"To cross-seed or not to cross-seed": a pilot study using metallo-β-lactamases as drug targets for antibiotic resistance.

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ABSTRACT.

Knowledge of protein structures is of central importance in modern drug discovery and molecular biology, but to be useful the structures, including those obtained in the crystalline state, must be biologically relevant. Small variations in crystallisation conditions can lead to alternative crystal forms, conformations and oligomerization states, causing changes which can lead to altered fold and active site architectures. In the determination of protein structures by X-ray crystallography, crystallisation is an essential prerequisite and remains a major bottleneck in the drug discovery. Although many methods have been tried in an attempt to improve the production of protein crystals, it is still largely a 'trial and error' process. To our knowledge, crystallisation by cross-seeding using homologous proteins has previously only been successful for proteins with greater than 61-74% sequence identity. In the study presented here, we explore the effect of low sequence similarity, but fold homology, ??? on cross seeding using metallo-β-lactamases with sequence identities as low as 19% and sequence similarity of only 36%, byt wth homologous core folds. Despite the low sequence identities, the results show that Micro-Cross-Seeding Matrix Screening (MCMS) can increase the number of hits obtained, and shorten crystallization time. It can also help in the identification of new crystallization conditions and different crystal forms.

1. Introduction

Protein structures inform on all aspects of molecular biology and are pivotal to the success of rational drug design. X-ray crystallography is currently the most effective approach to their solution at the atomic level. Determining the structures of targets and/or related proteins is often highly desirable in drug discovery programmes (Caliandro *et al.*, 2013). The information so gained is useful in structure and fragment based drug design, as well as for protein engineering and vaccine design. For the purpose of drug discovery, it is desirable that crystals are reproducibly obtained and that they routinely diffract X-rays to a resolution better than 2.5 Å (Davies *et al.*, 2012). Furthermore, crystals must be robust enough to survive soaking with known and putative ligands. It is also desirable to explore several conformations in order to ensure knowledge of 'biologically relevant' conformations and that the sites suitable for drug binding are not occluded by protein-protein crystal contacts (Davies *et al.*, 2012) which might prevent successful ligand soaking experiments. Crystallisation has also emerged as a large-scale process both for the direct recovery of proteins from fermentation broths, cross-linked enzyme crystals are used as industrial biocatalysts (Lee *et al.*, 2000, Ali *et al.*, 2013, Bush, 2013). All these applications require reproducible crystallisation conditions which result in high quality crystals with minimal loss of functional activity. However, obtaining highly-ordered diffraction-quality crystals remains a major bottleneck in X-ray structure determination. Failure to produce crystals that diffract to sufficient resolution is a major obstacle towards solving protein structures by X-ray crystallography. It has been estimated that greater than 60% of the overall cost of structure determination efforts is attributable to failed attempts (Slabinski *et al.*, 2007).

Despite increased interest in research into the systematics of protein crystallisation (crystallogenesis), determining suitable crystallisation conditions for a particular protein is still

substantially an empirical process (Giegé, 2013). Most biomacromolecules are very sensitive to their chemical environment. Changes in pH, salt concentration, temperature, and other parameters can induce significant changes in macromolecular conformation and hydration (McPherson, 1990). As a result, macromolecular crystals can often be sensitive to changes in conditions during their growth (Bush, 2013). There is usually an extremely limited volume of biomaterial available to screen all the desired possible combinations of potential crystallisation conditions (Lee *et al.*, 2000, Cudney *et al.*, 1994). Furthermore, for the purpose of drug discovery where the aim is normally to co-crystallise with a ligand, protein degradation as well as chemical changes of the ligand upon prolonged incubation can present major obstacles to success (Stewart *et al.*, 2002, Blundell *et al.*, 2004).

To overcome the challenge of crystal formation, various protein crystal growing strategies have been explored to increase the success rate (Landsberg *et al.*, 2006). Working with readily crystallisable homologous proteins as models, surface engineering of proteins as well as changes in construct length and the presence/absence of tags can improve the probability of obtaining crystals and also their quality, by altering crystal-packing interactions (Roos *et al.*, 2006, Qiu *et al.*, 2009). However, whilst a crystal structure of a variant can provide answers to questions posed in the particular study, in some cases structural knowledge of the wild type protein is vital for the correct interpretation of the biochemical and biophysical characteristics (Roos *et al.*, 2006, Abuhammad *et al.*, 2013). Small changes in sequence, although possibly allowing the protein to exhibit distinct crystallization behaviour, can result in differences in conformation as well as in active site geometry and function (Wei *et al.*, 2007).

In the crystallization of purified biomolecules, two critical steps determine the quality of the final crystal: the nucleation of an initial seed and its enlargement. To produce well-ordered single

crystals, the growth rate must simultaneously be slow enough to avoid excessive defect formation, yet fast enough to ensure significant crystal growth before undesirable competing additional nuclei form. The contradiction inherent in meeting the requirements of optimal nucleation and growth severely limits the efficiency of most crystal growth protocols, and has been proposed as a root cause of unsuccessful crystallization attempts (Lee *et al.*, 2000).

One way to increase the chances of finding the right crystallisation condition is via decoupling of the crystal growth step from the nucleation step in order to grow large regular crystals (Bergfors, 2003). This can sometimes be achieved using the seeding technique, which is a powerful tool for biomolecular crystallization that is based on knowledge of the phase diagram and permits the decoupling of crystal nucleation and growth (Bancel *et al.*, 1998, Bergfors, 2003, Mac Sweeney *et al.*, 2009). Heterogeneous nucleation has been investigated for crystal growth on surfaces, including hydrophobic glass, mineral surfaces and many other materials (Chayen *et al.*, 2001, Govada *et al.*, 2016, Ino *et al.*, 2011, Chayen *et al.*, 2014).

Micro-seeding, which involves the introduction of microscopic crystal fragments into a prepared protein solution, has been useful for obtaining diffraction-quality crystals and has enabled the determination of many structures (e.g.(Mac Sweeney *et al.*, 2009, Bergfors, 2003, Abuhammad *et al.*, 2013, Guddat, 1994, Bourne, 1999, Fong, 2004, Christine *et al.*, 2008). These successes have led to the emergence of an automated microseeding matrix screening (MMS) approach, in which seeds are systematically transferred into new conditions to promote crystal growth (D'Arcy *et al.*, 2007, Ireton *et al.*, 2004, Shaw, 2011).

Micro-cross-seeding using heterogeneous protein nucleants has been utilised successfully to facilitate the crystallization of structurally of fold`??? related proteins. These include variants of the same protein (Roos *et al.*, 2006, Sanishvili *et al.*, 1994), recombinantly modified protein with

multi-histamine ?histidine?? tag (Luang *et al.*, 2010), selenomethionyl-substituted proteins (Bottomley, 1994, Vassylyeva, 2006, Hattori, 2007), macromolecular complexes ligands (Oswald *et al.*, 2008), different monoclonal antibody fragments (Shan, 1993, Obmolova, 2010), and homologous proteins from different organisms (Vijay-Kumar *et al.*, 1987, Abuhammad *et al.*, 2013, Sanchez-Weatherby, 2006).

We have previously obtained encouraging initial results for a hitherto unexplored crystallization method with arylamine N-acetyltransferase from *M. tuberculosis* (TBNAT). Despite prolonged and wide-ranging trials to crystallize TBNAT, an anti-tubercular drug target, no crystals were obtained. In an alternative approach, cross-seeding of TBNAT protein with micro-crystalline seeds from a homologous NAT from *M. marinum* (MMNAT) (74 % sequence identity) surprisingly resulted in a single 20 µm sized TBNAT crystal that diffracted to 2.1 Å and enabled determination of the TBNAT structure (Abuhammad et al., 2013).

Here we report a systematic study to probe the minimum sequence identity required for such successful cross-seeding, using a set of metallo-β-lactamases (MBLs), some of which had thus far proved recalcitrant to all previous crystallisation attempts.

β-Lactamases are a large structurally heterogeneous family of bacterial enzymes catalysing the cleavage of β-lactam antibiotics, which represent ~60% of all antibiotics in clinical use. The production of β-lactamases in Gram-negative bacteria is the most important mechanism of resistance to antibiotics, a phenomenon which represents a major public health issue (Bush, 2013).

In mechanistic and structural terms, β-lactamases can be divided into the serine β-lactamases (SBL) and the metallo-β-lactamases (Bush *et al.*, 2010). MBLs are a distinct group of β-lactamases that utilise zinc ions in their active site for hydrolytic activity (Meini *et al.*, 2015, Cornaglia *et al.*, 2011). MBLs are divided into three subfamilies (B1-3), with the B1 enzymes being the most clinically relevant (Bebrone, 2007, Galleni *et al.*, 2001), and they are not inactivated by any of the currently available SBL inhibitors (Walsh *et al.*, 2005). Further, due to variations in MBL structures, including with respect to mobile loops surrounding the active site, a major challenge in their inhibition is the development of compounds with the breadth of selectivity necessary for clinical utility (Brem *et al.*, 2015). This challenge is further exacerbated by the emergence of MBL variants which can have altered inhibitor profiles (Liénard *et al.*, 2008, Ghavami *et al.*, 2015, Brem *et al.*, 2016, Fast *et al.*, 2013). The São Paulo MBL (SPM-1) was first identified in *Pseudomonas aeruginosa* and has spread to enable widespread resistance in Brazil and elsewhere(Toleman *et al.*, 2002) SPM-1 is a current medicinal chemistry target and is of structural interest because has features from different MBL subfamilies. SPM-1 shares moderate sequence similarity (~30%) with MBLs from both the B1 and B2 MBL-subclasses (Murphy *et al.*, 2003, Murphy *et al.*, 2006, Brem *et al.*, 2015). Although overall SPM-1 is closest to the class B1 MBLs, its unusual structural properties, especially loops around its active site which resemble those of B2 MBLs, distinguish it from other B1 enzymes of equivalent medical relevance (Brem *et al.*, 2015).

Interest in structural studies of MBLs has escalated recently due to their increased prevalence which has promoted increased interest in inhibitor design for them. To aid the medicinal chemistry efforts of MBLs inhibitors, a constant supply of crystals is required to determine the 3D structure of ligand-protein complexes.

Some MBLs, such as BcII (from *Bacillus cereus*) and VIM-2 (from *Pseudomonas aeruginosa*) are readily crystallisable and the structures of several protein-ligand complexes have been determined for them, and are available in the Protein Data Bank (PDB) (www.rcsb.org) (Lauretti *et al.*, 1999, Murphy *et al.*, 2006, Brem *et al.*, 2015). One report has suggested that cross-seeding MBLs (i.e. streak seeding CphA and Imp-1) may be achievable (Sharma *et al.*, 2005). However, several MBLs and their variants have been recalcitrant to crystallisation attempts. We have identified a set of MBLs for which crystallisation conditions either have never been established or conditions are available but irreproducible and/or impractical (e.g. very long crystallisation times). To try to address this problem, we have investigated micro-cross-seeding by applying the MMS approach to test the impact of low sequence identity between the seed protein and the target protein on identifying efficient reproducible crystallisation conditions. To the best of our knowledge, this is the first systematic study on micro-cross-seeding using homologous proteins.

2. Materials and Methods

All chemicals and reagents were purchased from Sigma Aldrich (Poole, Dorset, UK), unless otherwise stated.

Protein Expression and Purification

All MBLs utilised in this study had been previously successfully produced and purified in recombinant form. IMP-1, SPM-1 and NDM-1 Δ42, and were prepared and purified as described previously (van Berkel *et al.*, 2013). Almost identical procedures were applied to both B2-IMP-1 and SPM-1-L2 (details will be published elsewhere). VIM-1 and VIM-2 were produced and purified as described previously (Makena *et al.*, 2013). The proteins used in this study are summarized in Table 1.

Size-Exclusion Chromatography and Multi-Angle Light Scattering (SEC-MALS) for Determination of the Oligomeric State

Size exclusion measurements were carried out for NDM-1 and NDM-1-Δ42 using a Superdex 200 10/300 GL column (GE Healthcare) equilibrated in 25 mM Tris-HCl, pH 7.5, and 150 mM NaCl at 0.4 mL/min on an AKTA system (Pharmacia). The column was followed in-line by a Dawn Heleos-II light scattering detector (Wyatt Technologies) and an Optilab-rex refractive index monitor (Wyatt Technologies). Molecular mass calculations were performed using ASTRA 5.3.4.14 (Wyatt Technologies) assuming a refractive index increment (*dn/dc* value) of 0.186 mL/g.

Crystallisation

Protein crystallisation was carried out using the sitting-drop vapour-diffusion technique. Sitting drops were set up in 96-well plates (SwissCI) containing commercially available sparse matrix and systematic grid screen conditions as detailed below. The proteins were subjected to a pre-crystallization test (PCT, Hampton) to check that their concentrations were suitable prior to committing the bulk of the sample to a full crystallization screen.

Optimisation of the Crystallisation Conditions

The seed crystals were obtained as described previously (Brem *et al.*, 2016, Green *et al.*, 2011, Brem *et al.*, 2015). The BcII, VIM-2, NDM-Δ42 starter crystals were freshly grown at 293 K by the vapour diffusion method using established conditions (Table 1) (Brem *et al.*, 2016, Green *et al.*, 2011). Optimisation of the conditions (± 10% on concentrations of the precipitant) was carried out in 24 well plates using 4 μL drops containing 2 μL protein and 2 μL crystallisation liquor (Figure S1 in Supplementary Information). SPM-1 crystals were obtained in a condition

containing 0.2 M potassium nitrate and 20% PEG 3350. These crystals were grown at 277 K over a period of at least 6 months and were of poor diffraction quality (~8 Å as tested on an in-house FRE+ SuperBright X-ray generator with Osmic HF optics and a Saturn 944+ CCD detector, Rigaku). Hence we refer to these as old crystals.

