Increasing the soluble expression and crystallization of the *Escherichia coli* quorum-sensing protein LsrK

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LsrK is one of the key components of the *luxS*-regulated (*lsr*) operon in *Escherichia coli* and plays an important role during the quorum-sensing (QS) process mediated by autoinducer-2 (AI-2). The AI-2 molecule is imported into the cell by the LsrACB transporter and is subsequently phosphorylated (to AI-2-P) by LsrK. AI-2-P binds to the repressor protein of the *lsr* operon (LsrR) and triggers various cellular responses related to QS by dissociating LsrR from the DNA. Although a large amount of purified LsrK is required for structural studies, recombinant GST-LsrK was mostly expressed in an insoluble form. To enhance the soluble expression of LsrK, an attempt was made to increase the expression of the cellular chaperone proteins that are well known to support proper protein folding. Transformed *E. coli* was cultured in high-salt LB medium and heat shock was applied prior to subsequent IPTG induction at 20°C. These procedures increased the yield of purified LsrK by about tenfold compared with standard IPTG induction at 20°C. The expressed LsrK was readily purified by GST-affinity chromatography. Crystals of LsrK were grown by the hanging-drop vapour-diffusion method. The X-ray diffraction data of the crystal were processed in a primitive hexagonal space group to 2.9 Å resolution.

1. Introduction

Quorum sensing (QS) is a type of cell-to-cell communication used by bacteria that regulates many bacterial phenotypes by sensing inter-species or intra-species populations. This process is accomplished by small signalling molecules called auto-inducers (AIs), which are produced by bacteria and accumulate in the extracellular medium (Fuqua *et al.*, 1994). The AI molecules are classified into several families according to the bacterial species and their processing mechanisms: *N*-acyl L-homoserine lactones (AHLs; AI-1), autoinducer-2 (AI-2; Gamby *et al.*, 2012) and auto-inducing peptides (AIPs; Gospodarek *et al.*, 2009). Unlike AI-1 (AHLs) and AIPs, AI-2 seems to mediate inter-species communication as the *luxS* gene has been identified in both Gram-positive and Gram-negative bacteria and the LuxS protein is a key enzyme for AI-2 synthesis. The AI-2 molecule (4*S*)-4,5-dihydroxy-2,3-pentanedione (DPD) is synthesized from *S*-adenosylhomocysteine by *S*-ribosylhomocysteinase (LuxS) and methylthioadenosine nucleosidase (Roy, Smith *et al.*, 2010). AI-2 signalling is regulated by the *lsr* (*luxS*-regulated) operon in the enteric bacteria *Salmonella enterica* serovar Typhimurium and *Escherichia coli*. LsrK plays an important role during QS by phosphorylating the imported AI-2 molecule to AI-2-P, which eventually initiates QS signalling by binding to LsrR (Keller & Surette, 2006). Both LsrK and LsrR are promising drug targets for the development of novel antibiotics, since deletion of the

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Table 1
Macromolecule-production information.

Source organism	<i>E. coli</i> (K-12 strain)
DNA source	<i>E. coli</i> (K-12 strain)
Forward primer	5'-CCGAGATCTATGGCTCGACTCTTTACCCCTT-CAG-3'
Reverse primer	5'-CCGCTCGAGCTATAACCCAGGCGCTTCCAT-AAC-3'
Cloning vector	pGEX-4T-1 (GE Healthcare)
Expression vector	pGEX-4T-1 (GE Healthcare)
Expression host	<i>E. coli</i> BL21 (DE3)
Complete amino-acid sequence of the construct produced	MARLFTLSESKYYLMALDAGTGSIRAVIFDLEGN-QIAGVQAEWRHLAVPDVPGSMEFDLNKNWQLA-CECMRQALHNAGIAPEYIAAVSACSMREGIVL-YNNEGAPIWACANVDARAAREVSELKELHNNT-FENEVYRATGQTLALSAIPRLWLHHRSDIY-RQASTITMISDWLAYMLSGELAVDPSNAGTGLLDLTTRDWKPALDMAGLRADILSPVKETGT-LLGVVSSQAELCGLKAGTPVVVGGGDVQLGC-LGLGVVRPAQTAVLGTFWQQVNNLAAPVTDPEMNVVRNPHVIPGMVQAESISFFTGLTMRWFR-DAFCAEEKLIAERLGIDTYTLLEEMASRVPPG-SWGVMPIFSMDRMFKTYHAAPSFINLSIDPD-KCNKATLFRALAEENAAIVSACNLQIADFSNI-KPSSLVFAGGGSGKGLWSQILADVSGLPVNIIP-VVKEATALGCAIAAGVGAGIFSSMAETGERLV-RWERTHTPDPEKHELYQDSRDKWQAVYQDQLG-LVDHGLTTLTLWKAPGL

