Acta Crystallographica Section F
Structural Biology and Crystallization Communications
ISSN 1744-3091

Cloning, expression, purification, crystallization and preliminary X-ray analysis of *Ea*Lsc, a levansucrase from *Erwinia amylovora*

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Received 5 February 2013
Accepted 19 April 2013

The Gram-negative bacterium *Erwinia amylovora* is a destructive pathogen of Rosaceae. During infection, *E. amylovora* produces the exopolysaccharide levan, which contributes to the occlusion of plant vessels, causing the wilting of shoots. Levan is a fructose polymer that is synthesized by multifunctional enzymes called levansucrases. The levansucrase from *E. amylovora* (*Ea*Lsc) was heterologously expressed as a GST-fusion protein in *Escherichia coli*, purified and crystallized after tag removal. The protein crystallized in space group $P2_12_12$. X-ray diffraction data were acquired to 2.77 Å resolution. The structure of the enzyme was solved by molecular replacement. The asymmetric unit contains eight enzyme molecules, giving a solvent content of 58.74% and a Matthews coefficient of 2.98 Å^3 Da^{-1}.

1. Introduction

Erwinia amylovora is a Gram-negative bacterium belonging to the Enterobacteriaceae family which causes the devastating disease called fire blight in rosaceous plants such as apple, pear, raspberry, cotoneaster and pyracantha. *E. amylovora* infects the host plants primarily through the nectarthodes, the stoma-like openings in the flowers from which nectar is secreted, or through wounds in succulent tissues. After infection has been established, the bacteria can move through the vascular system of the plant, thus spreading the infection to other tissues. *E. amylovora* synthesizes two different types of exopolysaccharides, amylovoran and levan, which cause occlusion of the plant vessels, resulting in the characteristic fire-blight wilting (Gross *et al.*, 1992).

Levan is a β-2,6-linked fructose polymer that is synthesized by the enzyme levansucrase. Mutants for levansucrase displayed reduced virulence, demonstrating that levan formation plays a role in the virulence of *E. amylovora* (Geier & Geider, 1993). The importance of levan in virulence is also underpinned by the up-regulation of levansucrase during the infection of immature pear tissue (Zhao *et al.*, 2005). However, to date the molecular mechanisms by which levan elicits its function are not fully understood. Analogy with other bacterial species suggests that levan might represent an initial protection against the plant defence mechanisms (Cote & Ahlgren, 1993).

Levansucrases (EC 2.4.1.10) are multifunctional enzymes that catalyze both sucrose-hydrolysis and transfructosylation reactions. In the transferase reaction, the fructose residue released from sucrose hydrolysis can be linked to a variety of acceptors such as another sucrose molecule, leading to oligofructoside synthesis, or fructans (polymerase reaction). Fructooligosaccharides have received attention as low-calorie and noncariogenic sweeteners and as selective energy sources and as 'pre-biotics' for beneficial microorganisms in the intestinal flora (Salminen *et al.*, 1996).

Based on structural and functional similarity, levansucrases are classified into glycosyl hydrolase family 68 (GH 68) according to the Carbohydrate-Active Enzymes (CAZy) database (http://www.cazy.org; Henrissat, 1991). These enzymes are closely related at the amino-acid sequence level to inulosucrases (EC 2.4.1.9), which form inulin-type β-2,1-fructans. Three crystal structures of levansucrases from the

Gram-positive bacteria *Bacillus subtilis* (Meng & Fütterer, 2003), *B. megaterium* (Strube *et al.*, 2011) and *Arthrobacter* sp. K-1 (Tonozuka *et al.*, 2012) and one from the Gram-negative bacterium *Gluconacetobacter diazotrophicus* (Martínez-Fleites *et al.*, 2005) have been reported, revealing the structural determinants of substrate recognition and the spatial organization of three key catalytic acidic residues, DED, in the active site acting as a nucleophile, as a proton donor and as a transition-state stabilizer, respectively (Meng & Fütterer, 2008). The core structure is highly conserved across the organisms and features a five-bladed β-propeller topology. Levansucrases are usually monomeric enzymes. However, reports of dimeric levansucrases can be found in the literature (Yanase *et al.*, 1992; Goldman *et al.*, 2008; Ohtsuka *et al.*, 1992; Kang *et al.*, 2005).