Seed Stock Preparation

The seed stocks were propagated as described by Shaw Stewart *et al.* (2011). In summary, the starter crystals in each crystallisation drop were thoroughly crushed *in-situ* in the crystallisation plate using a glass probe with a 0.25 mm glass bead melted on the end. For each, 2 µL of the reservoir solution was added and mixed with the entire contents of the crystal containing subwell, by withdrawing and dispensing the drop from the pipette tip several times. For each drop, the mixture was then transferred to a single Eppendorf tube containing one Seed Bead (Hampton Research) and 20 µL of reservoir solution. The mixture was transferred back and forth between the Eppendorf tube and the drop subwell several times to ensure that the majority of crystalline material had been transferred from the drop subwell to the tube. The tube contents were vortexed for 2 min and then kept on ice. The plastic bead was removed, and the seed stock was used immediately without further dilution. All seed stocks were stored at -80 Cº.

Micro-Cross-Seeding Matrix Screening

All proteins were prepared in 50 mM HEPES and 150 mM NaCl pH 7.5 at the concentrations shown in Table 1. Micro-cross-seeding matrix screening (MCMS) was carried out using a Mosquito liquid handler (TTP Labtech). Vapor diffusion sitting-drop experiments were dispensed to 96-well three-subwell plates (SwissCI).

For the "first tier" of test, crystallisation drops had a total volume of 0.2 μL. For one set of controld drops (control-1) drops, those without seed stock added volumes consisted of 0.1 μL protein plus 0.1 μL precipitant condition. For drops with added seed stock, volumes were 0.08 μL protein, 0.1 μL screen and 0.02 μL seed stock. The initial screens were carried out using JCSG + sparse matrix screen (Newman *et al.*, 2005).

Duplicate plates were set up for each protein with the addition of the seed stocks produced (Section 2.3.2). In a second set of control drops (control-2), the seed solution was replaced with seed-reservior solution; the drop thus consisted of each protein solution, precipitant and seed-reservior solution. All the plates were incubated at 293 K and inspected daily for crystal growth.

Where protein crystals appeared from the first tier tests of MCMS, these crystals were used to produce "second tier" seed stocks. These second tier seed stocks were used for: a) micro-seeding optimisation carried out by varying the precipitant concentrations of each first tier hit component by ± 10% (optimisation was carried out as described in 2.3.1; Figure S2 in Supplementary Information), and b) MCMS using the JCSG + screen (Table 2). The crystallisation strategy used in this study is summarized in Figure 1.

Protein crystals were identified using either a UV microscope (Membrane Protein Laboratory, Diamond Light Source, UK) or by testing for diffraction.

Crystal Packing Analysis

Sequence alignments were carried out using the Fold and Function Assignment System (FFAS) Server (Jaroszewski *et al.*, 2005). The root mean square deviations of C-atoms (RMSD) between the structures were obtained using the jFATCAT_flexible application on the PDB website (Prlić *et al.*, 2010). Interface analysis was carried out using PISA (Krissinel *et al.*, 2007)

(http://www.ebi.ac.uk/msd-srv/prot_int/pistart.html) and PCalign (Cheng *et al.*, 2015). Structural figures and graphical renderings were made with either PYMOL (Schrodinger, 2010) or Discovery Studio Visualizer 3.5 (Accelrys, 2012).

3. Results

The MBLs used in this study were categorised into two subgroups. The first was the "seed" subset, and it encompassed proteins for which reliably reproducible crystallisation conditions had previously been identified (BcII, VIM-2, NDM-1Δ42 and SPM-1)**refs**. The second "target" subset consisted of proteins for which crystallisation conditions were either not yet available (i.e. recalcitrant to all previous crystallisation attempts: VIM-1, B2-IMP-1, SPM-1-L2 (H108N) and NDM-1) or which had suboptimal crystallisation conditions (IMP-1, SPM-1 and NDM-1Δ42) and which for a variety of reasons were not ideal for medicinal chemistry applications (i.e. irreproducible and/or impractical). The latter set of crystals were of poor resolution, grew with an infrequent success rate, or needed a long duration to appear, as shown in Table 2. The table also shows a comparison of the sequence identity between seed proteins and target proteins.

Of the seven MBL protein constructs used in this study, four (SPM-1, SPM-1-L2, IMP-1, B2-IMP-1) were crystallised successfully by the application of MCMS. The crystals of two of these enzymes (SPM-1-L2 and IMP-1) were of suitable diffraction quality for structure determination, whilst the diffraction qualities of B2-IMP-1 and SPM-1 were not as good. However, NDM-1, NDM1-Δ42 and VIM-1 failed to crystallise in a useful form using our MCMS method.

Protein Preparation for Crystallisation

All proteins were subjected to the PCT Pre-Crystallization Test (Hampton Research) to identify reasonable concentrations for crystallisation, as shown in Table 1.

The MBLs used as the seed subset had been previously crystallised in our laboratory**refs** (Table 1). Optimisation of these conditions was carried out for all seed crystals, except SPM-1, prior to seeding. This step was included to ensure that sufficient quantities of the seed crystals

were available in isomorphous forms and with relatively homogenous size distributions (Figure 2). “Old” SPM-1 crystals were available from previous efforts to crystallise the protein.

First Tier MCMS

First tier screening using the JCSG+ sparse matrix screen generated hits within one week for four of the target MBLs:, IMP-1 and SPM-1, and variants B2-IMP-1 (IMP1 with extra residues GP on the N-terminal) and SPM-1-L2 (H108N) (Table 2, Table S1 in Supplementary Information, Figure 3 and 4). However, none of these crystals were of diffraction quality. Addition of seed stocks from BcII, VIM-2 and SPM-1 all resulted in SPM-1 crystals, which grew in condition JCSG+/C11 (0.1 M sodium acetate, pH 4.6, 2.0 M ammonium sulphate) (Figure 4). Self-seeding with a seed stock derived from the old SPM-1 crystals resulted in the identification of a new condition for SPM-1: JCSG+/G8 (0.15 M DL-malic acid, 20% w/v PEG 3350). Both of these conditions are different from the previously identified condition from which the seeds were obtained. The crystals of SPM-1-L2 (H108N) variant were produced using BcII and NDM-1-Δ42 seeds from JCSG+/C11 (0.1 M sodium acetate, pH 4.6, 2.0 M ammonium sulfate) (Figure 4).

Interestingly, several seed stocks led to the crystallisation of IMP-1 and its single residue variant B2-IMP-1. Crystals of IMP-1were obtained in condition JCSG+/G9 (0.1 M potassium thiocyanate, and 30% w/v PEG 2000 MME) using the SPM-1 seed stock (Figure 4). However, both the BcII and NDM-1-Δ42 seed stocks produced crystals of B2-IMP-1 in two different conditions: for BcII JCSG+/G10 (0.15 M potassium bromide, 30 % w/v PEG 2K MME) (Figure 4) and JCSG+/E2 (0.2 M sodium chloride, 0.1 M sodium cacodylate, pH 6.5, 2.0 M ammonium sulphate), respectively. It is worth noting that around 20 to 40 % of the conditions contained salt crystals as determined by UV and/ or X-ray analysis. Crystals of SPM-1 and NDM-1 grew in the absence of seeding (but with the addition of the seed stock reservoir

condition). None of the crystals mentioned above showed diffraction suitable for structure determination.

Optimisation of the Initial Hits

Optimisation of condition JCSG+/C11 for both SPM-1 and SPM-1-L2 was carried out as described in Methods and on average resulted in crystals in 25% of the wells (Table 3, Tables S2 and S3 in Supplementary Information). However, crystals were obtained to the same extent regardless of whether either seeds stock or reservoir buffer (without seeds) were added to the crystallisation drops. Optimisation of condition JCSG+/G8 resulted in no hits. None of the crystals so obtained were of diffraction quality (highest resolution diffraction was only 3 Å).

Optimisation was carried out for the conditions shown in Table 3 together with the addition of seed various seeds that had proven successful in the first tier tests. For the IMP-1 JCSG+/G9 crystallisation condition, the self-seeding case generated crystals in 96% of the wells for (Table 3 and Table S4 in Supplementary Information), whilst the addition of VIM-2 seed stock resulted in crystals in 63% of the wells. Only 25 % of the wells were observed to produce crystals when the crystallisation was carried out using the buffer condition without seeds. Diffraction quality crystals were obtained and data were collected to 1.3 Å at I04-1, Diamond Light Source. Since optimal conditions were identified for the wild type IMP-1, further optimisation of the crystallisation conditions for its mutant, B2-IMP-1 was not carried out.

Second Tier MCMS

In the second tier of MCMS screening which used seed stocks from hits from the first tier of MCMS, a new crystallisation condition was obtained for SPM-1 upon seeding with VIM-2: JCSG+/E8 (0.1 M sodium acetate, pH 4.5, 1.0 M di-ammonium hydrogen phosphate). In

addition, condition JCSG+/C11 produced SPM-1 crystals using VIM-2 seed stock, and similar results were obtained using the VIM-2 reservoir buffer without seeds. Self-seeding SPM-1 with either old or fresh SPM-1 seed stock resulted in crystals in condition JCSG+/C11. However, none of these crystals diffracted to a resolution higher than 4 Å. Crystals of SPM-1-L2 were observed in new conditions, including JCSG+/A5 (0.2 M magnesium formate, 20% w/v PEG 3350) and JCSG+/A10 (0.2 M potassium formate, 20% w/v PEG 3350) using SPM-1 seed stock. An SPM-1-L2 crystal from condition JCSG+/A5 diffracted to 2.6 Å resolution and was used to obtain a structure of this enzyme (PDB code XXXX, details to be published elsewhere). Self-seeding SPM-1-L2 using seeds obtained from condition JCSG+/C11 seeded with BcII, resulted in crystals in JCSG+/D11 (0.14 M calcium chloride, 0.07 M sodium acetate, pH 4.6, 14% v/v 2-propanol, 30% v/v glycerol). The addition of reservoir buffer alone without seeds to the crystallisation drop in the place of seed stock also resulted in crystal growth in control wells. Several new crystallisation conditions using the JCSG+ sparse matrix screen for IMP-1 were identified by self-seeding, including: JCSG+/H3 (0.1 M Bis Tris, pH 5.5, 25% w/v PEG 3350/G4/B4), JCSG+/H8 (0.2 M sodium chloride, 0.1 M Bis Tris, pH 5.5, 25% w/v PEG 3350), JCSG+/A10 (0.2 M potassium formate, 20% w/v PEG 3350) and JCSG+/A12 (0.2 M potassium nitrate, 20% w/v PEG 3350). However, seeding of IMP-1 with the SPM-1 seed stock resulted in IMP-1 crystals in condition JCSG+/G2 (0.02 M magnesium chloride, 0.1 M HEPES, pH 7.5, 22% w/v polyacrylic acid 5100 sodium salt). No IMP-1 crystals were observed in the absence of seed stocks in all these new conditions.

Interestingly, the addition of only reservoir buffer without seeds from the BcII seed stock to the B2-IMP-1 crystallisation drops led to crystals in JCSG+/D11 (0.14 M calcium chloride, 0.07 M sodium acetate, pH 4.6, 14% v/v 2-propanol/ 30 % v/v glycerol) and JCSG+/E11 (0.16 M

calcium acetate, 0.08 M sodium cacodylate, pH 6.5, 14.4 % w/v PEG 8K/ 20 % v/v glycerol). On the other hand, self-seeding of B2-IMP-1 resulted in crystals for conditions JCSG+/H7 (0.2 M ammonium sulfate, 0.1 M bis tris, pH 5.5, 25 % w/v PEG 3350), and JCSG+/H9 (0.2 M lithium sulfate, 0.1 M bis tris, pH 5.5, 25 % w/v PEG 3350), in addition to conditions JCSG+/C11 and JCSG+/G10. The presence of BcII or B2-IMP-1 seeds appeared necessary for B2-IMP-1 crystal growth. Table S5 in Supplementary Information summaries these results.

SEC-MALS Analysis

The hydrodynamic properties of proteins (a measure of their homogeneity) have an extremely strong influence on the success of crystallization (Price *et al.*, 2009). The oligomeric states of two of the MBLs: NDM-1 and NDM-1-Δ42 were investigated by SEC-MALS. NDM-1, which failed to crystallise in the first tier MCMS showed micro-heterogeneity, with a tetramer being observed in addition to the main dimer peak (Figure S3 in Supplementary Information). By contrast, the N-terminally truncated NDM-1-Δ42, which was used as seed in this study showed high micro-homogeneity (Figure S3 in Supplementary Information). The observed polydispersity of NDM-1 is likely to hamper crystallisation. Further characterization of the other MBLs used in this study (NAME IF POSSIBLE) will be published separately.

Comparison of Seed Stock Crystal Packing to Target Protein Crystal Packing

Sequence and 3D RMSD comparisons between the proteins used as seed stocks and the crystallisation target proteins, both in terms of identity and similarity, were carried out using the jFATCAT_flexible application on the PDB website (Prlić *et al.*, 2010) (Table 2). Surprisingly, seed stocks of homologous proteins with sequence identity as low as 33-19% (name??), and sequence similarity as low as 36-45% resulted in successful crystallisation. Seven of the eight

successful cross-micorseeding conditions resulted from seed stock proteins with less than 33% sequence identity to the crystallised target proteins. Using NDM-1-Δ42 (19% sequence identity) seed stock enhanced SPM-1-L2 crystallisation. However, all proteins share a similar overall fold as indicated by the RMSD values despite their low sequence similarity (Table 2). It may be inferred from these results that, at least under some conditions, high sequence similarity/identity is not essential for successful cross seeding with proteins with a similar fold. Regarding the small subset of MBLs used in this study, it is likely that the 3D similarities between the seed proteins and the crystallisation target proteins have contributed to these observations. However, further studies using proteins with lower 3D structure similarity are required to validate this proposal.

Hydrogen bonds and salt bridges play an important role in crystal packing interactions (Xu *et al.*, 1997, Abuhammad *et al.*, 2013). Therefore, involvement of similar interfaces may be important for successful cross-micro-seeding, given the limited number of degrees of translational and rotational freedom (only three of each) available to the fully folded globular proteins to achieve their most favourable packing configuration in a crystal (Xu *et al.*, 1997, Norel *et al.*, 1994).

