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Expression, crystallization and preliminary crystallographic data analysis of PigF, an *O*-methyltransferase from the prodigiosin-synthetic pathway in *Serratia*

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Prodigiosin, which is a member of the prodiginines, is a red linear tripyrrole compound. A gene cluster for the biosynthesis of prodigiosin has been identified in *Serratia* and most genes in the cluster have been functionally assigned. A bifurcated biosynthetic pathway for prodigiosin has previously been determined. The last step in the biosynthetic pathway of 4-methoxy-2,2′-bipyrrole-5-carbaldehyde (MBC) is catalyzed by PigF, which transfers a methyl group to 4-hydroxy-2,2′-bipyrrole-5-carbaldehyde (HBC) to form the terminal product MBC, but its catalytic mechanism is not known. To elucidate its mechanism, recombinant PigF was purified and crystallized. The crystals belonged to space group $P2_1$, with unit-cell parameters $a = 69.4$, $b = 52.4$, $c = 279.2$ Å, $\beta = 96.8^\circ$. The native crystals may contain six molecules in the asymmetric unit, with a V_M of 2.17 Å^3 Da^{-1} and a solvent content of 43.43%. A full data set was collected at 2.6 Å resolution using synchrotron radiation on beamline BL17U of Shanghai Synchrotron Radiation Facility (SSRF), People's Republic of China. Molecular replacement was unsuccessful. To solve the structure of PigF by experimental phasing, selenomethionine-derivativized protein crystals were prepared from a condition with 0.01 *M* spermidine as an additive. One crystal diffracted to 1.9 Å resolution and a full data set was collected on beamline BL17U at SSRF. The crystal belonged to space group $P2_1$, with unit-cell parameters $a = 69.0$, $b = 52.9$, $c = 93.4$ Å, $\beta = 97.3^\circ$. Heavy-atom substructure determination and phasing by SAD clearly showed that the crystal contains two molecules in the asymmetric unit, with a V_M of 2.19 Å^3 Da^{-1} and a solvent content of 43.82%.

1. Introduction

Serratia spp. are Gram-negative bacteria that are classified into the large family Enterobacteriaceae (Giri *et al.*, 2004). *Serratia* spp. occur in the natural environment (soil, water and plant surfaces) or as opportunistic human pathogens (Grimont & Grimont, 2005). *Serratia* spp. can secrete nucleases, lipases, chitinases and proteases (Hines *et al.*, 1988) and these enzymes can be used to distinguish the *Serratia* genus from others in the Enterobacteriaceae family. In addition to the secreted proteins, many strains of *Serratia* can produce a red pigment determined to be prodigiosin (Rapoport & Holden, 1962; Wasserman *et al.*, 1960); this is also a characteristic feature of *Serratia*. Prodigiosin is a member of the prodiginines and is a classic secondary metabolite that is only produced in the stationary stage of bacterial growth (Williams, 1973). Prodiginines are a family of red linear tripyrrole compounds produced by some *Serratia* species, actinomycetes and other bacteria (Williamson *et al.*, 2006) and mainly consist of four broad structural classes of compounds (Williamson *et al.*, 2006). The physiological function of prodigiosin is still not known. However, previous studies have illustrated that prodigiosin has antifungal, antibacterial, antiprotozoal/antimalarial, immunosuppressive and anticancer activities (Campàs *et al.*, 2003; D'Alessio *et al.*, 2000; Montaner *et al.*, 2000; Mortellaro *et al.*, 1999; Pérez-Tomás *et al.*, 2003; Williamson *et al.*, 2006; Zhou *et al.*, 2010). Several biosynthetic gene clusters for prodiginines have been cloned from various microorganisms (Cerdeño *et al.*, 2001; Harris *et al.*, 2004; Jeong *et al.*, 2005; Malpartida *et al.*, 1990; Thomson *et al.*, 2000). Bifurcated pathways for prodiginines have also been proposed. 2-Methyl-3-*n*-amyl-pyrrole

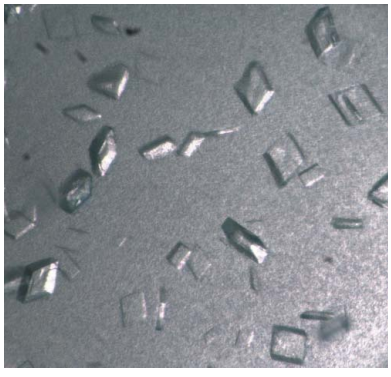

Table 1
Data-collection and processing statistics.