lsrK and *lsrR* genes was found to alter the expression of 146 and 149 genes, respectively, and greatly reduced biofilm formation by *E. coli* (Roy, Fernandes *et al.*, 2010; Li *et al.*, 2007). Our group recently showed that the binding of AI-2-P to LsrR destabilizes the tetrameric structure of LsrR, which triggers dissociation of LsrR from the target DNA, while the binding of phosphorylated isobutyl DPD (an AI-2-P antagonist) does not. Moreover, a different LsrR-binding mode utilized by ribose 5-phosphate was also identified, which can be exploited for the design of different antagonists (Ha *et al.*, 2013). Similarly, the structure of LsrK will be very useful in developing a drug that inhibits phosphorylation.

The three-dimensional structure of LsrK has not yet been elucidated, and the exact binding sites for the substrates (DPD and ATP) have not yet been characterized even at the level of the amino-acid sequence. A larger amount of protein is required for the determination of the three-dimensional structure. However, recombinant LsrK is barely expressed in *E. coli*, and overexpression mostly results in insoluble LsrK. There have been many reports on increasing the soluble expression of proteins in *E. coli*. It has been shown that heat shock prior to IPTG induction can prevent some proteins from moving to the inclusion body (Chen *et al.*, 2002). Exposure of *E. coli* to heat stress induces the expression of various heat-shock proteins (HSPs), many of which function as molecular chaperones, assisting protein folding by disassembling the aggregated proteins or by preventing the aggregation of proteins. Chaperones that are co-expressed with a target protein are able to prevent aggregation or to arrange the disulfide bonds of the recombinant protein correctly in several cases (Schlieker *et al.*, 2002). It has also been reported that the addition of osmolytes or salts to the growth medium enhances the soluble expression of proteins (Bolen & Baskakov, 2001; Thomas & Baneyx, 1996; Oganessian *et al.*, 2007).

In this study, we carried out the overexpression, purification and crystallization of LsrK to solve its three-dimensional structure. An increased amount of LsrK was obtained through osmotic shock and heat shock prior to overnight IPTG induction at 20°C. The purified recombinant LsrK protein was crystallized and X-ray diffraction data were obtained at 2.9 Å resolution. Further improvement of the LsrK crystals and additional *in vitro* biochemical experiments can be facilitated by increasing the amount of purified LsrK.

2. Materials and methods

2.1. Cloning of the *lsrK* gene and expression of the LsrK protein

The gene encoding LsrK was amplified from the genomic DNA of *E. coli* (K-12 strain). The polymerase chain reaction (PCR) was performed using 5' and 3' primers containing BglII (AGATCT) and XhoI (CTCGAG) endonuclease sites, respectively, because the *lsrK* gene contains a BamHI (GGATCC) site. All restriction enzymes were purchased from New England Biolabs, Massachusetts, USA. The resulting PCR product was cloned into the pGEX-4T-1 expression vector (GE Healthcare, Pennsylvania, USA) using the BamHI and XhoI sites, and recombinant LsrK was expressed as an N-terminal GST-fusion protein (Table 1).

The *lsrK* expression plasmid was transformed into *E. coli* BL21 (DE3) cells and the antibiotic ampicillin (100 µg ml⁻¹) was used for *E. coli* to maintain the plasmid. A seed culture with a 1/100th volume of the main culture was prepared by growing the transformed cells in Luria–Bertani (LB) growth medium (Duchefa Biochemie, The Netherlands) overnight at 37°C. The amount of LsrK expressed was tested with the following three different incubation conditions using a 2.8 l scaled baffled flask containing 1 l LB medium. (i) The cells were grown in LB medium at 37°C until the OD of the culture at 600 nm (OD₆₀₀) reached 0.7–0.8 and induction of LsrK expression was then performed with 1.0 mM isopropyl β-D-1-thiogalactopyranoside (IPTG; Duchefa Biochemie, The Netherlands) for 4 h. (ii) The cells were grown in LB medium containing 0.4% glucose at 37°C until the OD₆₀₀ reached 0.5–0.6 and the temperature of the incubator was then changed to 20°C. When the temperature reached 20°C, IPTG induction was performed for 20 h at the same temperature. (iii) For the expression of LsrK in the presence of osmotic shock and heat shock, the cells were grown in LB medium containing an additional 250 mM NaCl and 0.4% glucose at 37°C. When the culture OD₆₀₀ reached 0.5–0.6, the culture flasks were immediately transferred to a different shaking incubator with the temperature preset to 42°C. After 1 h, the cells were cooled on ice for 30 min and IPTG induction was then performed at 20°C for an additional 20 h. The cells were then harvested by centrifugation at 2500g and 4°C for 30 min.