Taking our observations thus far into account, we carried out crystal packing analysis of the structures of MBLs used as seeds in this study (Table 2). This analysis was aided by the PISA web server (http://www.ebi.ac.uk/msd-srv/prot_int/cgi-bin/piserver) (Krissinel *et al.*, 2007) which enabled the identification of crystal packing interfaces. PISA gives a Complexation Significance Score (CSS), which is a measure of the significance that the interface plays in the formation of assemblies. For the MBL structures analysed here, ligands in particular zinc ions, contribute to binding in the analysed MBL structures more than do protein-protein interfaces (see Figure 5 for CSS results). Crystal packing could be affected by the introduction of various additives to the crystallisation cocktails, since if they bind they can modify the surface properties

of the protein, and thus mediate interactions. A summary of the crystal packing interface analyses including the CSSs, number of interfaces, interface surface areas, and the hydrogen bonds and salt bridges within the interfaces are given in Figure 5.

The SPM-1 seed crystals have a large number of intermolecular interfaces that have the highest surface area of the MBLs used as seeds in this study. In these SPM-1 crystals, only 25% of crystal packing is protein mediated, whilst 42% and 33% are mediated by Zn ions (both active site and non-active site ions) and other crystallisation components (i.e. Cl^-, glycerol, Co^{2+}, Ni^{2+}), respectively. These crystals only succeeded as self-seeds and in seeding IMP-1. These observations suggest that high surface area of intermolecular interfaces has no impact on the success of the seeding process.

The BcII seed stock resulted in crystals for three different MBLs (Figure 4 and Table 2). Crystal packing interfaces within BcII seed crystals mainly consist of protein interfaces (46%; Figure 5B), which are well maintained by hydrogen bonds and only a few salt bridges.

This might in part explain why BcII is better for cross-seeding compared to SPM-1, in which the interfaces are well maintained by both hydrogen bonds and salt bridges. When buried in the protein interface, an isolated charge not involved in a salt bridge could substantially destabilize the association owing to desolvation effects. Therefore, a change in residues among different MBL strains in these regions may well have a substantial impact on the forces that dominate potential crystal contacts, since this is known to affect crystallisation success (Goldschmidt *et al.*, 2014).

NDM-1-Δ42 produced crystals when seeded with B2-IMP-1 or SPM-1-L2, with which it has sequence identities of 30% and 19%, respectively. ???Whilst, VIM-2 seeds resulted only in crystals of SPM-1 despite the low sequence identity (24%). ***CHECK…It is not obvious

whether any of the seeds interface factors compared here contributed to the success of the seeding procedure in inducing crystallisation.

To further explore the potential effects of seed crystal packing interface properties on the success of cross-seeding, using PCalign we compared the crystal packing in an SPM-1-L2 structure obtained in this work with the crystal packing in all seed crystals analysed. PCalign is a software program that recognizes spatial and chemical similarity between protein-protein interfaces. It carries out a structural alignment of two given interfaces and provides a score that quantifies the physicochemical similarity. This normalized score (PC-score) is normalized between 0 and 1, with a value of 1 indicating identical interfaces. The PC similarity scores obtained for the different interfaces are shown in Table 4.

The structure of SPM-1-L2 was determined using a protein crystal obtained in the second tier MCMS using SPM-1 crystals obtained from the first tier MCMS. Despite the residue variation between the two proteins, their crystals share only one common crystal packing interface (Figure 6) with a PC similarity score of 0.886 (Table S6 in Supplementary Information). In the WT SPM-1 seed this crystal-packing interface is well maintained by six salt-bridges enforced by hydrogen bonds. However, this interface was not observed in the other available SPM-1 structure (PDB code 2fhx).

4. Discussion and Conclusions

This study was aimed at obtaining proof of principle for the benefit of cross-seeding using structurally homologous proteins of variable sequence similarity to aid crystallisation of target proteins. We set out to investigate several factors that might contribute to the success of seeding and affect the selection of suitable seeds. Seeding is expected to increase the overall success rate and lower the cost of the entire crystallisation process in term of materials and time. Self-seeding using poor protein crystals has been used successfully to induce better protein crystallisation. However, some proteins are so recalcitrant to crystallisation that crystals fail to grow even in these conditions. Therefore, the use of crystallisable homologous proteins as seeds represents an attractive alternative in such scenarios. However, there is a dominant belief that such cross seeding is only achievable in the presence of high sequence similarity between the seed protein and target protein.

Crystallisation depends on interface interactions between protein molecules. Crystal packing interactions have to be selective and specific, otherwise aggregation and precipitation may occur. Thus the role of crystal packing interactions in crystallisation might explain the success or otherwise of seeding. Crystal packing interactions depend on many protein properties, including hydrogen bonding and electrostatic and 3D shape complementarities. Crystal packing can make use of essentially all regions of the protein surface, aligning them in ways that are compatible with the symmetry and lattice parameters that characterise each crystal form (Crosio *et al.*, 1992). We have already observed conservation of crystal packing interfaces in a previous cross-microseeding experiment successful in crystallising TBNAT (Abuhammad *et al.*, 2013). The crystal packing promoted by the seed protein (MMNAT) utilised several intermolecular interacting residues also present in the target protein (TBNAT). Two crystal packing interfaces

were conserved between the MMNAT seed crystal form and the TBNAT crystal form, despite them having lowest sequence similarity relative to the other crystal packing interfaces. However, the intermolecular interfaces were maintained by hydrogen bonds and salt bridges indicating the influence of these stabilising interactions (Abuhammad *et al.*, 2013).

The results presented here show that the use of homologous proteins in cross-microseeding can improve the rate and conditions of crystallisation. Crystals in all successful experiments grew within three to seven days at the convenient temperature of 20 °C. This represents a significant improvement, especially in the case of SPM-1 crystals which previously took more than six months to grow at 4 °C. Furthermore, it was observed that cross seeding resulted in the formation of relatively large crystals (>100 µm) of uniform size and shape within each crystallisation drop. This behaviour is highly desirable in fragment-based drug discovery, and also for other purposes such as commercial biocatalyst production. However, none of the crystals obtained in the first tier MCMS screen were of good enough diffraction quality for structure determination as needed for drug discovery purposes. Most of the crystals obtained only diffracted to a resolution of ~4 Å and showed diffraction patterns characteristic of overlapping lattices. It is worth noting that in some cases, such as SPM-1, this resolution is an improvement over crystals previously obtained, which had never diffracted to a resolution better than 8 Å. Further optimisation of four of the identified MCMS conditions for IMP-1 resulted in crystals giving improved diffraction (1.3 Å). However the diffraction resolution of SPM-1 crystals was only marginally improved from 4 to 3 Å.

In this study only as a single dilution of the seed stock was used. Further trials using serial dilution of the seed stock to find the optimum concentration could lead to diffraction quality crystals. Optimisation of the seeding experiment is required in order to control crystallisation

kinetics and thus improve the diffraction quality of the resulting crystals (Till *et al.*, 2013). However, care must be taken, since seeding can also enhance the formation of salt crystals (Shaw, 2011).

The crystallizability of a protein is greatly affected by the chemical and conformational purity, and also by the oligomeric homogeneity of the sample, both of which are affected by solution components. In fact it has been reported previously that seeding is not capable of overcoming issues related to sample purity or microhomogeneity (Bergfors, 2003), and this was indeed observed in our study for NDM-1 (refer to Results).

It is difficult to speculate on the precise mechanism by which heterogeneous protein seeding may induce crystallisation. The seeds may either act as real nuclei to which molecules adhere, so increasing in size. It is possible that protein molecules from the seed crystal are also contained within the target-protein crystal. The probability of this will depend on the structural similarity and interface complementarity in terms of shape and surface properties (charge, hydrophilicity, solvation… etc). It has also been observed that the presence of contaminants can affect protein crystallisation (Bergfors, 2003, Vekilov *et al.*, 1996, Judge *et al.*, 1998, Matsui *et al.*, 2006, Burke *et al.*, 2001). Thus it is possible that the hetero-nuclei dissolve and act as contaminants in the solution. Other additives of the seed drop can also affect crystallisation by acting as contaminants.

Additionally the nuclei could act as ‘sticky templates’ that attract target protein molecules and support epitaxial growth, thus acting to catalyse the formation of target protein critical nuclei. A study of heteroseeding in colloidal model system shows that the crystallites first grow on the seed but then, on reaching a critical size, detaches from it (Allahyarov *et al.*, 2015). The detached and relaxed crystallite then continues to grow, except close to the seed,??check a process which

now prevents crystallization. Thus in this case, crystallization seeds facilitate crystallization only during the initial growth phase and then act as impurities that prevent further crystallisation (Allahyarov *et al.*, 2015).

In our study, despite low sequence similarity between the seeds and target proteins, cross-seeding allowed for the identification of crystallisation conditions for proteins that are known to be recalcitrant to crystallisation. Cross-seeding also resulted in the acceleration of crystal growth, since SPM-1 crystals were observed within 3-7 days as opposed to taking at least 6 months to grow previously: a significant improvement. Furthermore, the seeded crystals grew at room temperature, which is convenient for crystal handling and manipulation. The seeding has also provided us with a source of crystals that can be used in several further cycles of iterative seeding and crystallisation. These experiments are not only important in optimising crystallisation conditions, but also for the identification of new crystallisation conditions resulting in crystals with different crystal packing. These crystals might have improved access to active sites and thus be more suitable for ligand soaks necessary in drug discovery.

In conclusion, although a minimal amount of data was examined in this pilot study, the results indicate the potential for the MCMS method to produce crystals which otherwise were not attainable. Further studies are required to confirm the usefulness of this technique on a much larger scale, including cross-seeding between totally unrelated proteins. However it is clear that MCMS is a technique that is very well worth attempting.

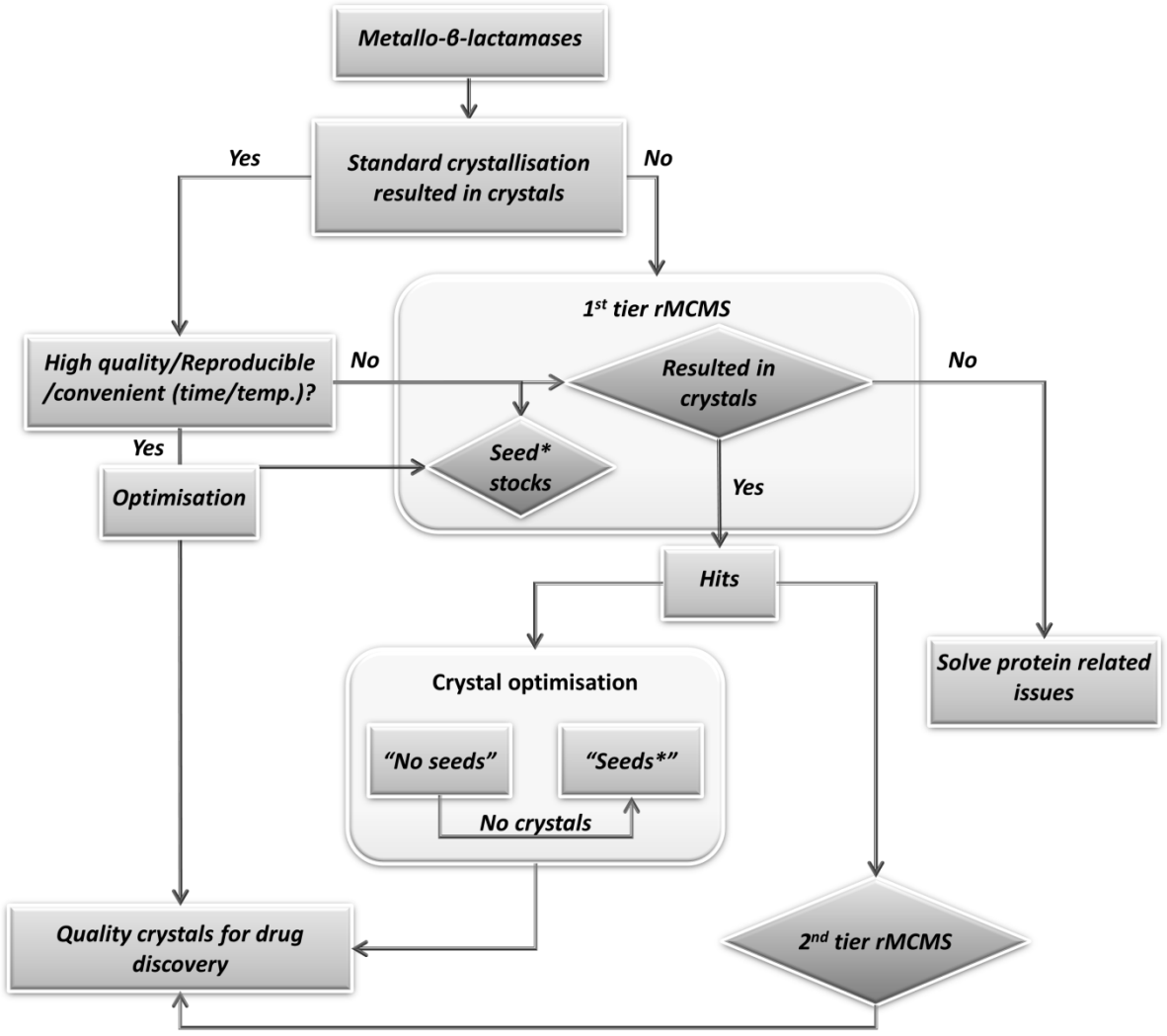

Figure 1 Workflow diagram of the crystallisation strategies used for metallo-βlactamases.* self-seeding was carried out when applicable.