Values in parentheses are for the highest resolution shell.

	Native	SeMet†
Wavelength (Å)	0.9796	0.9793
Temperature (K)	100	100
Crystal-to-detector distance (mm)	220	220
Rotation range per image (°)	1.0	1.0
Total rotation range (°)	180	360
Space group	$P2_1$	$P2_1$
Unit-cell parameters (Å, °)	a = 69.4, b = 52.4, c = 279.2, $\alpha = \gamma = 90$, β = 96.8	a = 69.4, b = 52.4, c = 93.4, $\alpha = \gamma = 90$, β = 97.3
Resolution range (Å)	158520 (14063)	367696 (51696)
Unique reflections	56891 (5734)	102407 (14627)
Multiplicity	2.79 (2.45)	3.59 (3.53)
$R_{\rm merge}$‡ (%)	20.2 (81.0)	11.3 (57.4)
Completeness (%)	91.3 (87.3)	99.1 (99.4)
$\langle I/\sigma(I)\rangle$	6.09 (2.11)	11.66 (3.33)

† Friedel pairs were treated as different reflections during scaling in *XDS* for the SeMet data set. ‡ $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ and $\langle I(hkl)\rangle$ are the observed intensity of measurement i and the mean intensity of the reflection with indices hkl, respectively.

(MAP) or 2-undecylpyrrole and 4-methoxy-2,2′-bipyrrole-5-carbaldehyde (MBC) are synthesized in two different pathways and are then condensed to form prodigiosin or undecylprodigiosin. Most of the genes in the biosynthetic gene cluster participating in the synthesis of prodiginines are known (Harris *et al.*, 2004; Williamson *et al.*, 2005). The enzymes PigB, PigD and PigE from *Serratia* synthesize MAP, while PigA, PigG, PigH, PigI and PigJ have been demonstrated *in vitro* to comprise the assembly-line portion of the pathway that synthesizes 4-hydroxy-2,2′-bipyrrole-5-methanol (HBM; Garneau-Tsodikova *et al.*, 2006). HBM is then converted to 4-hydroxy-2,2′-bipyrrole-5-carbaldehyde (HBC) by PigM. The last step in the pathway to MBC is catalyzed by PigF, which transfers a methyl group to HBC to form the final product MBC (Williamson *et al.*, 2005). To elucidate the mechanism of the final step of MBC formation, we cloned and expressed the PigF gene from the previously isolated *Serratia* sp. FS14 (Zheng *et al.*, 2011) in *Escherichia coli*; the protein was then purified and crystallized.

2. Materials and methods

2.1. Gene cloning, protein expression and purification

The PigF gene was amplified using the chromosomal DNA of *Serratia* sp. FS14 as a template with the primers cgcacatATGCCTTTAACCAAGCAAGATGC and actctcgagTTTTTCGCCGACGATCAGG corresponding to the 5′ and 3′ ends, respectively, and *Pfu* polymerase. These primers introduced restriction sites for the endonucleases *Nde*I and *Xho*I at the 5′ and 3′ ends of the PCR product, respectively. The amplified PCR product was digested with *Nde*I and *Xho*I and then ligated into the expression vector pET24b to create the expression construct. This step introduced eight amino acids containing six consecutive histidines at the C-terminus of the target protein (LEHHHHHH). The construct was verified by restriction-enzyme digestion and DNA sequencing; the encoded protein sequence was identical to the previously reported sequence of PigF (GenBank CAH55651.1). The plasmid containing PigF was then transformed into *E. coli* C43 (DE3) cells for expression (Miroux & Walker, 1996). The freshly transformed colony was inoculated and grown at 310 K in Luria–Bertani (LB) medium containing kanamycin (30 µg ml^{-1}) overnight to an OD_{600} of 1.0; the cells were then induced with 0.5 m*M* IPTG and grown for a further 3 h at 303 K. The induced cells were harvested by centrifugation and the pellet was resuspended in binding buffer consisting of 50 m*M* Tris–HCl pH 8.0, 300 m*M* NaCl, 5 m*M* imidazole, 5% glycerol and supplemented with 1 m*M* PMSF just prior to sonication. Sonication was carried out on ice with a 200 W output for a total time of 30 min with a 1 s pulse-on and 3 s pulse-off sequence. The cell lysate was clarified by centrifugation for 30 min at 15 000 rev min^{-1} (Hitachi R20A2 rotor) and the target protein was purified by IMAC on Ni–NTA Superflow (Qiagen). The protocol used was as follows. The supernatant was loaded onto an Ni–NTA column previously equilibrated with binding buffer. After washing the column with six bed volumes of binding buffer containing 5, 20 and 50 m*M* imidazole, the target protein was eluted with elution buffer containing 100 and 250 m*M* imidazole. Fractions containing PigF were combined and concentrated to approximately 1 ml by centrifugation with an Amicon Ultra-15 filter (Millipore). The concentrated protein sample was then loaded onto a Superdex G-200 (Pharmacia Biotech) column pre-equilibrated with 20 m*M* Tris–HCl pH 8.0 containing 300 m*M* NaCl and 5% glycerol, and the column was eluted with 1.2 bed volumes of the same buffer. The elution pattern showed two protein peaks, which were analyzed by SDS–PAGE. The purity was checked by staining the gels with Coomassie Brilliant Blue R250. Since the first peak to elute appeared at the position of the void volume of the column, we pooled the fractions corresponding to the second peak together. The cells used for the expression of the selenomethionine-derivatized (SeMet) PigF were grown as described previously (Van Duyne *et al.*, 1993) and were induced as for native PigF; the SeMet PigF was purified following the same protocol as used for the native PigF. The protein purification was performed at 277 K.