2.2. Purification of LsrK

The harvested cells were resuspended in a buffer consisting of 50 mM Tris–HCl, 0.5 M NaCl, 10 mM β-mercaptoethanol,

Table 2
Crystallization.

Method	Hanging-drop vapour diffusion
Plate type	VDX 24-well plates
Temperature (K)	293
Protein concentration (mg ml ⁻¹)	10
Buffer composition of protein solution	10 mM Tris-HCl, 200 mM NaCl, 5 mM β -mercaptoethanol, 2 mM DPD, 2 mM MgSO ₄ , 2 mM ATP- γ -S, pH 7.5
Composition of reservoir solution	0.1 M sodium citrate tribasic, 20–24% PEG 300, 0.1 M lithium chloride, pH 5.5
Volume and ratio of drop	2 μ l (1:1)
Volume of reservoir (μ l)	1000

0.1% Tween 20 at pH 7.5. Additional protease inhibitors (1.0 mM benzamidine and 1.0 mM phenylmethylsulfonyl fluoride; Sigma-Aldrich) were added before the cells were lysed by sonication in ice. The cell debris was removed by centrifugation at 25 000g and 4°C for 1 h and the supernatant was filtered with 0.4 μ m syringe filters (Sartorius Stedim Biotech). The GST-fused LsrK protein (GST-LsrK) was purified by affinity chromatography using a GSTrap FF column (GE Healthcare). The presence of 0.5–1.0 M NaCl in the cell lysate increased the binding of GST-LsrK to the column. The column was pre-equilibrated with binding buffer (25 mM Tris-HCl, 100 mM NaCl, 10 mM β -mercaptoethanol, pH 7.5) and the protein was eluted with an elution buffer consisting of 25 mM Tris-HCl, 100 mM NaCl, 10 mM reduced L-glutathione at pH 7.5. The pH of the elution buffer was adjusted after the addition of 10 mM glutathione. 50% glycerol was slowly added to the protein eluate to make a 2.5% solution, and thrombin digestion was then performed at 4°C overnight. The digested protein solution was concentrated using a centrifugal concentrator with a 30 kDa molecular-weight cutoff (Millipore), and gel-permeation chromatography (GPC) on a HiLoad 16/26 Superdex 200 column was then performed in GPC buffer (10 mM Tris-HCl, 200 mM NaCl, 5 mM β -mercaptoethanol, pH 7.5). A GSTrap FF column was directly attached to the end of the GPC column to remove the cleaved GST protein that co-eluted with LsrK. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to identify the presence of LsrK. The purified LsrK was concentrated (to $\sim$ 10 mg ml⁻¹) and the dispensed protein aliquots were stored in a freezer at -80°C after quick freezing using liquid nitrogen. The concentration of the LsrK protein was estimated using UV absorption at 280 nm with an extinction coefficient of 93 390 M⁻¹ cm⁻¹ (<http://web.expasy.org/protparam/>).

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2.3. Crystallization of LsrK and collection of X-ray diffraction data

2 mM 4,5-dihydroxy-2,3-pentanedione (DPD), 2 mM adenosine 5'-(γ -thio)triphosphate (ATP- γ -S; Sigma-Aldrich) and 2 mM MgCl₂ were added to the LsrK solution before crystallization setup. The DPD was a D/L mixture as previously used for the crystallization of LsrR (Ha *et al.*, 2013). The slowly hydrolyzed ATP analogue ATP- γ -S was used instead of ATP. Screening of crystallization conditions was performed using kits from Hampton Research (Crystal Screen, Crystal Screen 2, SaltRx, SaltRx 2, Index, PEGRx, PEGRx 2 and PEG/Ion) and Rigaku Reagents (Wizard I, II, III and IV) via the sitting-drop vapour-diffusion method using a Mosquito instrument (TTP LabTech) at 20°C.