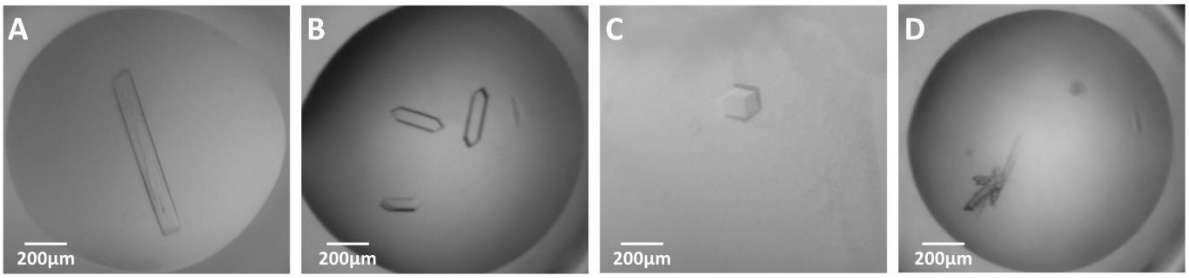

Figure 2 MBL crystals used to produce seed stocks. A) BcII is B) VIM-2 C) NDM-1 D) SPM-1. Details provided in Table 1.

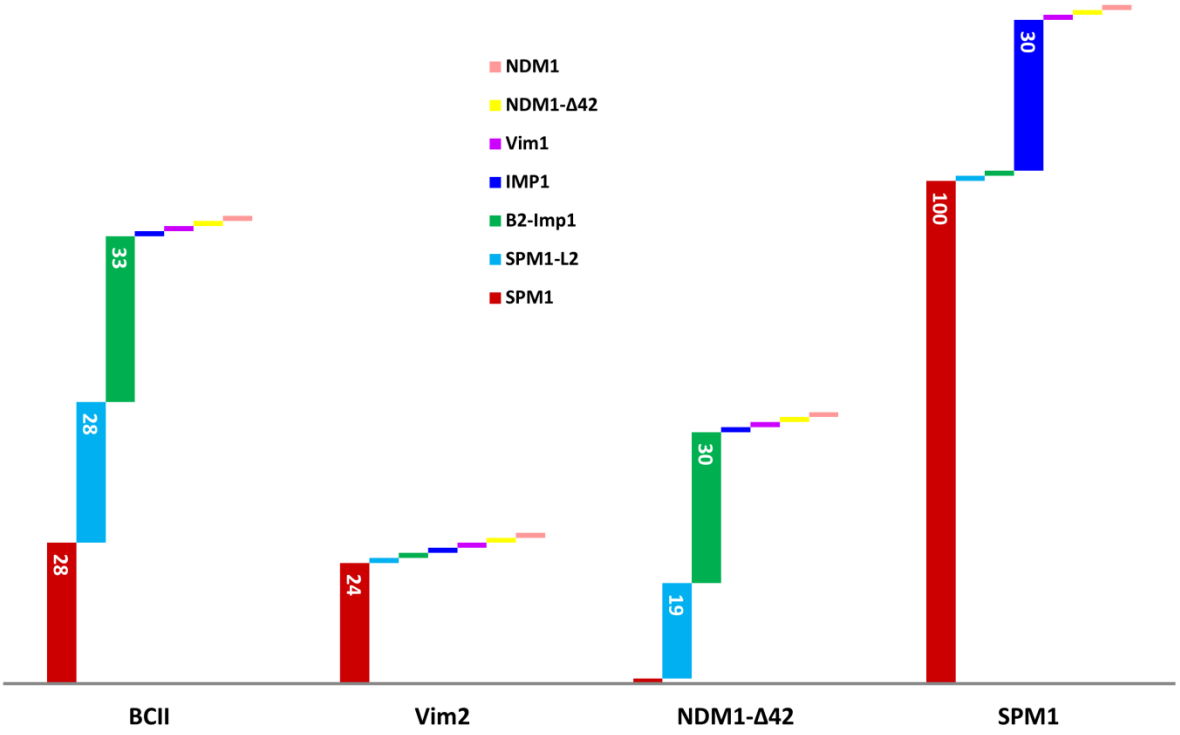

Figure 3 Schematic representation of the first tier MCMS screen results. MBLs used as seeds are indicated on the *x*-axis. Successful seeding attempts resulting in protein crystals (regardless of quality) are indicated by the bars and labelled with the percentage sequence identity between seed and target proteins (*height of each column*, *y*-axis). Horizontal lines indicate the failure of seeding in inducing crystallisation.

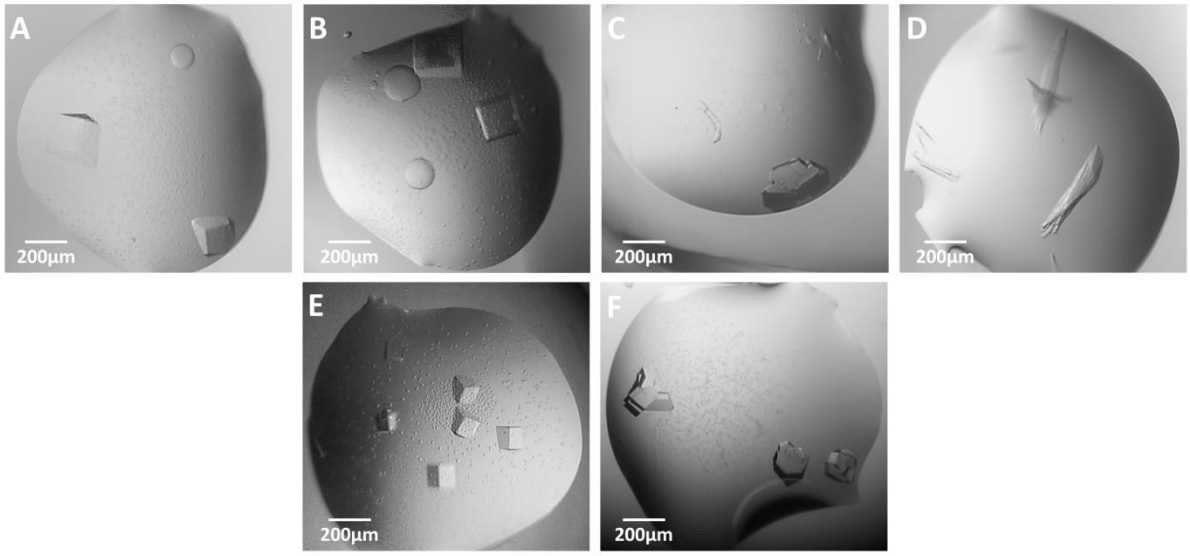

Figure 4 MBLs crystals obtained in the first tier MCMS (A, B, C and D) and after optimisation (E and F). A) SPM-1 crystals grew in condition JCSG+/C11 using BcII seeds. B) SPM-1-L2 crystals grew in condition JCSG+/C11 using BcII seeds. C) IMP-1 crystals grew in condition JCSG+/G9 using SPM-1 seeds. D) IMP-1-B2 crystals grew in condition JCSG+/G10 using BcII. E) SPM-1 crystals obtained upon optimisation of condition JCSG+/C11 using self-seeding. F) Crystals of IMP-1 obtained upon optimisation of condition JCSG+/G9 using self-seeding.

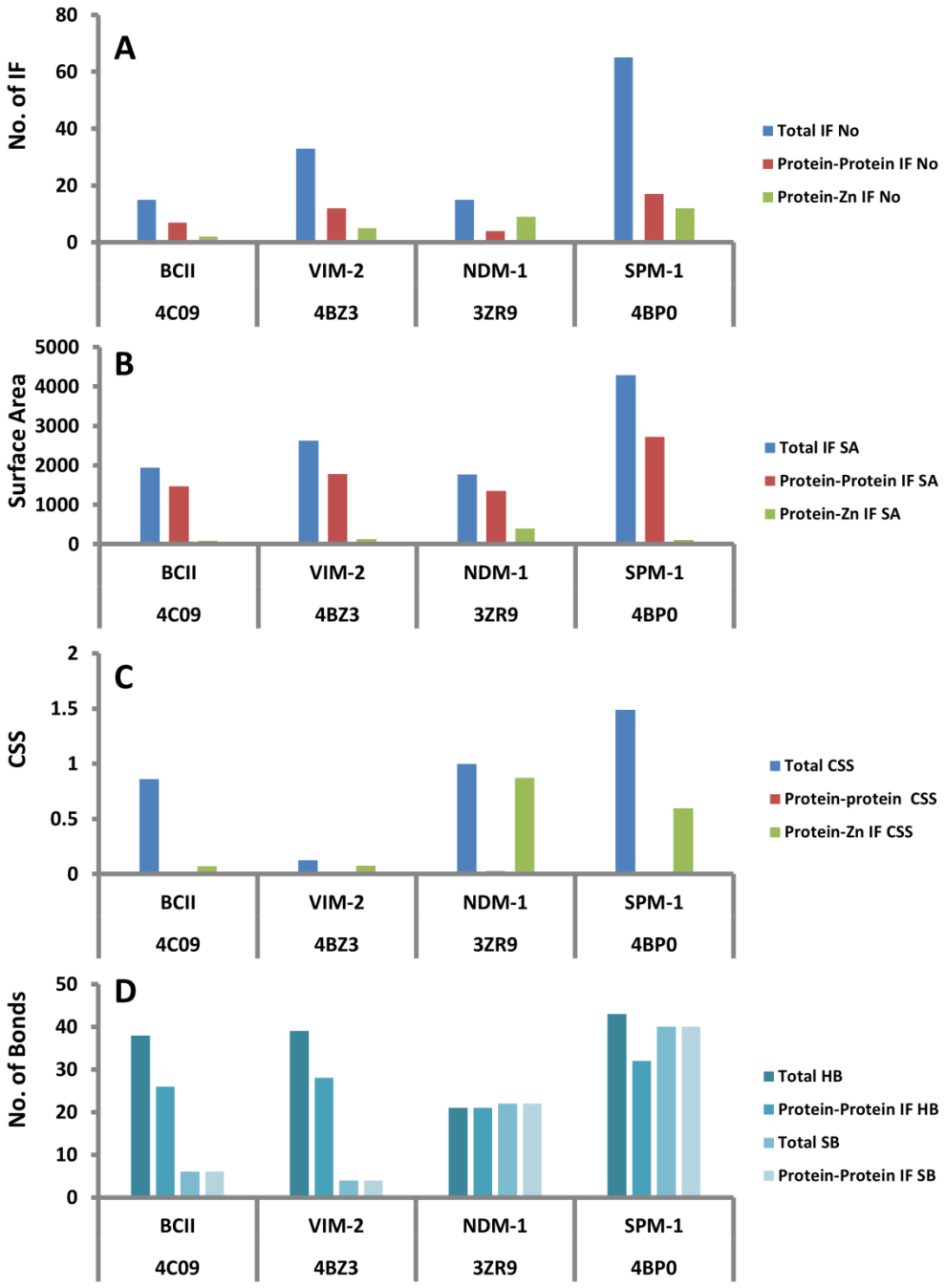

Figure 5 Analysis of the interfaces in each of the seed crystals using PISA. A) comparison of the total number of interfaces, B) The surface area (SA) of interfaces, C) Complexation Significance Score (CSS), which indicates the significance of the interface for assembly formation (maximal fraction of the total free energy of binding that belongs to the interface in stable assemblies). For example, if the only stable assembly found is a tetramer with 2 interfaces called #1 with ΔG=-10 kcal/M and 2 interfaces called #2 with ΔG=-5 kcal/M, then the CSS for interface #1 is $2\times10/(2\times10+2\times5)=2/3$. If, in addition, a dimer with interface #1 is found to be stable, then the CSS for interface #1 is max(1,2/3)=1. D) Number of hydrogen bonds (HB) and salt bridges (SB) in total IFs and in crystal packing interfaces.

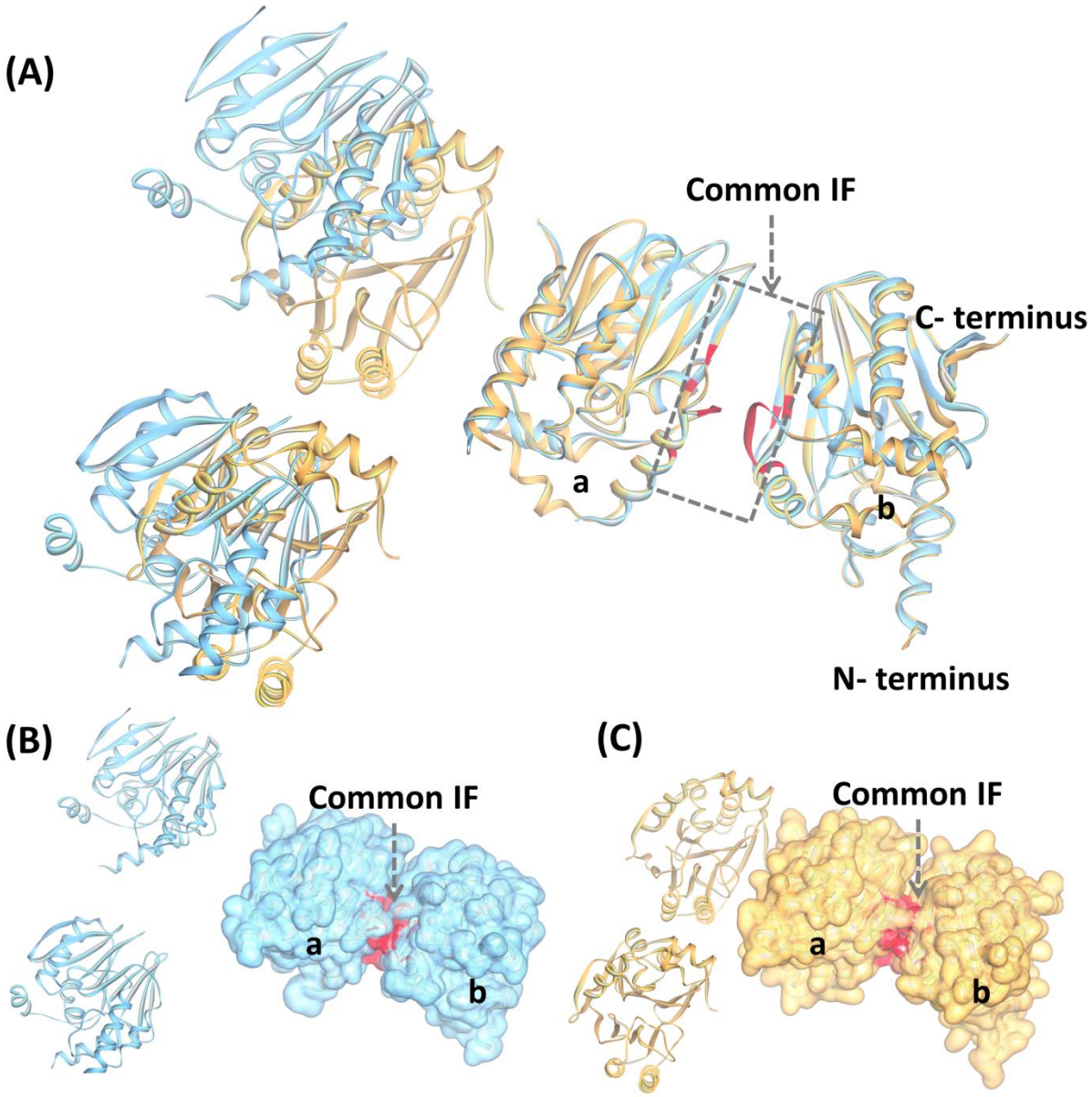

Figure 6 Common packing interactions in SPM-1 and SPM-1 variants. The red surface highlights common intermolecular interfaces that are conserved only within known mycobacterial structures. A) Ribbon representation of the superimposed SPM-1 seed (PDB 4bp0; gold) and SPM-1-L2 (PDB XXX; blue) structures demonstrate substantial overlap in crystal packing involving a common interface despite the fact that they crystallize in different space groups. (B) SPM-1. (C) SPM-1-L2.

