Supplementary Material (ESI) for Chemical Communications

New Insight into the Mechanism of Methyl Transfer during the Biosynthesis of Fosfomycin

Supporting Information

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MATERIALS AND METHODS

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, *E. coli* strains WM4489 and WM3608 were obtained from the Metcalf group (UIUC). All chemicals were obtained from Sigma-Aldrich unless otherwise indicated. Fosfomycin disodium salt was obtained from Fluka. Proteinase K and lysozyme were obtained Sigma-Aldrich. ISP2, ISP4, YM, agar, beef extract, yeast extract, malt extract and other reagents required for cell culture were obtained from Difco. MuA Transposase and Phusion polymerase from Finnzymes. Buffer G was obtained from Epicentre. Micro biospin gel filtration columns were obtained from Bio-Rad.

Creating a library of transposon insertions. A library of transposon insertions was generated in an *in vitro* reaction of the gel purified BglII-fragment of pAE5 that contained a kanamycin (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. WM4489 is an *E. coli* DH10B derivative that contains the *trfA33* gene under the control of a rhamnose-inducible promoter. Colonies were picked into library plates containing 1.5 mL LB + 25 μg/ml apromycin (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 at Urbana-Champaign 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).

Gene disruption. The disruptions consisted of the selectable transposon utilized for sequencing. Fosmid containing clones of 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. Fosmid DNA was isolated from these strains, checked for expected BamHI restriction pattern and used to transform *E. coli* WM3608.[1] These transformants were then used as donors

for conjugal transfer of the fosmids to *S. lividans* 66 following the "high-throughput" protocol described previously[2] with the exception that the entire *E. coli*/*S. lividans* mixture was spotted on R2 no sucrose media in 2.5 µL aliquots. After 16-20 h 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.

Bioassay for fosfomycin production by *S. lividans* and chemical complementation. *S. lividans* exconjugants were grown on HT agar[3] at 30 °C for 2-3 days and assayed for the ability to produce fosfomycin. For chemical complementation bioassays, the exconjugants were grown on HT agar containing 1 mM 2-aminoethylphosphonate or 1 mM 2-hydroxyethylphosphonate. Two strains were used for bioauthentication. *E. coli* WM5923 is very sensitive to fosfomycin (MIC~1 µg/ml) due to the native *E. coli PhnCDE* phosphonate transporter integrated into the chromosome under an IPTG inducible promoter. *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.[4] An overnight culture of WM5923 or BL21 pET20-MurA C115D was diluted 10-fold in water and 50 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 *S. lividans* on HT agar were placed on the surface. After overnight incubation at 30 °C the plates were analyzed for inhibition zones.

Synthesis of 2-hydroxyethylphosphonate. To a solution of dibenzyl phosphite (0.524 g) in DMF (20 mL) was added cesium carbonate (1.96 g) and tetrabutylammonium iodide (TBAI) (2.42 g). The suspension was stirred at room temperature for 1 h, followed by addition of benzyl 2-bromoethyl ether (216 mg). The mixture was stirred at room temperature for two days. The resulting white suspension was diluted with water (30 mL) and extracted with ethyl acetate (3×30 mL). The organic layer was washed with water (3 × 30 mL) and brine (30 mL). The crude product was purified by silica gel chromatography eluting with hexane/EtOAc (1:2) to give a colorless liquid (326 mg, 83%). To this product (103 mg) was added Pd/C (20 mg) in methanol (5 mL). The suspension was stirred at room temperature under hydrogen atmosphere overnight. The resulting suspension was filtered, and the filtrate was concentrated to give a colorless liquid (31 mg, 97%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.60 (dt, J = 12.4, 7.2 Hz, 2H), 1.85 (dt, J = 18.0, 7.2 Hz, 2H). ^{31}P NMR (162 MHz, $CDCl_3$) δ 27.7.

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