Two crystallization conditions were initially selected after screening, (i) 0.4 M ammonium phosphate monobasic and (ii) 0.1 M sodium acetate trihydrate, 30% PEG 300, pH 4.5, and a third condition was later selected for further optimization.

Figure 1

Expression and purification of GST-LsrK. The amounts of LsrK expressed and their elution from GST-affinity chromatography were analyzed by SDS-PAGE for three different culture conditions. (a, b) IPTG induction of LsrK was performed at 37°C for 5 h (a) and at 20°C for 20 h (b). (c) Induction of LsrK was performed after heat shock (42°C); osmotic shock was also applied by adding an additional 0.25 M NaCl to the LB medium. The samples were loaded onto the SDS-PAGE gel as labelled at the top (lane 1, total cell lysate before IPTG induction; lane 2, total cell lysate after induction; lane 3, the pellet of the lysate resuspended in a 1/5 volume of the original total cell lysate; lane 4, supernatant of the cell lysate; lane 5, elution fraction from GST-affinity chromatography).

After additional screening of PEGRx condition A4 using the Additive Screen HT kit (Hampton Research), an LsrK crystal was grown in a buffer consisting of 0.1 M sodium citrate tribasic, 20–24% PEG 300, 0.1 M lithium chloride pH 5.5; Table 2) *via* hanging-drop vapour diffusion. The cryoprotectant solution contained 38% 1,6-hexanediol, and the LsrK crystals were flash-cooled in liquid nitrogen. An additional 2 mM DPD, MgSO₄ and ATP- γ -S were constantly maintained in the crystal washing and cryoprotectant solutions. X-ray diffraction data were collected in a cold helium stream (100 K) on the BL-17A beamline at the Photon Factory, Tsukuba, Japan and the diffraction data were processed using *HKL-2000* (HKL Research). Data-collection and processing statistics are shown in Table 3.

3. Results

3.1. Soluble expression of LsrK

It was very difficult to obtain properly folded GST-fused LsrK protein (GST-LsrK) in an *E. coli* expression system. Although IPTG induction at 37°C resulted in a small increase in the protein band in the SDS-PAGE gel corresponding to the molecular weight of GST-LsrK (84 kDa), LsrK was barely detected in the eluate from GST affinity chromatography (Fig. 1a). Therefore, it was likely that the soluble GST-LsrK expressed at 37°C was not suitable to bind to the GST-affinity column. It is well known that IPTG induction at low temperature increases the solubility of the expressed proteins (Sørensen & Mortensen, 2005), and we tried to express LsrK at 20°C for ~20 h. The amount of GST-LsrK expressed seemed to be similar to the case for induction at 37°C, but most of the LsrK (more than 90%) changed into an insoluble form (Fig. 1b). Nevertheless, the eluate from GST-affinity column chromatography showed an unexpected increase in the amount of GST-LsrK. Previously, we identified that N-terminally His-tagged LsrK obtained by His-tag affinity column chromatography was prone to aggregation and could not be concentrated at all. It was also confirmed that the

Table 3

Data collection and processing.

Values in parentheses are for the outer shell.

Diffraction source	BL-17A, Photon Factory
Wavelength (Å)	0.9800
Temperature (K)	100
Detector	Pilatus CBF CCD
Crystal-to-detector distance (mm)	577.0
Rotation range per image (°)	1.0
Total rotation range (°)	180
Exposure time per image (s)	4.0
Space group	<i>P</i> 6 ₂ 22
<i>a</i> , <i>b</i> , <i>c</i> (Å)	102.26, 102.26, 343.75
α , β , γ (°)	90.0, 90.0, 120.0
Mosaicity (°)	0.63
Resolution range (Å)	50.0–2.9 (3.0–2.9)
Total No. of reflections	309482
No. of unique reflections	24341
Completeness (%)	98.9 (98.8)
Multiplicity	12.7 (8.1)
$\langle I/\sigma(I) \rangle$	42.8 (2.8)
<i>R</i> _{r.i.m.}	0.051 (0.370)
Overall <i>B</i> factor from Wilson plot (Å ²)	84.4

majority of this tentatively soluble His-tagged LsrK eluted in the void volume during GPC (data not shown). It was likely that GST-LsrK expressed at 37°C did not precipitate even though it remained in a soluble form. However, the misfolded GST-LsrK was likely to have precipitated when it was expressed at 20°C overnight. The easy separation of the well folded LsrK from the misfolded soluble protein using GST-affinity column chromatography led us to use the GST-LsrK fusion for further optimization studies.