Table 1 The set of proteins used in this study.

Protein	Concentration (mg/mL)	PDB code	Residues	Construct	Seed crystallisation conditions
BcII	15	4C09 (C 1 2 1)	227	Full length	23-25% PEG 3350; 0.1 M bis-tris pH 5.5; 0.15-0.2 M $(NH4)_2SO4$
VIM-2	15	4BZ3 (C 1 2 1)	242	Full length	20-22% PEG 3350; 0.2-0.25 M Mg-formate
NDM-Δ42	15	3ZR9 (I 4 2 2)	230	Truncated (G42-R270)	15-17% PEG 3350; 0.1 M HEPES pH 7.5; 0.007-0.008 M salt mix ($CoCl_2$, $NiCl_2$, $MgCl_2$, $CdCl_2$)
SPM-1	15	4BP0 (P 2_1 2_1 2_1)	250	Full length	20% PEG 3350, 0.2 M potassium nitrate
IMP-1	15			Full length	NA
B2-IMP-1	20			Extra GP-N-terminal	NA
SPM-1-L2	20			H108N	NA
VIM-1	10			Full length	NA
NDM-1	20			Full length	NA

Table 2 Percentage sequence identities (upper right triangle) and similarities (lower left shaded triangle) of β-lactamases used in this study. RMSD values are given between brackets. Seeds resulting in successful growth of crystals (regardless of resolution) are shown with red borders.

		Low resolution, long time to crystallise, or irreproducible			**No prior crystals**			
Seed \ **Target**		**IMP-1**	**SPM-1**	**NDM1-Δ42**	**VIM-1**	**B2-IMP1**	**SPM-1-L2**	**NDM-1**
BcII		33(1.57)	28(1.83)	28(1.65)	37	33	28	28
		43	43	47	50	43	43	42
VIM-2		28(1.56)	24(1.96)	30(2.63)	90	28	24	32
		45	36	47	95	45	36	51
NDM-1-Δ42		30(1.82)	19(2.05)	100(0)	33	30	19	87
		45	41	100	48	45	41	87
SPM-1		30(1.73)	100(0)	22(2.05)	23	30	99	19
		42	100	45	38	42	100	36

Table 3 Results of crystal growth optimisation showing the percentage of wells within each 24-well plate that gave protein crystals (regardless of diffraction quality).

	IMP-1 (G9)		**SPM-1 (C11)**		**SPM-1 (G8)**		**SPM-1-L2 (C11)**	
	Seed	**Seed Reservoir**	**Seed**	**Seed Reservoir**	**Seed**	**Seed Reservoir**	**Seed**	**Seed Reservoir**
BcII	-	-	33	13	-	-	-	-
VIM-2	63	-	38	42	-	-	38	33
NDM-1-Δ42			21	25	-	-	-	-
SPM-1	-	1	29	54	-	-	17	8
Self-seeding	96	-	21	13	-	-	17	13
No seed*	25	-	13	17	-	-	-	-

*For no seeding condition, a volume of the crystallisation condition reservoir was used instead of the seed solution.

Supporting Information

Figure S1. A schematic representation of the customised-screen plates designed to optimise the seed crystals. A) BcII, B) VIM-2, C) NDM-Δ42.

Figure S2. A schematic representation of the customised-screen plates designed to optimise the crystals obtained out of the first tier MCMS. A) JCSG+/C11, B) JCSG+/G8, C) JCSG+/G9.

Figure S3. Representative SEC-MALS analyses of A) NDM-1 and B) NDM-1-Δ42.

Table S1. Conditions resulted in crystals in the first tier MCMS.

Table S2. Conditions resulted in crystals upon optimization of condition JCSG+/C11 of SPM-1-L2 crystals obtained in the first tier MCMS.

Table S3. Conditions resulted in crystals upon optimization of condition JCSG+/C11of SPM-1 crystals obtained in the first tier MCMS.

Table S4. Conditions resulted in crystals upon optimization of condition JCSG+/G9 of IMP-1 crystals obtained in the first tier MCMS.

Table S5. Conditions resulted in crystals in the second tier MCMS.

Table S6. Interfaces comparison between the SPM-1-L2 structure and the seed crystals.

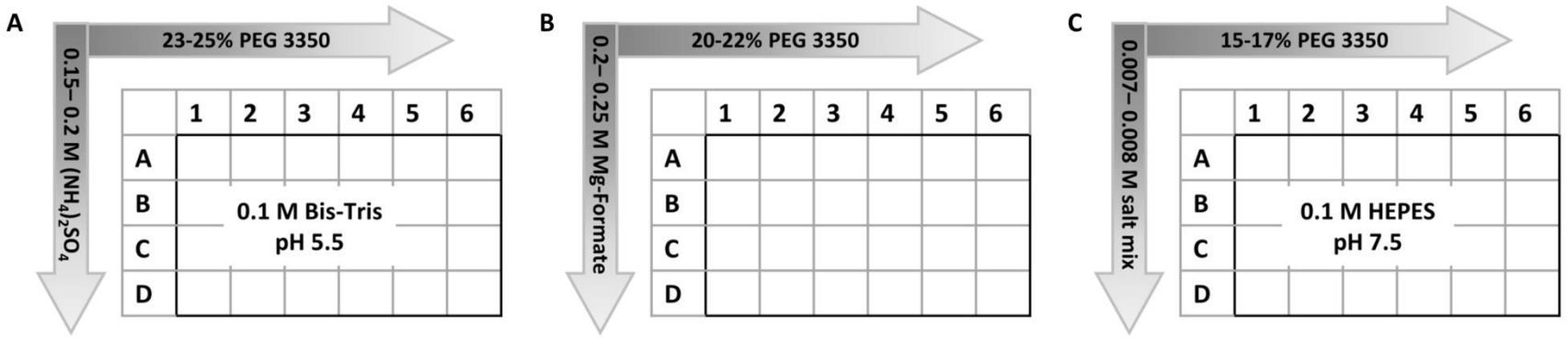

Figure S1. A schematic representation of the customised-screen plates designed to optimise the seed crystals. A) BcII, B) VIM-2, C) NDM-Δ42.

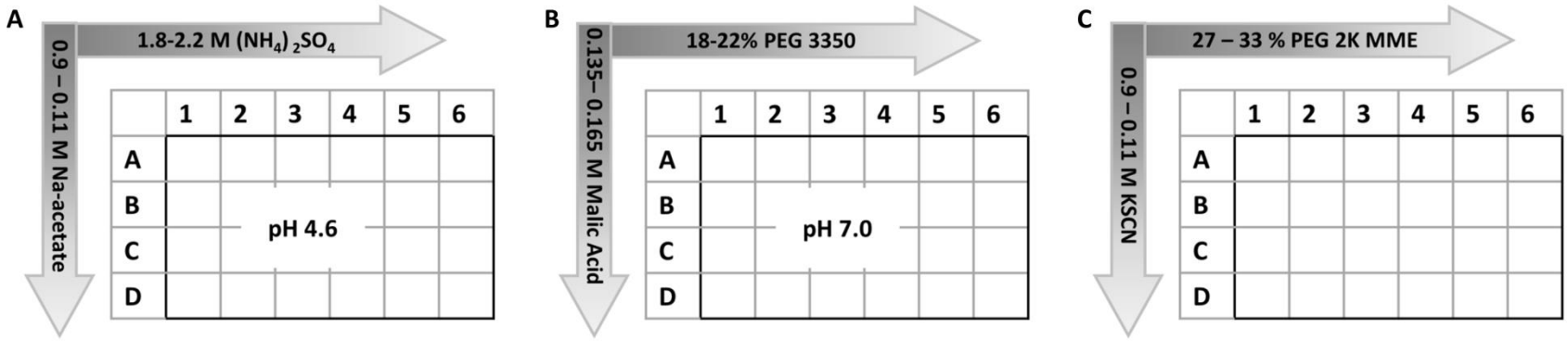

Figure S2. A schematic representation of the customised-screen plates designed to optimise the crystals obtained out of the first tier MCMS. A) JCSG+/C11, B) JCSG+/G8, C) JCSG+/G9.

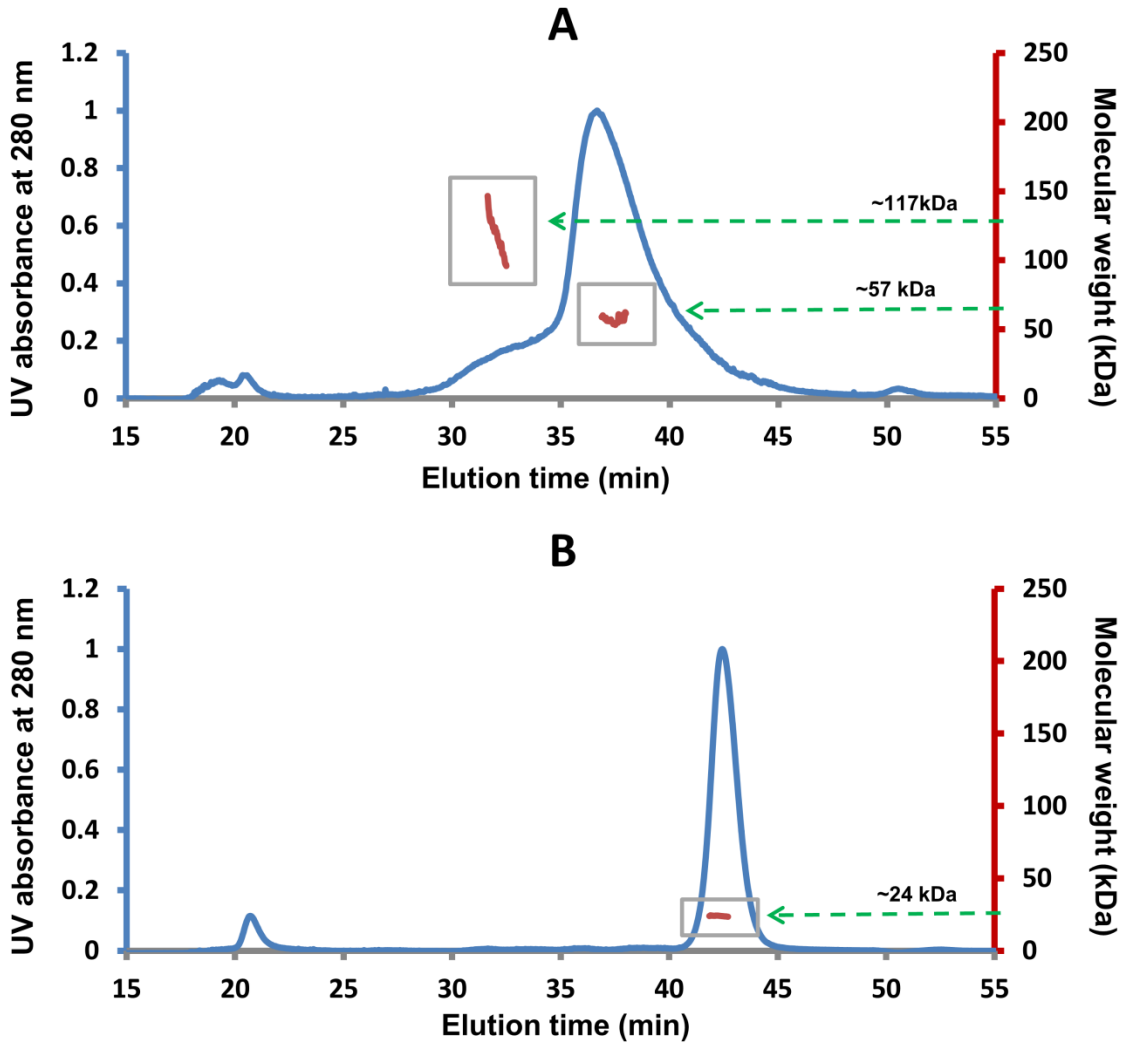

Figure S3. Representative SEC-MALS analyses of A) NDM-1 and B) NDM-1-Δ42. Analysis of NDM-1/ NDM-1-Δ42 was carried out by size exclusion chromatography followed by in-line multi-angle light scattering. The right axis represents the molecular mass at any given point in the elution profile, and the segments of the curve within each peak are expanded in a box and labelled with the average measured molecular mass. The elution profile of NDM1 demonstrates an abundant, stable, concentration-independent dimer and a lower fraction of a more dynamic concentration-dependent tetramer. The column volume is 23 mL (57 min), the void volume is 7.5 mL (18.75 min).