Whilst sucrose hydrolysis by levansucrases is well characterized, less is known about the transfructosylation reactions that they catalyze. In fact, despite their having the same active-site architecture, the product spectrum of these enzymes varies significantly from high-molecular-weight fructans to oligosaccharides, suggesting that the enzymes can employ a processive or nonprocessive type of reaction based on structural determinants (Kralj *et al.*, 2008; Ozimek *et al.*, 2006). Biotechnological interest in the production of fructooligosaccharides has engaged researchers in the search for these determinants, which are apparently localized outside the core structure (Anwar *et al.*, 2012).

The structure–function relationships in bacterial levansucrases, and in bacterial fructansucrases more generally, are therefore still not completely understood and further effort is required in their biochemical and structural characterization. In this paper, we report the cloning, expression, purification, crystallization and preliminary X-ray analysis of a levansucrase from *E. amylovora*, a key enzyme in the virulence of the pathogen and a potential biocatalyst for the biotechnological production of fructooligosaccharides.

2. Materials and methods

2.1. Cloning, overexpression and purification of *E. amylovora* levansucrase

The levansucrase gene (ENA accession No. CBJ48143.1) was amplified by PCR from genomic DNA isolated from *E. amylovora* strain Ea273 (ATCC 49946) using the following primers: Lsc-F, 5′-CGATCA**CCATGG**GCAGATTATAATTATAAACCAACGC, and Lsc-R, 5′-CGA**GAATTC**TTATTTTAAAGTAATGTCTTTCATTGC. These primers included *Nco*I and *Eco*RI restriction sites, respectively (shown in bold). PCR was performed using KOD Hot Start DNA polymerase (EMD4Biosciences, Germany) with the following cycling profile: 368 K for 2 min followed by 30 cycles of 368 K for 30 s, 325 K for 20 s and 345 K for 20 s and finally followed by an extension cycle at 345 K for 2 min. Reaction products were visualized by agarose gel electrophoresis stained with GelRed (Biotium, USA) and were purified from the gel using a QIAquick Gel Extraction Kit (Qiagen, Germany) according to the manufacturer's instructions. The purified PCR product was treated for 3 h at 310 K with *Nco*I and *Eco*RI (NEB, USA) for double digestion, purified using a QIAquick PCR Purification Kit (Qiagen, Germany) and ligated into pETM-30 vector (Dümmler *et al.*, 2005), which contains a sequence for the expression of a His_6-GST-tagged fusion protein containing a TEV protease-cleavage site. The construct was propagated in *Escherichia coli* NovaBlue cells (EMD4Biosciences, Germany), purified using a DNA Miniprep Kit (Sigma, USA) and sequenced by Microsynth AG (Switzerland) to test the correctness of the gene sequence.

E. coli BL21 (DE3) chemically competent cells (EMD4Biosciences, Germany) were transformed with the pETM-30::Lsc construct for expression of the recombinant protein. After initial small-scale expression trials, which demonstrated good levels of protein expression, the transformed cells were grown overnight in 10 ml 2×YT medium containing kanamycin (30 µg ml^{-1}) at 310 K. The culture was used to seed 1 l medium (1:100 dilution) and was grown at 310 K for 3 h (to an OD of 0.8); the temperature was then decreased to 293 K and the culture was left to equilibrate for 1 h before induction with 1 m*M* IPTG for 16 h. Cells were harvested by centrifugation at 4500*g* for 15 min at 277 K, resuspended in 100 ml ice-cold PBS and centrifuged again, yielding 6 g of wet cell paste. The cells were resuspended in 50 ml ice-cold PBS containing 0.2 mg ml^{-1} lysozyme and a protease-inhibitor tablet (Roche, Switzerland), stirred for 30 min at room temperature and lysed by sonication (Soniprep, MSE, UK) on ice for 2 min using 10 s cycles (15.6 MHz). Cell debris was removed by centrifugation at 18 000*g* for 20 min at 277 K. After filtration through a 0.45 µm cellulose acetate filter, the cell extract was loaded onto a GSTrap HP 5 ml column (GE Healthcare, Sweden) equilibrated with PBS at a flow rate of 1.5 ml min^{-1}. The column was then washed with PBS until the A_{280} reached baseline and the enzyme was eluted with 10 m*M* reduced glutathione in 50 m*M* Tris–HCl buffer pH 8.0. The enzyme solution was treated with 0.4 mg of a solubility-enhanced L56V/S135G TEV protease (Cabrita *et al.*, 2007) at room temperature for 2 h for removal of the GST tag. Since both the recombinant TEV and the GST-tagged protein contained a His tag, the reaction mixture was passed through a HisTrap HP 1 ml column to remove the tagged proteins, including the uncleaved GST-fusion levansucrase, from the solution. The flowthrough containing the cleaved enzyme was concentrated and loaded onto a Sephadex S75 column (1.6 × 60 cm) equilibrated with 25 m*M* Tris–HCl buffer pH 7.5 containing 150 m*M* NaCl at a flow rate of 1 ml min^{-1}. All purification steps were carried out at room temperature. The protein purity was confirmed by SDS–PAGE.