2.2. Crystallization

Purified PigF was concentrated to 39 mg ml^{-1} in elution buffer using an Amicon Ultra-15 filter (Millipore) and was stored at 277 K. The concentration of PigF was determined from the absorbance at 280 nm, assuming an ε_{280} of 1.138 for a 1.0 mg ml^{-1} protein solution. Initial crystallization screens were performed using six different screening kits from Hampton Research (Index, MembFac, Crystal Screen, Crystal Screen 2, Crystal Screen Lite and Crystal Screen Cryo) employing the sitting-drop vapour-diffusion method with protein concentrations of 30 and 15 mg ml^{-1}. The crystallization drop was formed of 1 µl protein solution and 1 µl reservoir solution. All trials were stored at 295 K. The initial screen yielded thick plate-shaped crystals from the following conditions: 0.2 *M* $CaCl_2$, 0.1 *M* sodium acetate pH 4.6, 15%(*w*/*v*) 2-methyl-2,4-pentanediol (Crystal Screen Lite condition No. 1) and 0.1 *M* NaCl, 0.1 *M* sodium acetate pH 4.6, 12%(*w*/*v*) 2-methyl-2,4-pentanediol (MembFac condition No. 1). The data sets collected from the initial crystals could not be indexed. Both conditions were used for further optimization. The conditions were optimized by grid screening of the MPD concentration and the pH of the crystallization condition and the use of additive screens, detergent screens and heavy-atom screens. Crystals suitable for data processing were grown by mixing a 1 µl droplet of 15 mg ml^{-1} protein solution with the same amount of reservoir solution supplemented with 2 m*M* $K_2Pt(CN)_4$ and equilibrating against 200 µl reservoir solution [0.1 *M* NaCl, 0.1 *M* sodium acetate pH 4.6, 12%(*w*/*v*) MPD] using the hanging-drop method in 24-well plates. SeMet protein was crystallized by mixing a 1 µl droplet of 15 mg ml^{-1} protein solution with the same amount of reservoir solution supplemented with additives (Hampton Research) and equilibrating against 200 µl reservoir solution [0.1 *M* NaCl, 0.1 *M* sodium acetate pH 4.6, 12%(*w*/*v*) MPD] using the hanging-drop

method in 24-well plates. SeMet protein crystals used for data collection were obtained from a condition with 0.01 *M* spermidine as an additive. All crystals reached maximum dimensions after 2 d.

2.3. Data collection

Prior to cryocooling, crystals were looped out from the crystallization drop and sequentially transferred into fresh mother liquor with 10–20% glycerol in 5% steps for several seconds. After equilibration in the final solution, the crystal was flash-cooled in liquid nitrogen. Complete X-ray diffraction data sets were collected on beamline BL17U of Shanghai Synchrotron Radiation Facility (SSRF), Shanghai, People's Republic of China using a MAR 225 detector. The best crystal of native PigF diffracted to a resolution of 2.6 Å. A 1.9 Å resolution data set was collected from an SeMet PigF crystal. Each frame was exposed for 1.2 s with a rotation range of 1.0°.