Table S1. Conditions resulted in crystals in the first tier MCMS.

MBL		JCSG+ Screen		Resolution** (Å)
Target	**Seed MBL***	**Code**	**Condition composition**	
SPM-1	SPM-1/O	G8	0.15 M DL-malic acid; pH 7.0; 20 % w/v PEG 3350	4.5
	BcII	C11	0.1 M sodium acetate; pH 4.6; 2.0 M ammonium sulfate	~8
	SPM-1/O	C11	0.1 M sodium acetate; pH 4.6; 2.0 M ammonium sulfate	~7
	VIM-2	C11	0.1 M sodium acetate; pH 4.6; 2.0 M ammonium sulfate	~5
	NS	C11	0.1 M sodium acetate; pH 4.6; 2.0 M ammonium sulfate	~7
SPM-1-L2	BcII	B3	0.1 M bicine; pH 9.0; 20 % w/v PEG 6K	ND
	NDM-Δ42	C11	0.1 M sodium acetate; pH 4.6; 2.0 M ammonium sulfate	ND
	SPM-1/O	C11	0.1 M sodium acetate; pH 4.6; 2.0 M ammonium sulfate	3-5
IMP-1	VIM-2	F6	0.1 M bicine; pH 9.0; 10 % v/v MPD	ND
	NS	G9	0.1 M potassium thiocyanate; 30 % w/v PEG 2K MME	1.5
	SPM-1	G9	0.1 M potassium thiocyanate; 30 % w/v PEG 2K MME	1.6
	BcII	C12	10 % w/v PEG 1000/ 10 % w/v PEG	ND
B2-IMP-1	NDM-Δ42	E2	0.2 M NaCl; 0.1 M sodium cacodylate; pH 6.5; 2.0 M ammonium sulfate	2.2
	BcII	G10	0.15 M potassium bromide; 30 % w/v PEG 2K MME	2.2-3
VIM-1	BcII	A4	0.02 M calcium chloride; 0.1 M sodium acetate; pH 4.6; 30 % v/v MPD	ND

*NS: no seed and O: old. **ND is no diffraction; crystals which did not diffract were not considered as a crystallisation hit.

Table S2. Conditions resulted in crystals upon optimization of condition JCSG+/C11 of SPM-1-L2 crystals obtained in the first tier MCMS.

Well code	Seed MBL*	Condition Composition
A5	VIM-2	0.09 M sodium acetate; pH 4.6; 2.12 M ammonium sulfate
A5	VIM-2	0.09 M sodium acetate; pH 4.6; 2.12 M ammonium sulfate
A5	VIM-2/R	0.09 M sodium acetate; pH 4.6; 2.12 M ammonium sulfate
A5	VIM-2/R	0.09 M sodium acetate; pH 4.6; 2.12 M ammonium sulfate
A6	VIM-2	0.09 M sodium acetate; pH 4.6; 2.2 M ammonium sulfate
A6	SS	0.09 M sodium acetate; pH 4.6; 2.2 M ammonium sulfate
A6	SS	0.09 M sodium acetate; pH 4.6; 2.2 M ammonium sulfate
A6	VIM-2	0.09 M sodium acetate; pH 4.6; 2.2 M ammonium sulfate
A6	SPM-1	0.09 M sodium acetate; pH 4.6; 2.2 M ammonium sulfate
A6	SPM-1	0.09 M sodium acetate; pH 4.6; 2.2 M ammonium sulfate
A6	VIM-2/R	0.09 M sodium acetate; pH 4.6; 2.2 M ammonium sulfate
A6	SS/R	0.09 M sodium acetate; pH 4.6; 2.2 M ammonium sulfate
A6	SS/R	0.09 M sodium acetate; pH 4.6; 2.2 M ammonium sulfate
A6	VIM-2/R	0.09 M sodium acetate; pH 4.6; 2.2 M ammonium sulfate
A6	SPM-1/R	0.09 M sodium acetate; pH 4.6; 2.2 M ammonium sulfate
A6	SPM-1/R	0.09 M sodium acetate; pH 4.6; 2.2 M ammonium sulfate
B4	VIM-2	0.097 M sodium acetate; pH 4.6; 2.04 M ammonium sulfate
B4	VIM-2	0.097 M sodium acetate; pH 4.6; 2.04 M ammonium sulfate
B5	VIM-2	0.097 M sodium acetate; pH 4.6; 2.12 M ammonium sulfate
B5	VIM-2	0.097 M sodium acetate; pH 4.6; 2.12 M ammonium sulfate
B5	VIM-2/R	0.097 M sodium acetate; pH 4.6; 2.12 M ammonium sulfate
B5	VIM-2/R	0.097 M sodium acetate; pH 4.6; 2.12 M ammonium sulfate
B6	VIM-2	0.097 M sodium acetate; pH 4.6; 2.2 M ammonium sulfate
B6	SS	0.097 M sodium acetate; pH 4.6; 2.2 M ammonium sulfate
B6	SS	0.097 M sodium acetate; pH 4.6; 2.2 M ammonium sulfate
B6	VIM-2	0.097 M sodium acetate; pH 4.6; 2.2 M ammonium sulfate
B6	SPM-1	0.097 M sodium acetate; pH 4.6; 2.2 M ammonium sulfate
B6	SPM-1	0.097 M sodium acetate; pH 4.6; 2.2 M ammonium sulfate
B6	VIM-2/R	0.097 M sodium acetate; pH 4.6; 2.2 M ammonium sulfate
B6	SS/R	0.097 M sodium acetate; pH 4.6; 2.2 M ammonium sulfate
B6	SS/R	0.097 M sodium acetate; pH 4.6; 2.2 M ammonium sulfate
B6	VIM-2/R	0.097 M sodium acetate; pH 4.6; 2.2 M ammonium sulfate
C5	VIM-2	0.1 M sodium acetate; pH 4.6; 2.12 M ammonium sulfate
C5	VIM-2	0.1 M sodium acetate; pH 4.6; 2.12 M ammonium sulfate
C5	VIM-2/R	0.1 M sodium acetate; pH 4.6; 2.12 M ammonium sulfate
C5	VIM-2/R	0.1 M sodium acetate; pH 4.6; 2.12 M ammonium sulfate
C6	VIM-2	0.1 M sodium acetate; pH 4.6; 2.2 M ammonium sulfate

C6	SS	0.1 M sodium acetate; pH 4.6; 2.2 M ammonium sulfate
C6	SS	0.1 M sodium acetate; pH 4.6; 2.2 M ammonium sulfate
C6	VIM-2	0.1 M sodium acetate; pH 4.6; 2.2 M ammonium sulfate
C6	SPM-1	0.1 M sodium acetate; pH 4.6; 2.2 M ammonium sulfate
C6	SPM-1	0.1 M sodium acetate; pH 4.6; 2.2 M ammonium sulfate
C6	VIM-2/R	0.1 M sodium acetate; pH 4.6; 2.2 M ammonium sulfate
C6	SS/R	0.1 M sodium acetate; pH 4.6; 2.2 M ammonium sulfate
C6	SS/R	0.1 M sodium acetate; pH 4.6; 2.2 M ammonium sulfate
C6	VIM-2/R	0.1 M sodium acetate; pH 4.6; 2.2 M ammonium sulfate
C6	SPM-1/R	0.1 M sodium acetate; pH 4.6; 2.2 M ammonium sulfate
C6	SPM-1/R	0.1 M sodium acetate; pH 4.6; 2.2 M ammonium sulfate
D5	VIM-2	0.11 M sodium acetate; pH 4.6; 2.12 M ammonium sulfate
D5	VIM-2	0.11 M sodium acetate; pH 4.6; 2.12 M ammonium sulfate
D5	VIM-2/R	0.11 M sodium acetate; pH 4.6; 2.12 M ammonium sulfate
D5	VIM-2/R	0.11 M sodium acetate; pH 4.6; 2.12 M ammonium sulfate
D6	VIM-2	0.11 M sodium acetate; pH 4.6; 2.2 M ammonium sulfate
D6	SS	0.11 M sodium acetate; pH 4.6; 2.2 M ammonium sulfate
D6	SS	0.11 M sodium acetate; pH 4.6; 2.2 M ammonium sulfate
D6	VIM-2	0.11 M sodium acetate; pH 4.6; 2.2 M ammonium sulfate
D6	SPM-1	0.11 M sodium acetate; pH 4.6; 2.2 M ammonium sulfate
D6	SPM-1	0.11 M sodium acetate; pH 4.6; 2.2 M ammonium sulfate
D6	VIM-2/R	0.11 M sodium acetate; pH 4.6; 2.2 M ammonium sulfate
D6	VIM-2/R	0.11 M sodium acetate; pH 4.6; 2.2 M ammonium sulfate

*R: reservoir, SS: self-seed, NS, no seed.

Table S3. Conditions resulted in crystals upon optimization of condition JCSG+/C11of SPM-1 crystals obtained in the first tier MCMS.

Well code	Seed MBL	Condition Composition
A1	NS/R	0.09 M sodium acetate; pH 4.6; 1.8 M ammonium sulfate
A1	NS/R	0.09 M sodium acetate; pH 4.6; 1.8 M ammonium sulfate
A4	SPM-1/R	0.09 M sodium acetate; pH 4.6; 2.04 M ammonium sulfate
A4	SPM-1/R	0.09 M sodium acetate; pH 4.6; 2.04 M ammonium sulfate
A5	SPM-1/R	0.09 M sodium acetate; pH 4.6; 2.12 M ammonium sulfate
A5	SPM-1/R	0.09 M sodium acetate; pH 4.6; 2.12 M ammonium sulfate
A5	VIM-2/R	0.09 M sodium acetate; pH 4.6; 2.12 M ammonium sulfate
A5	VIM-2/R	0.09 M sodium acetate; pH 4.6; 2.12 M ammonium sulfate
A5	SPM-1	0.09 M sodium acetate; pH 4.6; 2.12 M ammonium sulfate
A5	SPM-1	0.09 M sodium acetate; pH 4.6; 2.12 M ammonium sulfate
A5	VIM-2	0.09 M sodium acetate; pH 4.6; 2.12 M ammonium sulfate
A5	SS	0.09 M sodium acetate; pH 4.6; 2.12 M ammonium sulfate
A5	NS	0.09 M sodium acetate; pH 4.6; 2.12 M ammonium sulfate
A5	NS	0.09 M sodium acetate; pH 4.6; 2.12 M ammonium sulfate
A5	SS	0.09 M sodium acetate; pH 4.6; 2.12 M ammonium sulfate
A5	VIM-2	0.09 M sodium acetate; pH 4.6; 2.12 M ammonium sulfate
A6	BcII/R	0.09 M sodium acetate; pH 4.6; 2.2 M ammonium sulfate
A6	NDM-Δ42/R	0.09 M sodium acetate; pH 4.6; 2.2 M ammonium sulfate
A6	SPM-1/R	0.09 M sodium acetate; pH 4.6; 2.2 M ammonium sulfate
A6	BcII/R	0.09 M sodium acetate; pH 4.6; 2.2 M ammonium sulfate
A6	NDM-Δ42/R	0.09 M sodium acetate; pH 4.6; 2.2 M ammonium sulfate
A6	SPM-1/R	0.09 M sodium acetate; pH 4.6; 2.2 M ammonium sulfate
A6	VIM-2/R	0.09 M sodium acetate; pH 4.6; 2.2 M ammonium sulfate
A6	NS/R	0.09 M sodium acetate; pH 4.6; 2.2 M ammonium sulfate
A6	NS/R	0.09 M sodium acetate; pH 4.6; 2.2 M ammonium sulfate
A6	VIM-2/R	0.09 M sodium acetate; pH 4.6; 2.2 M ammonium sulfate
A6	BcII	0.09 M sodium acetate; pH 4.6; 2.2 M ammonium sulfate
A6	NDM-Δ42	0.09 M sodium acetate; pH 4.6; 2.2 M ammonium sulfate
A6	SPM-1	0.09 M sodium acetate; pH 4.6; 2.2 M ammonium sulfate
A6	BcII	0.09 M sodium acetate; pH 4.6; 2.2 M ammonium sulfate
A6	NDM-Δ42	0.09 M sodium acetate; pH 4.6; 2.2 M ammonium sulfate
A6	SPM-1	0.09 M sodium acetate; pH 4.6; 2.2 M ammonium sulfate
A6	VIM-2	0.09 M sodium acetate; pH 4.6; 2.2 M ammonium sulfate
A6	SS	0.09 M sodium acetate; pH 4.6; 2.2 M ammonium sulfate
A6	NS	0.09 M sodium acetate; pH 4.6; 2.2 M ammonium sulfate
A6	NS	0.09 M sodium acetate; pH 4.6; 2.2 M ammonium sulfate
A6	SS	0.09 M sodium acetate; pH 4.6; 2.2 M ammonium sulfate