2.2. Activity assay

Sucrose hydrolysis was evaluated in 0.1 *M* potassium phosphate buffer pH 6.5 containing 10 m*M* sucrose. 1 µg enzyme was added to the reaction mixture (1 ml total volume) and the sample was incubated at 310 K for 30 min. The release of glucose and fructose was measured using a D-glucose/D-fructose enzymatic assay (Megazyme).

2.3. Thermofluor assay

A thermal shift assay (Ericsson *et al.*, 2006) was performed in 100 m*M* HEPES buffer pH 7.5 containing 100 m*M* NaCl using SYPRO Orange (Invitrogen) as the fluorescent dye. Thermal denaturation was measured between 281 and 368 K in an iCycler iQ5 Multicolor Real Time PCR Detection System (Bio-Rad, USA).

2.4. Crystallization

The protein was used for crystallization trials at a concentration of 10 mg ml^{-1} in 25 m*M* Tris–HCl buffer pH 7.5 containing 150 m*M* NaCl. The protein concentration was determined by direct UV measurement at 280 nm on a NanoVue spectrophotometer using an extinction coefficient of 86 540 M^{-1} cm^{-1} as determined by *ProtParam*. Protein crystallization trials were carried out at 293 K using the microbatch-under-oil method in 96-well MRC plates (Cambridge, England) and volatile oil (catalogue No. MD2-06, Molecular Dimensions). The crystallization wells were protected

from drying using adhesive ClearView sheets (Molecular Dimensions). Drops of 1 µl protein solution were added to 15 µl volatile oil, immediately followed by 1 µl of precipitant from the crystallization kits (PACT *premier* HT-96 and JCSG-*plus* HT-96, Molecular Dimensions) using a multichannel pipette. The best crystals appeared after three weeks in conditions G9 (0.1 *M* KSCN, 30% PEG 2000 MME) and G10 (0.15 *M* KBr, 30% PEG 2000 MME) of the JCSG-*plus* screen and were reproduced by hanging-drop vapour diffusion by adding 1 µl 35% PEG 2000 MME and either 0.2 *M* KBr or 0.1 *M* KSCN to 1 µl protein solution. The drops were equilibrated against 1 ml precipitant solution using a 24-well Linbro plate (Hampton Research). Protein crystals appeared as stacked plates within three weeks and grew to maximum dimensions of about 0.3 × 0.3 × 0.1 mm. The crystallization solution provided sufficient cryoprotection and crystals were scooped out from the mother liquor using CryoLoops and flash-cooled in liquid nitrogen for storage in a dewar for subsequent transport to the beamline.

2.5. Data collection and processing

Diffraction data were collected at 100 K using synchrotron radiation on the EMBL P13 beamline at the PETRA III storage ring, c/o DESY, Hamburg, Germany. The wavelength was set to 0.968 Å using an Si(III) crystal monochromator (FMB, Oxford). Data were collected using an unfocused beam collimated with a 50 µm diameter aperture. The beamline was equipped with a PILATUS 6M detector (DECTRIS) and an MD2 goniometer (EMBL) with a horizontal spindle axis. The data were processed using *XDS* (Kabsch, 2010) and were scaled with *SCALA* (Evans, 2006). The structure was solved by molecular replacement using the *BALBES* pipeline (Long *et al.*, 2008). From the structures in the PDB, *BALBES* chose *G. diazotrophicus* levansucrase (Martínez-Fleites *et al.*, 2005; PDB entry 1w18), which has a sequence identity of 41.2% to *Ea*Lsc, as the best model for molecular replacement.