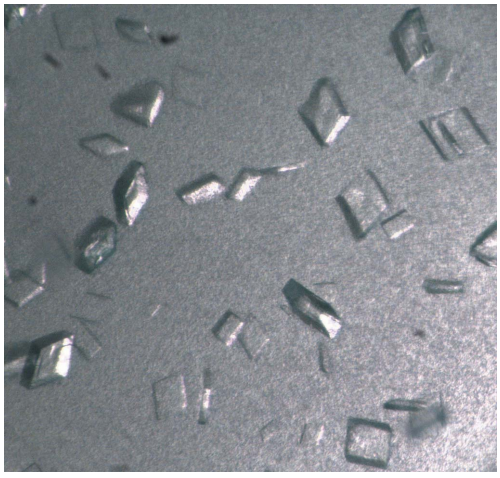

Figure 1
Crystals of PigF.

The data were processed using the *XDS* package (Kabsch, 2010*a*,*b*). The data-collection and processing statistics are summarized in Table 1.

3. Results and discussion

Recombinant PigF with a His_6 tag at the C-terminus was overexpressed in *E. coli* in a soluble form with a yield of about 20 mg of purified protein per litre of culture. Thick plate-shaped crystals appeared in the hanging drops at 295 K within 6 h and reached maximum dimensions in 2 d. Finally, single crystals with dimensions of 50 × 50 × 10 µm were obtained using 0.1 *M* NaCl, 0.1 *M* sodium acetate pH 4.6, 12%(*w*/*v*) MPD supplemented with 2 m*M* $K_2Pt(CN)_4$ as precipitant in 2 d at 295 K (Fig. 1). The best diffraction data were collected to 2.6 Å resolution using synchrotron X-rays. The crystals belonged to space group $P2_1$, with unit-cell parameters a = 69.4, b = 52.4, c = 279.2 Å, $\alpha = \gamma = 90$, $\beta = 96.8^\circ$ (Fig. 2). No anomalous signal from the Pt compound was detected in the native data.

Crystal structure determination of PigF was attempted by molecular replacement (MR) using the crystal structure of mitomycin 7-*O*-methyltransferase from *Streptomyces lavendulae* (PDB entry 3gwz; 25% amino-acid sequence identity; Singh *et al.*, 2011) or that of aclacinomycin-10-hydroxylase from *S. purpurascens* (PDB entry 1qzz; 28% amino-acid sequence identity; Jansson *et al.*, 2003) as a search model. MR was not successful. We therefore prepared SeMet PigF crystals and collected a complete 1.9 Å resolution data set to solve the crystal structure. The crystal belonged to space group $P2_1$, with unit-cell parameters a = 69.0, b = 52.9, c = 93.4 Å, $\beta = 97.3^\circ$ (Fig. 3). Experimental phasing was performed with *SHELXD*/*SHELXE* (Sheldrick, 2008) using *HKL2MAP* (Pape & Schneider, 2004). *SHELXD* detected six of 18 Se atoms with occupancy higher than 0.59; a clear decrease in occupancy was detected for the seventh Se atom (occupancy 0.27). The electron-density map after density modification by *SHELXE* showed that the asymmetric unit contained two molecules of PigF. The corresponding Matthews coefficient V_M