A6	VIM-2	0.09 M sodium acetate; pH 4.6; 2.2 M ammonium sulfate
B3	BcII	0.097 M sodium acetate; pH 4.6; 1.96 M ammonium sulfate
B3	BcII	0.097 M sodium acetate; pH 4.6; 1.96 M ammonium sulfate
B4	SPM-1/R	0.097 M sodium acetate; pH 4.6; 2.04 M ammonium sulfate
B4	SPM-1/R	0.097 M sodium acetate; pH 4.6; 2.04 M ammonium sulfate
B5	SPM-1/R	0.097 M sodium acetate; pH 4.6; 2.12 M ammonium sulfate
B5	SPM-1/R	0.097 M sodium acetate; pH 4.6; 2.12 M ammonium sulfate
B5	VIM-2/R	0.097 M sodium acetate; pH 4.6; 2.12 M ammonium sulfate
B5	VIM-2/R	0.097 M sodium acetate; pH 4.6; 2.12 M ammonium sulfate
B5	VIM-2	0.097 M sodium acetate; pH 4.6; 2.12 M ammonium sulfate
B5	VIM-2	0.097 M sodium acetate; pH 4.6; 2.12 M ammonium sulfate
B6	NDM-Δ42/R	0.097 M sodium acetate; pH 4.6; 2.2 M ammonium sulfate
B6	SPM-1/R	0.097 M sodium acetate; pH 4.6; 2.2 M ammonium sulfate
B6	NDM-Δ42/R	0.097 M sodium acetate; pH 4.6; 2.2 M ammonium sulfate
B6	SPM-1/R	0.097 M sodium acetate; pH 4.6; 2.2 M ammonium sulfate
B6	VIM-2/R	0.097 M sodium acetate; pH 4.6; 2.2 M ammonium sulfate
B6	SS/R	0.097 M sodium acetate; pH 4.6; 2.2 M ammonium sulfate
B6	SS/R	0.097 M sodium acetate; pH 4.6; 2.2 M ammonium sulfate
B6	VIM-2/R	0.097 M sodium acetate; pH 4.6; 2.2 M ammonium sulfate
B6	BcII	0.097 M sodium acetate; pH 4.6; 2.2 M ammonium sulfate
B6	NDM-Δ42	0.097 M sodium acetate; pH 4.6; 2.2 M ammonium sulfate
B6	SPM-1	0.097 M sodium acetate; pH 4.6; 2.2 M ammonium sulfate
B6	BcII	0.097 M sodium acetate; pH 4.6; 2.2 M ammonium sulfate
B6	NDM-Δ42	0.097 M sodium acetate; pH 4.6; 2.2 M ammonium sulfate
B6	SPM-1	0.097 M sodium acetate; pH 4.6; 2.2 M ammonium sulfate
B6	VIM-2	0.097 M sodium acetate; pH 4.6; 2.2 M ammonium sulfate
B6	SS	0.097 M sodium acetate; pH 4.6; 2.2 M ammonium sulfate
B6	NS	0.097 M sodium acetate; pH 4.6; 2.2 M ammonium sulfate
B6	NS	0.097 M sodium acetate; pH 4.6; 2.2 M ammonium sulfate
B6	SS	0.097 M sodium acetate; pH 4.6; 2.2 M ammonium sulfate
B6	VIM-2	0.097 M sodium acetate; pH 4.6; 2.2 M ammonium sulfate
C4	BcII/R	0.1 M sodium acetate; pH 4.6; 2.04 M ammonium sulfate
C4	BcII/R	0.1 M sodium acetate; pH 4.6; 2.04 M ammonium sulfate
C4	VIM-2/R	0.1 M sodium acetate; pH 4.6; 2.04 M ammonium sulfate
C4	VIM-2/R	0.1 M sodium acetate; pH 4.6; 2.04 M ammonium sulfate
C4	BcII	0.1 M sodium acetate; pH 4.6; 2.04 M ammonium sulfate
C4	BcII	0.1 M sodium acetate; pH 4.6; 2.04 M ammonium sulfate
C4	VIM-2	0.1 M sodium acetate; pH 4.6; 2.04 M ammonium sulfate
C4	VIM-2	0.1 M sodium acetate; pH 4.6; 2.04 M ammonium sulfate
C5	NDM-Δ42/R	0.1 M sodium acetate; pH 4.6; 2.12 M ammonium sulfate
C5	SPM-1/R	0.1 M sodium acetate; pH 4.6; 2.12 M ammonium sulfate

C5	NDM-Δ42/R	0.1 M sodium acetate; pH 4.6; 2.12 M ammonium sulfate
C5	SPM-1/R	0.1 M sodium acetate; pH 4.6; 2.12 M ammonium sulfate
C5	VIM-2/R	0.1 M sodium acetate; pH 4.6; 2.12 M ammonium sulfate
C5	VIM-2/R	0.1 M sodium acetate; pH 4.6; 2.12 M ammonium sulfate
C5	NDM-Δ42	0.1 M sodium acetate; pH 4.6; 2.12 M ammonium sulfate
C5	SPM-1	0.1 M sodium acetate; pH 4.6; 2.12 M ammonium sulfate
C5	NDM-Δ42	0.1 M sodium acetate; pH 4.6; 2.12 M ammonium sulfate
C5	SPM-1	0.1 M sodium acetate; pH 4.6; 2.12 M ammonium sulfate
C5	VIM-2	0.1 M sodium acetate; pH 4.6; 2.12 M ammonium sulfate
C5	SS	0.1 M sodium acetate; pH 4.6; 2.12 M ammonium sulfate
C5	SS	0.1 M sodium acetate; pH 4.6; 2.12 M ammonium sulfate
C5	VIM-2	0.1 M sodium acetate; pH 4.6; 2.12 M ammonium sulfate
C6	NDM-Δ42/R	0.1 M sodium acetate; pH 4.6; 2.2 M ammonium sulfate
C6	SPM-1/R	0.1 M sodium acetate; pH 4.6; 2.2 M ammonium sulfate
C6	NDM-Δ42/R	0.1 M sodium acetate; pH 4.6; 2.2 M ammonium sulfate
C6	SPM-1/R	0.1 M sodium acetate; pH 4.6; 2.2 M ammonium sulfate
C6	VIM-2/R	0.1 M sodium acetate; pH 4.6; 2.2 M ammonium sulfate
C6	SS/R	0.1 M sodium acetate; pH 4.6; 2.2 M ammonium sulfate
C6	NS/R	0.1 M sodium acetate; pH 4.6; 2.2 M ammonium sulfate
C6	NS/R	0.1 M sodium acetate; pH 4.6; 2.2 M ammonium sulfate
C6	SS/R	0.1 M sodium acetate; pH 4.6; 2.2 M ammonium sulfate
C6	VIM-2/R	0.1 M sodium acetate; pH 4.6; 2.2 M ammonium sulfate
C6	NDM-Δ42	0.1 M sodium acetate; pH 4.6; 2.2 M ammonium sulfate
C6	SPM-1	0.1 M sodium acetate; pH 4.6; 2.2 M ammonium sulfate
C6	NDM-Δ42	0.1 M sodium acetate; pH 4.6; 2.2 M ammonium sulfate
C6	SPM-1	0.1 M sodium acetate; pH 4.6; 2.2 M ammonium sulfate
C6	VIM-2	0.1 M sodium acetate; pH 4.6; 2.2 M ammonium sulfate
C6	SS	0.1 M sodium acetate; pH 4.6; 2.2 M ammonium sulfate
C6	SS	0.1 M sodium acetate; pH 4.6; 2.2 M ammonium sulfate
C6	VIM-2	0.1 M sodium acetate; pH 4.6; 2.2 M ammonium sulfate
D2	SPM-1/R	0.11 M sodium acetate; pH 4.6; 1.88 M ammonium sulfate
D2	SPM-1/R	0.11 M sodium acetate; pH 4.6; 1.88 M ammonium sulfate
D3	SPM-1/R	0.11 M sodium acetate; pH 4.6; 1.96 M ammonium sulfate
D3	SPM-1/R	0.11 M sodium acetate; pH 4.6; 1.96 M ammonium sulfate
D3	BcII	0.11 M sodium acetate; pH 4.6; 1.96 M ammonium sulfate
D3	BcII	0.11 M sodium acetate; pH 4.6; 1.96 M ammonium sulfate
D4	SPM-1/R	0.11 M sodium acetate; pH 4.6; 2.04 M ammonium sulfate
D4	SPM-1/R	0.11 M sodium acetate; pH 4.6; 2.04 M ammonium sulfate
D4	VIM-2/R	0.11 M sodium acetate; pH 4.6; 2.04 M ammonium sulfate
D4	VIM-2/R	0.11 M sodium acetate; pH 4.6; 2.04 M ammonium sulfate
D4	BcII	0.11 M sodium acetate; pH 4.6; 2.04 M ammonium sulfate

D4	SPM-1	0.11 M sodium acetate; pH 4.6; 2.04 M ammonium sulfate
D4	BcII	0.11 M sodium acetate; pH 4.6; 2.04 M ammonium sulfate
D4	SPM-1	0.11 M sodium acetate; pH 4.6; 2.04 M ammonium sulfate
D5	NDM-Δ42/R	0.11 M sodium acetate; pH 4.6; 2.12 M ammonium sulfate
D5	SPM-1/R	0.11 M sodium acetate; pH 4.6; 2.12 M ammonium sulfate
D5	NDM-Δ42/R	0.11 M sodium acetate; pH 4.6; 2.12 M ammonium sulfate
D5	SPM-1/R	0.11 M sodium acetate; pH 4.6; 2.12 M ammonium sulfate
D5	VIM-2/R	0.11 M sodium acetate; pH 4.6; 2.12 M ammonium sulfate
D5	VIM-2/R	0.11 M sodium acetate; pH 4.6; 2.12 M ammonium sulfate
D5	BcII	0.11 M sodium acetate; pH 4.6; 2.12 M ammonium sulfate
D5	NDM-Δ42	0.11 M sodium acetate; pH 4.6; 2.12 M ammonium sulfate
D5	BcII	0.11 M sodium acetate; pH 4.6; 2.12 M ammonium sulfate
D5	NDM-Δ42	0.11 M sodium acetate; pH 4.6; 2.12 M ammonium sulfate
D5	VIM-2	0.11 M sodium acetate; pH 4.6; 2.12 M ammonium sulfate
D5	VIM-2	0.11 M sodium acetate; pH 4.6; 2.12 M ammonium sulfate
D6	BcII/R	0.11 M sodium acetate; pH 4.6; 2.2 M ammonium sulfate
D6	NDM-Δ42/R	0.11 M sodium acetate; pH 4.6; 2.2 M ammonium sulfate
D6	SPM-1/R	0.11 M sodium acetate; pH 4.6; 2.2 M ammonium sulfate
D6	BcII/R	0.11 M sodium acetate; pH 4.6; 2.2 M ammonium sulfate
D6	NDM-Δ42/R	0.11 M sodium acetate; pH 4.6; 2.2 M ammonium sulfate
D6	SPM-1/R	0.11 M sodium acetate; pH 4.6; 2.2 M ammonium sulfate
D6	VIM-2/R	0.11 M sodium acetate; pH 4.6; 2.2 M ammonium sulfate
D6	SS/R	0.11 M sodium acetate; pH 4.6; 2.2 M ammonium sulfate
D6	NS/R	0.11 M sodium acetate; pH 4.6; 2.2 M ammonium sulfate
D6	NS/R	0.11 M sodium acetate; pH 4.6; 2.2 M ammonium sulfate
D6	SS/R	0.11 M sodium acetate; pH 4.6; 2.2 M ammonium sulfate
D6	VIM-2/R	0.11 M sodium acetate; pH 4.6; 2.2 M ammonium sulfate
D6	BcII	0.11 M sodium acetate; pH 4.6; 2.2 M ammonium sulfate
D6	SPM-1	0.11 M sodium acetate; pH 4.6; 2.2 M ammonium sulfate
D6	BcII	0.11 M sodium acetate; pH 4.6; 2.2 M ammonium sulfate
D6	SPM-1	0.11 M sodium acetate; pH 4.6; 2.2 M ammonium sulfate
D6	VIM-2	0.11 M sodium acetate; pH 4.6; 2.2 M ammonium sulfate
D6	VIM-2	0.11 M sodium acetate; pH 4.6; 2.2 M ammonium sulfate

*R: reservoir, SS: self-seed, NS, no seed

Table S4. Conditions resulted in crystals upon optimization of condition JCSG+/G9 of IMP-1 crystals obtained in the first tier MCMS.

Well code	Seed MBL*	Condition Composition
A1	SS	0.09 M potassium thiocyanate; 27 % w/v PEG 2K MME
A1	SS	0.09 M potassium thiocyanate; 27 % w/v PEG 2K MME
A2	SS	0.09 M potassium thiocyanate; 28.2 % w/v PEG 2K MME
A2	SS	0.09 M potassium thiocyanate; 28.2 % w/v PEG 2K MME
A3	SS	0.09 M potassium thiocyanate; 29.4 % w/v PEG 2K MME
A3	SS	0.09 M potassium thiocyanate; 29.4 % w/v PEG 2K MME
A3	SPM1/R	0.09 M potassium thiocyanate; 29.4 % w/v PEG 2K MME
A4	VIM-2	0.09 M potassium thiocyanate; 30.6 % w/v PEG 2K MME
A4	SS	0.09 M potassium thiocyanate; 30.6 % w/v PEG 2K MME
A4	SS	0.09 M potassium thiocyanate; 30.6 % w/v PEG 2K MME
A4	VIM-2	0.09 M potassium thiocyanate; 30.6 % w/v PEG 2K MME
A5	VIM-2	0.09 M potassium thiocyanate; 31.8 % w/v PEG 2K MME
A5	SS	0.09 M potassium thiocyanate; 31.8 % w/v PEG 2K MME
A5	SS	0.09 M potassium thiocyanate; 31.8 % w/v PEG 2K MME
A5	VIM-2	0.09 M potassium thiocyanate; 31.8 % w/v PEG 2K MME
A6	VIM-2	0.09 M potassium thiocyanate; 33% w/v PEG 2K MME
A6	SS	0.09 M potassium thiocyanate; 33% w/v PEG 2K MME
A6	SS	0.09 M potassium thiocyanate; 33% w/v PEG 2K MME
A6	VIM-2	0.09 M potassium thiocyanate; 33% w/v PEG 2K MME
B1	SS	0.097 M potassium thiocyanate; 27 % w/v PEG 2K MME
B1	NS	0.097 M potassium thiocyanate; 27 % w/v PEG 2K MME
B1	NS	0.097 M potassium thiocyanate; 27 % w/v PEG 2K MME
B1	SS	0.097 M potassium thiocyanate; 27 % w/v PEG 2K MME
B2	SS	0.097 M potassium thiocyanate; 28.2 % w/v PEG 2K MME
B2	NS	0.097 M potassium thiocyanate; 28.2 % w/v PEG 2K MME
B2	NS	0.097 M potassium thiocyanate; 28.2 % w/v PEG 2K MME
B2	SS	0.097 M potassium thiocyanate; 28.2 % w/v PEG 2K MME
B3	SPM-1/R	0.097 M potassium thiocyanate; 29.4 % w/v PEG 2K MME
B3	VIM-2	0.097 M potassium thiocyanate; 29.4 % w/v PEG 2K MME
B3	SS	0.097 M potassium thiocyanate; 29.4 % w/v PEG 2K MME
B3	SS	0.097 M potassium thiocyanate; 29.4 % w/v PEG 2K MME
B3	VIM-2	0.097 M potassium thiocyanate; 29.4 % w/v PEG 2K MME
B4	VIM-2	0.097 M potassium thiocyanate; 30.6 % w/v PEG 2K MME
B4	SS	0.097 M potassium thiocyanate; 30.6 % w/v PEG 2K MME
B4	SS	0.097 M potassium thiocyanate; 30.6 % w/v PEG 2K MME
B4	VIM-2	0.097 M potassium thiocyanate; 30.6 % w/v PEG 2K MME
B5	VIM-2	0.097 M potassium thiocyanate; 31.8 % w/v PEG 2K MME