3. Results and discussion

The levansucrase gene was amplified from genomic DNA and ligated into a pETM-30 expression vector between the *Nco*I and *Eco*RI restriction sites. The *Nco*I recognition sequence has an in-frame ATG codon that can be used for functional expression of the target protein, minimizing the number of non-native amino acids at the N-terminus. However, in order to complete the recognition sequence of *Nco*I a Gly residue is required after the AGT codon. This introduces a mutation in the amino acid immediately following the starting Met, which in our case was a Ser-to-Ala mutation. Removal of the GST tag resulted in only two extra amino acids (Gly and Ala) in the recombinant protein before the starting Met.

GST-tagged levansucrase was overexpressed in *E. coli*, yielding on average 25 mg protein per litre of culture. The presence of a His_6 tag on the GST domain and the use of recombinant His_6-tagged TEV greatly simplified the purification of the levansucrase after TEV protease treatment, allowing their removal together with residual

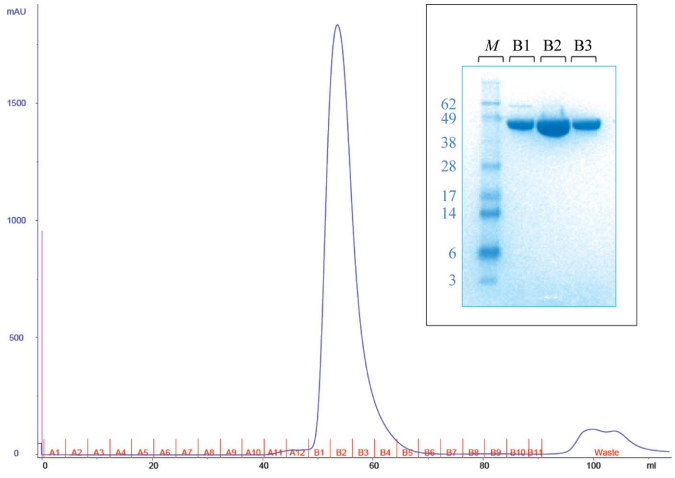

Figure 1
Purification of *Ea*Lsc. (*a*) Elution profile of *Ea*Lsc on size-exclusion chromatography (SEC) using a Superdex 75 16/60 column. (*b*) SDS–PAGE analysis of peak fractions from SEC purification, showing the homogeneity of *Ea*Lsc immediately prior to crystallization.

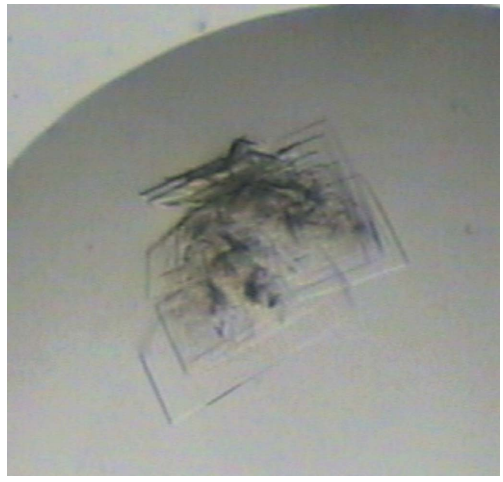

Figure 2
A cluster of *Ea*Lsc crystal plates obtained by hanging-drop vapour diffusion by adding 1 µl 35% PEG 2000 MME and 0.1 *M* KSCN to 1 µl protein solution followed by equilibration against 1 ml 35% PEG 2000 MME, 0.1 *M* KSCN.