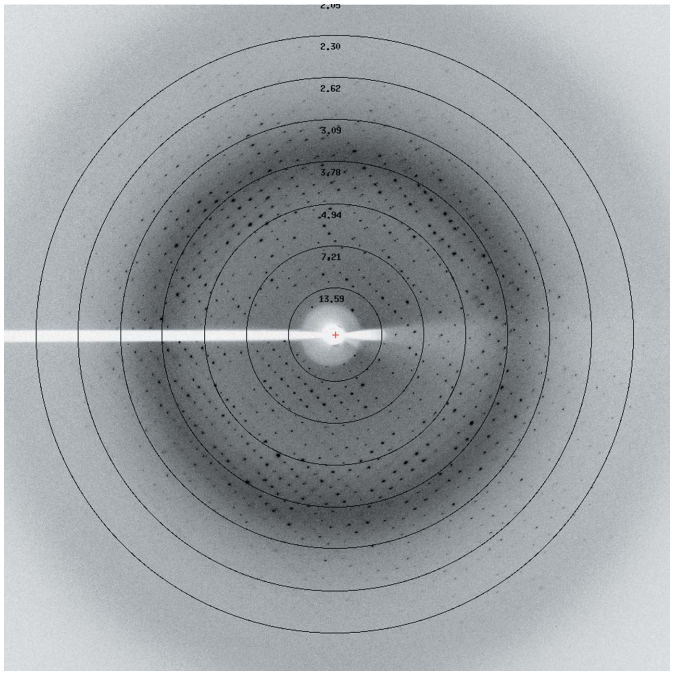

Figure 2
Diffraction image of a native PigF crystal.

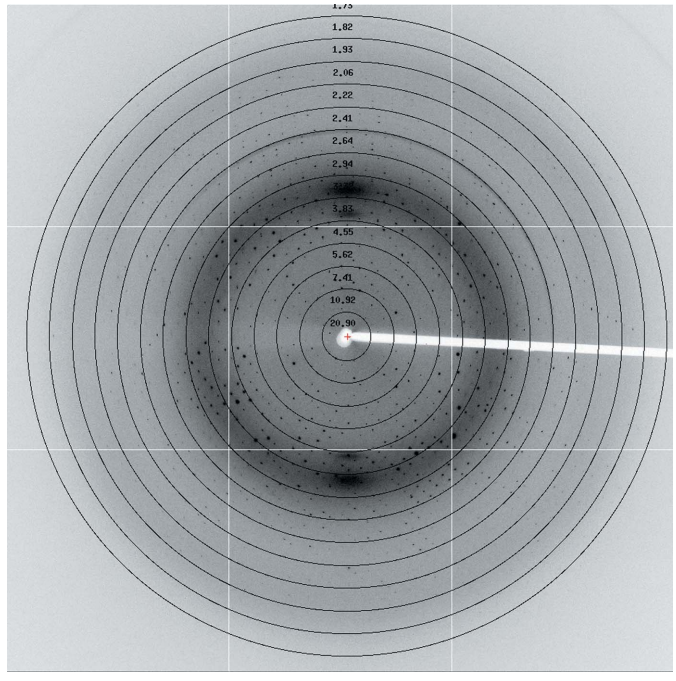

Figure 3
Diffraction image of an SeMet PigF crystal.

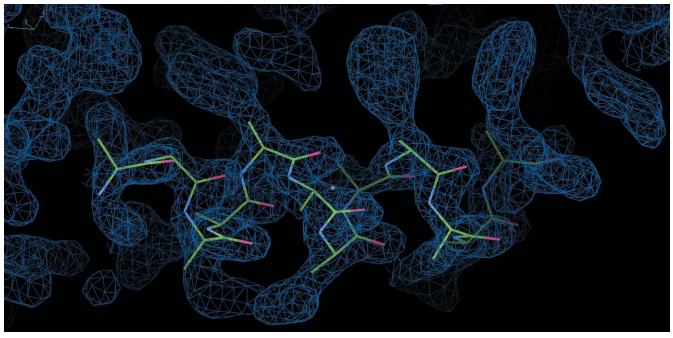

Figure 4
SAD electron-density map of PigF calculated from SeMet phases with a 1.5σ cutoff at 1.9 Å resolution using *SHELXC*/*D*/*E*.

(Matthews, 1968) was 2.19 Å^3 Da^{-1}, with a solvent content of 43.82%. Model building and structure refinement are in progress. Fig. 4 shows the electron density of a helix of PigF. From the results with SeMet PigF, we believe that six molecules per asymmetric unit is reasonable in the native crystal, giving a calculated Matthews coefficient V_M (Matthews, 1968) of 2.10 Å^3 Da^{-1}, corresponding to a solvent content of 41.48%.

