10-fold again with acetonitrile/methanol (9:1).

Mass Spectrometric Detection of MurA-Fosfomycin Adduct

Samples of 100-fold concentrated media extract prepared as described above for 45-*S. lividans* and WT-*S. lividans* were incubated with 100 μM MurA in the presence of 1 mM UDP-*N*-acetylglucosamine, respectively. After 1 hr at 25°C, small molecules were removed from the samples by gel filtration on micro biospin columns. Samples were submitted to the University of Illinois Mass Spectrometry Laboratory where they were desalted by C-4 zip-tip and analyzed by ESI-Q-TOF in positive ion mode with 0.1% formic acid.

Sequencing of Fosmid 45

A library of transposon insertions was generated in an in vitro reaction of the gel purified BglII-fragment of pAE5 that contained a Km selectable transposon, the pAE4-retrofitted fosmid 45, and MuA transposase according to the manufacturer's instructions. *E. coli* WM4489 was transformed with the reaction products, and successful insertions were selected on LB + 25 μg/ml Km. Colonies were picked into library plates containing 1.5 ml LB + 25 μg/ml Apr + 25 μg/ml Km + 0.2% rhamnose and grown overnight with shaking at 37°C. Fosmid purification and sequencing was carried out on 192 clones at the University of Illinois Biotechnology Center using TnFor (5′-CTT TCT AGA GAA TAG GAA CTT CGG G-3′) and TnRev (5′-GAC GAG TTC TTC TGA GCG G-3′) primers that anneal within the transposon. The resulting 384 sequence reads were assembled into contiguous fragments using Sequencher (Gene Codes Corp., Ann Arbor, MI). Remaining gaps were filled in by obtaining further sequence using specifically designed primers. Potential open reading frames were identified using BLAST analysis (http://www.ncbi.nlm.nih.gov) and visual inspection.

Deletion and Gene Disruption Analysis

To determine the boundaries of the fosfomycin cluster, various deletions were made by recombination between either the FRT or *loxP* site of the fosmid backbone and the matching site on the transposon. In each case, cells of *E. coli* WM4489 containing a transposon insertion product (selected with 25 μg/ml Km and 12 μg/ml Cm) were made electrocompetent and transformed with either pCP20 (expressing FLP recombinase under control of a temperature sensitive promoter) [64] or pAE19 (expressing Cre recombinase under control of the same promoter). Transformants were selected on LB + 100 μg/ml ampicillin (Amp) at 30°C, and subsequent single colonies

were restreaked on LB + 12 μg/ml Cm + 30 μg/ml Apr plates and grown at 42°C. The resulting colonies were again restreaked on LB + 12 μg/ml Cm + 30 μg/ml Apr plates, as well as LB + 50 μg/ml Km and LB + 100 μg/ml Amp plates and grown at 37°C to check for sensitivity. Correctly deleted clones did not grow on Km or Amp, indicating the Km marker had been deleted and the clone was cured of the recombinase encoding plasmid. For gene disruption experiments, the disruptions consisted of the randomly inserted Km selectable transposon utilized for sequencing. Fosmid containing clones of *E. coli* WM4489 with the transposon disrupting the desired gene were chosen with attention to location and polarity based on the sequencing reads obtained for each clone. For both disruption and deletion analysis, fosmid DNA was isolated from these strains, checked for correct deletion or disruption by BamHI restriction analysis, and integrated into *S. lividans* 66 using the previously described protocol. Exconjugants were assayed for the ability to produce fosfomycin on HT agar plates by the aforementioned bioassay with WM5923 as the indicating strain.

Supplemental Data

Supplemental Data include one table and are available at http://www.chembiol.com/cgi/content/full/13/11/1171/DC1/.

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