B5	SS	0.097 M potassium thiocyanate; 31.8 % w/v PEG 2K MME
B5	SS	0.097 M potassium thiocyanate; 31.8 % w/v PEG 2K MME
B5	VIM-2	0.097 M potassium thiocyanate; 31.8 % w/v PEG 2K MME
B6	VIM-2	0.097 M potassium thiocyanate; 33% w/v PEG 2K MME
B6	SS	0.097 M potassium thiocyanate; 33% w/v PEG 2K MME
B6	SS	0.097 M potassium thiocyanate; 33% w/v PEG 2K MME
B6	VIM-2	0.097 M potassium thiocyanate; 33% w/v PEG 2K MME
C1	SS	0.01 M potassium thiocyanate; 27 % w/v PEG 2K MME
C1	NS	0.01 M potassium thiocyanate; 27 % w/v PEG 2K MME
C1	NS	0.01 M potassium thiocyanate; 27 % w/v PEG 2K MME
C1	SS	0.01 M potassium thiocyanate; 27 % w/v PEG 2K MME
C2	SS	0.01 M potassium thiocyanate; 28.2 % w/v PEG 2K MME
C2	NS	0.01 M potassium thiocyanate; 28.2 % w/v PEG 2K MME
C2	NS	0.01 M potassium thiocyanate; 28.2 % w/v PEG 2K MME
C2	SS	0.01 M potassium thiocyanate; 28.2 % w/v PEG 2K MME
C3	VIM-2	0.01 M potassium thiocyanate; 29.4 % w/v PEG 2K MME
C3	SS	0.01 M potassium thiocyanate; 29.4 % w/v PEG 2K MME
C3	SS	0.01 M potassium thiocyanate; 29.4 % w/v PEG 2K MME
C3	VIM-2	0.01 M potassium thiocyanate; 29.4 % w/v PEG 2K MME
C4	VIM-2	0.01 M potassium thiocyanate; 30.6 % w/v PEG 2K MME
C4	SS	0.01 M potassium thiocyanate; 30.6 % w/v PEG 2K MME
C4	SS	0.01 M potassium thiocyanate; 30.6 % w/v PEG 2K MME
C4	VIM-2	0.01 M potassium thiocyanate; 30.6 % w/v PEG 2K MME
C5	VIM-2	0.01 M potassium thiocyanate; 31.8 % w/v PEG 2K MME
C5	SS	0.01 M potassium thiocyanate; 31.8 % w/v PEG 2K MME
C5	SS	0.01 M potassium thiocyanate; 31.8 % w/v PEG 2K MME
C5	VIM-2	0.01 M potassium thiocyanate; 31.8 % w/v PEG 2K MME
C6	VIM-2	0.01 M potassium thiocyanate; 33% w/v PEG 2K MME
C6	SS	0.01 M potassium thiocyanate; 33% w/v PEG 2K MME
C6	SS	0.01 M potassium thiocyanate; 33% w/v PEG 2K MME
C6	VIM-2	0.01 M potassium thiocyanate; 33% w/v PEG 2K MME
D1	SS	0.011 M potassium thiocyanate; 27 % w/v PEG 2K MME
D1	NS	0.011 M potassium thiocyanate; 27 % w/v PEG 2K MME
D1	NS	0.011 M potassium thiocyanate; 27 % w/v PEG 2K MME
D1	SS	0.011 M potassium thiocyanate; 27 % w/v PEG 2K MME
D2	SS	0.011 M potassium thiocyanate; 28.2 % w/v PEG 2K MME
D2	NS	0.011 M potassium thiocyanate; 28.2 % w/v PEG 2K MME
D2	NS	0.011 M potassium thiocyanate; 28.2 % w/v PEG 2K MME
D2	SS	0.011 M potassium thiocyanate; 28.2 % w/v PEG 2K MME
D3	VIM-2	0.011 M potassium thiocyanate; 29.4 % w/v PEG 2K MME
D3	VIM-2	0.011 M potassium thiocyanate; 29.4 % w/v PEG 2K MME

D4	VIM-2	0.011 M potassium thiocyanate; 30.6 % w/v PEG 2K MME
D4	SS	0.011 M potassium thiocyanate; 30.6 % w/v PEG 2K MME
D4	SS	0.011 M potassium thiocyanate; 30.6 % w/v PEG 2K MME
D4	VIM-2	0.011 M potassium thiocyanate; 30.6 % w/v PEG 2K MME
D5	VIM-2	0.011 M potassium thiocyanate; 31.8 % w/v PEG 2K MME
D5	SS	0.011 M potassium thiocyanate; 31.8 % w/v PEG 2K MME
D5	SS	0.011 M potassium thiocyanate; 31.8 % w/v PEG 2K MME
D5	VIM-2	0.011 M potassium thiocyanate; 31.8 % w/v PEG 2K MME
D6	VIM-2	0.011 M potassium thiocyanate; 33% w/v PEG 2K MME
D6	SS	0.011 M potassium thiocyanate; 33% w/v PEG 2K MME
D6	SS	0.011 M potassium thiocyanate; 33% w/v PEG 2K MME
D6	VIM-2	0.011 M potassium thiocyanate; 33% w/v PEG 2K MME

*R: reservoir, SS: self-seed, NS, no seed.

Table S5. Conditions resulted in crystals in the second tier MCMS.

Target MBL	JCSG+ code	Seed MBL	Condition Composition
SPM-1-L2	A5	SPM-1	0.2 M magnesium formate; 20 % w/v PEG 3350
	A10	SPM-1	0.2 M potassium formate; 20 % w/v PEG 3350
	D10	SS	0.2 M calcium acetate; 0.1 M Sodium cacodylate; pH 6.5; 40 % v/v PEG 300
	D10	SS/R	0.2 M calcium acetate; 0.1 M Sodium cacodylate; pH 6.5; 40 % v/v PEG 301
	D11	SS	0.14 M calcium chloride; 0.07 M Sodium acetate; pH 4.6; 14 % v/v 2-propanol/ 30 % v/v glycerol
	D11	SS/R	0.14 M calcium chloride; 0.07 M Sodium acetate; pH 4.6; 14 % v/v 2-propanol/ 30 % v/v glycerol
SPM-1	A7	SPM-1/O	0.1 M CHES; pH 9.5; 20 % w/v PEG 8K
	A10	SPM-1/O	0.2 M potassium formate; 20 % w/v PEG 3350
	B3	SPM-1/O	0.1 M Bicine; pH 9.0; 20 % w/v PEG 6K
	C11	SPM-1/O	0.1 M sodium acetate; pH 4.6; 2.0 M ammonium sulfate
	C11	VIM-2	0.1 M sodium acetate; pH 4.6; 2.0 M ammonium sulfate
	C11	SPM-1/O	0.1 M sodium acetate; pH 4.6; 2.0 M ammonium sulfate
	C11	VIM-2	0.1 M sodium acetate; pH 4.6; 2.0 M ammonium sulfate
	C11	SS	0.1 M sodium acetate; pH 4.6; 2.0 M ammonium sulfate
	D9	SS	0.17 M ammonium sulfate; 25.5 % w/v PEG 4K/ 15 % v/v glycerol
	D11	SS	0.14 M calcium chloride; 0.07 M Sodium acetate; pH 4.6; 14 % v/v 2-propanol/ 30 % v/v glycerol
	D11	SS	0.14 M calcium chloride; 0.07 M Sodium acetate; pH 4.6; 14 % v/v 2-propanol/ 30 % v/v glycerol
	D12	SPM-1/O	0.04 M potassium dihydrogen phosphate; 16 % w/v PEG 8K/ 20 % v/v glycerol
	D12	VIM-2	0.04 M potassium dihydrogen phosphate; 16 % w/v PEG 8K/ 20 % v/v glycerol
	E8	VIM-2	0.1 M sodium acetate; pH 4.5; 1.0 M di-ammonium hydrogen phosphate
	E8	VIM-2	0.1 M sodium acetate; pH 4.5; 1.0 M di-ammonium hydrogen phosphate
	E11	SS	0.16 M calcium acetate; 0.08 M sodium cacodylate; pH 6.5; 14.4 % w/v PEG 8K/ 20 % v/v glycerol
	H4	SS	0.2 M calcium chloride; 0.1 M Bis Tris; pH 5.5; 45 % v/v MPD
IMP-1	A10	SS	0.2 M potassium formate; 20 % w/v PEG 3350
	A12	SS	0.2 M potassium nitrate; 20 % w/v PEG 3350
	B2	SS	0.2 M sodium thiocyanate; 20 % w/v PEG 3350
	B4	SS	0.1 M HEPES; pH 7.5; 10 % w/v PEG 8K/ 8 % v/v ethylene

	B11	SPM-1/O	pH 6.5; 1.6 M tri-sodium citrate
	C12	SS	10 % w/v PEG 1000/ 10 % w/v PEG 8000
	D1	SS	24 % w/v PEG 1500/ 20 % v/v Glycerol
	G1	SS	0.1 M HEPES; pH 7.0; 30 % v/v Jeffamine ED-2001
	G2	SPM-1/O	0.02 M magnesium chloride; 0.1 M HEPES; pH 7.5; 22 % w/v polyacrylic acid 5100 sodium salt
	G4	SS	0.2 M tri-methylamine N-oxide; 0.1 M Tris; pH 8.5; 20 % w/v PEG 2K MME
	G9	SS	0.1 M potassium thiocyanate; 30 % w/v PEG 2K MME
	G10	SS	0.15 M potassium bromide; 30 % w/v PEG 2K MME
	H3	SS	0.1 M Bis Tris; pH 5.5; 25 % w/v PEG 3350
	H8	SS	0.2 M sodium chloride; 0.1 M Bis Tris; pH 5.5; 25 % w/v PEG 3350
B2-IMP-1	C11	SS	0.1 M sodium acetate; pH 4.6; 2.0 M ammonium sulfate
	D10	BcII	0.2 M calcium acetate; 0.1 M Sodium cacodylate; pH 6.5; 40 % v/v PEG 300
	D11	BcII	0.14 M calcium chloride; 0.07 M Sodium acetate; pH 4.6; 14 % v/v 2-propanol/ 30 % v/v glycerol
	E11	BcII	0.16 M calcium acetate; 0.08 M sodium cacodylate; pH 6.5; 14.4 % w/v PEG 8K/ 20 % v/v glycerol
	E11	BcII	0.16 M calcium acetate; 0.08 M sodium cacodylate; pH 6.5; 14.4 % w/v PEG 8K/ 20 % v/v glycerol
	F8	NDM-Δ42	pH 7.0; 2.1 M DL-malic acid
	F8	BcII	pH 7.0; 2.1 M DL-malic acid
	F9	NDM-Δ42	pH 7.0; 2.4 M sodium malonate
	F9	SS	pH 7.0; 2.4 M sodium malonate
	G10	SS	0.15 M potassium bromide; 30 % w/v PEG 2K MME
	H7	SS	0.2 M ammonium sulfate; 0.1 M Bis Tris; pH 5.5; 25 % w/v PEG 3350
	H9	SS	0.2 M lithium sulfate; 0.1 M Bis Tris; pH 5.5; 25 % w/v PEG 3350

*R: reservoir, SS: self-seed, NS: no seed, O: old.

Table S6. Interfaces comparison between the SPM-1-L2 structure and the seed crystals.

		BcII				**SPM-1**						**VIM-2**						**NDM**
	Interface Code	**AI**	**AF**	**AB**	**AC**	**AB**	**AC**	**Ac**	**AE**	**AN**	**AV**	**AG**	**AC**	**AD**	**AI**	**AB**	**AH**	**AB**
SPM-1-L2	**AB**	0.27	0.274	0.319	0.421	0.886	0.235	0.264	0.263	0.26	0.309	0.264	0.355	0.369	0.355	0.255	0.361	0.323
	AH	0.297	0.318	0.298	0.335	0.327	0.269	0.333	---	0.277	0.269	0.269	0.312	0.294	0.271	0.251	0.25	0.304
	AR	0.326	0.218	0.328	0.378	0.323	0.402	0.278	---	0.306	0.333	0.248	0.423	0.317	0.327	0.225	0.289	0.372

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Notes

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ABBREVIATIONS

MCMS, Micro-Cross-seeding Matrix Screening; MMS, microseeding matrix screening; 3D, three dimentional; TBNAT, arylamine N-acetyltransferase from *M. tuberculosis*; MMNAT, arylamine N-acetyltransferase from *M. marinum;* MBLs, metallo-β-lactamases; SBL, serine β-lactamases; PDB, Protein Data Bank; SEC-MALS, Size-Exclusion Chromatography and Multi-Angle Light Scattering; FFAS, Fold and Function Assignment System; RMSD, root mean square deviations; PEG, polyethylene glycol; MME, monomethyl ether; CSS, Complexation Significance Score;

BRIEFS .

SYNOPSIS

A systematic study of the minimum homology required for successful crystallisation of metallo-β-lactamases by the method of cross seeding is presented.

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