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References

Campàs, C., Dalmau, M., Montaner, B., Barragán, M., Bellosillo, B., Colomer, D., Pons, G., Pérez-Tomás, R. & Gil, J. (2003). *Leukemia*, **17**, 746–750.
Cerdeño, A. M., Bibb, M. J. & Challis, G. L. (2001). *Chem. Biol.* **8**, 817–829.
D'Alessio, R., Bargiotti, A., Carlini, O., Colotta, F., Ferrari, M., Gnocchi, P., Isetta, A., Mongelli, N., Motta, P., Rossi, A., Rossi, M., Tibolla, M. & Vanotti, E. (2000). *J. Med. Chem.* **43**, 2557–2565.
Garneau-Tsodikova, S., Dorrestein, P. C., Kelleher, N. L. & Walsh, C. T. (2006). *J. Am. Chem. Soc.* **128**, 12600–12601.
Giri, A. V., Anandkumar, N., Muthukumaran, G. & Pennathur, G. (2004). *BMC Microbiol.* **4**, 11.
Grimont, F. & Grimont, P. A. D. (2005). *Bergey's Manual of Systematic Bacteriology*, 2nd ed., edited by G. M. Garrity, D. J. Brenner, N. R. Krieg & J. T. Staley, pp. 685–693. New York: Springer.
Harris, A. K., Williamson, N. R., Slater, H., Cox, A., Abbasi, S., Foulds, I., Simonsen, H. T., Leeper, F. J. & Salmond, G. P. (2004). *Microbiology*, **150**, 3547–3560.
Hines, D. A., Saurugger, P. N., Ihler, G. M. & Benedik, M. J. (1988). *J. Bacteriol.* **170**, 4141–4146.
Jansson, A., Niemi, J., Lindqvist, Y., Mäntsälä, P. & Schneider, G. (2003). *J. Mol. Biol.* **334**, 269–280.
Jeong, H. *et al.* (2005). *Nucleic Acids Res.* **33**, 7066–7073.
Kabsch, W. (2010*a*). *Acta Cryst.* D**66**, 133–144.
Kabsch, W. (2010*b*). *Acta Cryst.* D**66**, 125–132.
Malpartida, F., Niemi, J., Navarrete, R. & Hopwood, D. A. (1990). *Gene*, **93**, 91–99.
Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
Miroux, B. & Walker, J. E. (1996). *J. Mol. Biol.* **260**, 289–298.
Montaner, B., Navarro, S., Piqué, M., Vilaseca, M., Martinell, M., Giralt, E., Gil, J. & Pérez-Tomás, R. (2000). *Br. J. Pharmacol.* **131**, 585–593.
Mortellaro, A., Songia, S., Gnocchi, P., Ferrari, M., Fornasiero, C., D'Alessio, R., Isetta, A., Colotta, F. & Golay, J. (1999). *J. Immunol.* **162**, 7102–7109.
Pape, T. & Schneider, T. R. (2004). *J. Appl. Cryst.* **37**, 843–844.
Pérez-Tomás, R., Montaner, B., Llagostera, E. & Soto-Cerrato, V. (2003). *Biochem. Pharmacol.* **66**, 1447–1452.
Rapoport, H. & Holden, K. G. (1962). *J. Am. Chem. Soc.* **84**, 635–642.
Sheldrick, G. M. (2008). *Acta Cryst.* A**64**, 112–122.
Singh, S., Chang, A., Goff, R. D., Bingman, C. A., Grüschow, S., Sherman, D. H., Phillips, G. N. & Thorson, J. S. (2011). *Proteins*, **79**, 2181–2188.
Thomson, N. R., Crow, M. A., McGowan, S. J., Cox, A. & Salmond, G. P. (2000). *Mol. Microbiol.* **36**, 539–556.
Van Duyne, G. D., Standaert, R. F., Karplus, P. A., Schreiber, S. L. & Clardy, J. (1993). *J. Mol. Biol.* **229**, 105–124.
Wasserman, H. H., McKeon, J. E. & Santer, U. V. (1960). *Biochem. Biophys. Res. Commun.* **3**, 146–149.
Williams, R. P. (1973). *Appl. Microbiol.* **25**, 396–402.
Williamson, N. R., Fineran, P. C., Leeper, F. J. & Salmond, G. P. (2006). *Nature Rev. Microbiol.* **4**, 887–899.
Williamson, N. R., Simonsen, H. T., Ahmed, R. A., Goldet, G., Slater, H., Woodley, L., Leeper, F. J. & Salmond, G. P. (2005). *Mol. Microbiol.* **56**, 971–989.
Zheng, C. C., Wang, C. L., Fu, L., Wang, W. W. & Xu, D. Q. (2011). *Microbiol. China*, **38**, 228–236.
Zhou, W., Jin, Z.-X. & Wan, Y.-J. (2010). *Appl. Microbiol. Biotechnol.* **88**, 1269–1275.