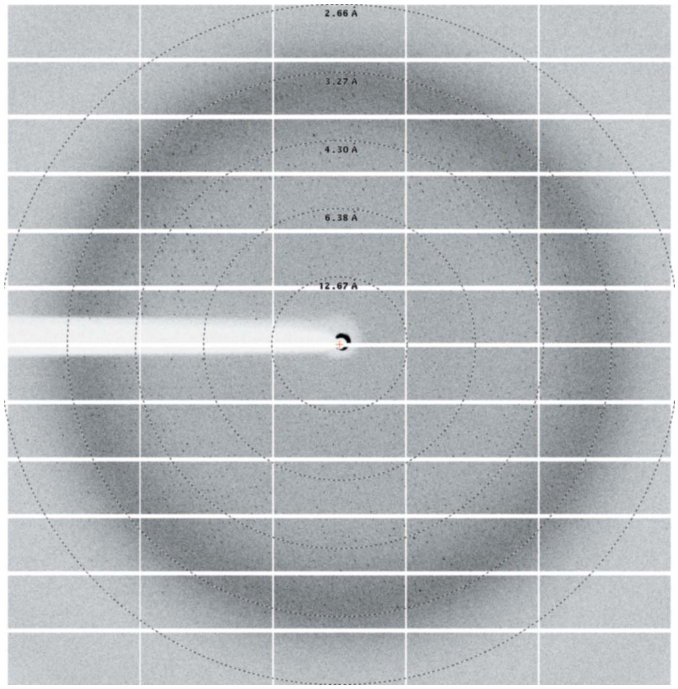

Figure 3
A diffraction image of an *Ea*Lsc crystal collected on a PILATUS 6M detector.

Table 1
X-ray data-collection statistics.

Values in parentheses are for the outermost resolution shell.

Beamline	P13, EMBL, Hamburg, Germany
Wavelength (Å)	0.968
No. of crystals	1
Detector	PILATUS 6M, DECTRIS
$\Delta\varphi$ per image (°)	0.1
No. of images	1600
Exposure time (s)	0.5
Crystal-to-detector distance (mm)	568.06
Space group	$P2_12_12$
Unit-cell parameters (Å)	
a	155.589
b	178.736
c	158.806
Resolution (Å)	178.74–2.77 (2.92–2.77)
Total No. of observations	678862
No. of unique observations	112787
$R_{\rm merge}$†	0.184 (0.591)
$R_{\rm p.i.m.}$‡	0.074 (0.235)
$\langle I/\sigma(I)\rangle$	7.5 (3.0)
Multiplicity	6.0 (6.2)
Completeness (%)	99.9 (99.9)
Crystal mosaicity§ (°)	0.102
B factor from Wilson plot (Å^2)	28.2

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is an individual intensity measurement and $\langle I(hkl)\rangle$ is the average intensity for this reflection. ‡ $R_{\rm p.i.m.} = \sum_{hkl}\{1/[N(hkl)-1]\}^{1/2}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is an individual intensity measurement and $\langle I(hkl)\rangle$ is the average intensity for this reflection. § REFLECTING_RANGE_E.S.D. as indicated by *XDS* (Kabsch, 2010).

uncleaved fusion protein by a quick immobilization on a nickel column. Nevertheless, size-exclusion chromatography (SEC) was necessary to remove the few contaminants still present in the enzyme solution and to provide information about the molecular weight of the enzyme. EaLsc eluted as a monomer with an apparent molecular weight of 47 kDa. The elution profile and the results of SDS–PAGE analysis of this last purification step are shown in Fig. 1. SDS–PAGE analysis of fractions B2 and B3 showed a single band migrating at approximately 47 kDa, which was in agreement with the theoretical molecular weight (46 536 Da) and the SEC analysis.

The hydrolytic activity of the recombinant enzyme was evaluated by measuring the concentrations of glucose and fructose released from sucrose. This experiment showed that the recombinant enzyme was indeed active under the assay conditions.

The thermal stability experiment showed that under the assay conditions the protein melting point was about 330 K and that the protein started to unfold at temperatures higher than 318 K, suggesting that the protein is stable at room temperature.

The crystals of levansucrase (Fig. 2) could be assigned to the orthorhombic space group $P2_12_12$ and diffracted to 2.77 Å resolution (Fig. 3). Data-collection statistics are reported in Table 1. The protein structure was solved by molecular replacement, with an initial R = 38.5% and $R_{\rm free}$ = 43.1%. The asymmetric unit contains eight molecules of *Ea*Lsc, giving a solvent content of 58.74% and a Matthews coefficient of 2.98 Å^3 Da^{-1}. Model building and refinement are currently under way.

We thank Dr M. Malnoy for providing genomic DNA from *E. amylovora* strain Ea273 and the Sample Preparation and Characterization (SPC) facility of EMBL Hamburg for performing the Thermofluor experiment. We thank also Professor Steve Bottomley from Monash University for kindly providing the solubility-enhanced L56V/S135G TEV protease clone. Plasmid pETM-30 was obtained from the European Molecular Biology Laboratory under a signed Material Transfer Agreement. Data were collected under European Molecular Biology Laboratory beam-time award No. MX-40. This work was financially supported by the Autonomous Province of Bolzano (project: A structural genomics approach for the study of the virulence and pathogenesis of *Erwinia amylovora*) and the Free University of Bolzano (project: *Erwinia amylovora*'s secretome).

References

Anwar, M. A., Leemhuis, H., Pijning, T., Kralj, S., Dijkstra, B. W. & Dijkhuizen, L. (2012). *FEBS J.* **279**, 3612–3621.
Cabrita, L. D., Gilis, D., Robertson, A. L., Dehouck, Y., Rooman, M. & Bottomley, S. P. (2007). *Protein Sci.* **16**, 2360–2367.
Cote, G. L. & Ahlgren, J. A. (1993). *Science and Technology of Fructans*, edited by M. Suzuki & N. J. Chatterton, pp. 141–168. Boca Raton: CRC Press.
Dümmler, A., Lawrence, A. M. & de Marco, A. (2005). *Microb. Cell Fact.* **4**, 34.
Ericsson, U. B., Hallberg, B. M., DeTitta, G. T., Dekker, N. & Nordlund, P. (2006). *Anal. Biochem.* **357**, 289–298.
Evans, P. (2006). *Acta Cryst.* D**62**, 72–82.
Geier, G. & Geider, K. (1993). *Physiol. Mol. Plant Pathol.* **42**, 387–404.
Goldman, D., Lavid, N., Schwartz, A., Shoham, G., Danino, D. & Shoham, Y. (2008). *J. Biol. Chem.* **283**, 32209–32217.
Gross, M., Geier, G., Rudolph, K. & Geider, K. (1992). *Physiol. Mol. Plant Pathol.* **40**, 371–381.
Henrissat, B. (1991). *Biochem. J.* **280**, 309–316.
Kabsch, W. (2010). *Acta Cryst.* D**66**, 125–132.
Kang, H. K., Seo, M. Y., Seo, E. S., Kim, D., Chung, S. Y., Kimura, A., Day, D. F. & Robyt, J. F. (2005). *Biochim. Biophys. Acta*, **1727**, 5–15.
Kralj, S., Buchholz, K., Dijkhuizen, L. & Seibel, J. (2008). *Biocatal. Biotransformation*, **26**, 32–41.
Long, F., Vagin, A. A., Young, P. & Murshudov, G. N. (2008). *Acta Cryst.* D**64**, 125–132.
Martínez-Fleites, C., Ortíz-Lombardía, M., Pons, T., Tarbouriech, N., Taylor, E. J., Arrieta, J. G., Hernández, L. & Davies, G. J. (2005). *Biochem. J.* **390**, 19–27.
Meng, G. & Fütterer, K. (2003). *Nature Struct. Biol.* **10**, 935–941.
Meng, G. & Fütterer, K. (2008). *BMC Struct. Biol.* **8**, 16.
Ohtsuka, K., Hino, S., Fukushima, T., Ozawa, O., Kanematsu, T. & Uchida, T. (1992). *Biosci. Biotechnol. Biochem.* **56**, 1373–1377.
Ozimek, L. K., Kralj, S., van der Maarel, M. J. & Dijkhuizen, L. (2006). *Microbiology*, **152**, 1187–1196.
Salminen, S., Isolauri, E. & Salminen, E. (1996). *Antonie Van Leeuwenhoek*, **70**, 347–358.
Strube, C. P., Homann, A., Gamer, M., Jahn, D., Seibel, J. & Heinz, D. W. (2011). *J. Biol. Chem.* **286**, 17593–17600.
Tonozuka, T., Tamaki, A., Yokoi, G., Miyazaki, T., Ichikawa, M., Nishikawa, A., Ohta, Y., Hidaka, Y., Katayama, K., Hatada, Y., Ito, T. & Fujita, K. (2012). *Enzyme Microb. Technol.* **51**, 359–365.
Yanase, H., Iwata, M., Nakahigashi, R., Kita, K., Kato, N. & Tonomura, K. (1992). *Biosci. Biotechnol. Biochem.* **56**, 1335–1337.
Zhao, Y., Blumer, S. E. & Sundin, G. W. (2005). *J. Bacteriol.* **187**, 8088–8103.