It has been reported that the presence of a chaperone protein increases the soluble expression of a target protein in the *E. coli* system (Oganesyan *et al.*, 2007). To increase the soluble expression of properly folded GST-LsrK by inducing the innate chaperone activity of *E. coli*, osmotic stress and heat shock were applied to the culture before IPTG induction. Indeed, the presence of both osmotic stress and heat shock greatly increased the soluble expression of GST-LsrK (Fig. 1c). Although the overall amount of GST-LsrK expressed was not changed compared with that obtained using IPTG

Figure 2

Purification of LsrK by GPC after thrombin cleavage. (a) LsrK (58 kDa) eluted at ~59 ml, which corresponds to 70 kDa. The column void and total volume were about 45 and 120 ml, respectively. The protein contained in the front peak is likely to have lost its functional structure. (b) SDS-PAGE analysis shows that the protein in the main elution peak forms a tight complex with 'Protein-X'. The remaining impurity band (cleaved GST) is indicated by an open arrow. The numbers above the figures are those of the elution fractions.

Table 4
Three different growth conditions for GST-LsrK.

1 l culture†	37°C	20°C	Osmotic stress/heat shock
Medium	LB	LB + G‡	LB + G/NaCl§
Induction time (h)	4	20	20
Cells (g)	3.2	4.1	4.3
Purity¶ (%)	—	85	95
LsrK†† (mg)	—	<0.2	>1.5

† The cultures were grown on a 6 l scale and the values were converted to a 1 l scale. ‡ 0.4% glucose was added to the LB medium to increase cell growth at low temperature. § 0.4% glucose and 0.25 M NaCl were additionally added to the LB medium. ¶ The purities of LsrK after the final GPC were roughly estimated by SDS-PAGE analysis. 'Protein-X' was not considered to be an impurity as it formed a complex with LsrK. †† Final yield of purified LsrK without the N-terminal GST tag.

induction at 20°C, the amount of purified GST-LsrK after GST-affinity chromatography was much greater (Figs. 1*b* and 1*c*). The effect of osmotic stress and heat shock on the yields of purified GST-LsrK did not appear to be additive, as only applying heat shock without osmotic stress resulted in a very small increase in the amount of purified GST-LsrK (data not shown).

3.2. Purification of LsrK

GST-LsrK did not bind well to the GST-affinity column, and thus repeated binding of the cell lysate to the column increased the yield of GST-LsrK. However, we accidentally discovered that the addition of more than 0.5 M NaCl to the cell lysate greatly increased the column-binding efficiency of GST-LsrK. The bound GST-LsrK was not dissociated from the column during washing with a low-salt binding buffer. We

added glycerol to the protein elution (2.5%) during cleavage of the GST tag using thrombin because GST-LsrK was prone to aggregation. The cleaved LsrK was finally purified by gel-permeation chromatography using a Superdex 200 column (Fig. 2). Interestingly, the fractions containing well folded LsrK contained another small protein (labelled 'Protein-X' in Fig. 2*b*), the band intensities of which were almost exactly correlated with those for LsrK. Therefore, 'Protein-X' appeared to form a tight complex with LsrK, and the characterization of 'Protein-X' will be investigated in future work. The overall procedures for protein expression and purification are summarized in Table 4. The purified LsrK was concentrated ($\sim 10 \text{ mg ml}^{-1}$) and the protein aliquots were frozen in liquid nitrogen for longterm storage.

3.3. X-ray diffraction of an LsrK crystal

LsrK crystals grew better when 2 mM ATP- γ -S and 2 mM MgCl₂ were included during crystallization (Fig. 3). Although the presence of DPD did not improve the crystallization of LsrK, we kept 2 mM DPD in the solutions containing the LsrK crystals with the hope of obtaining crystals of LsrK complexed with both ATP- γ -S and DPD. Through various optimizations of the crystallization condition and the screening of cryoprotectant solutions, we eventually obtained diffraction data to 2.9 Å resolution using synchrotron radiation at the Photon Factory (Fig. 3). The use of 1,6-hexanediol as the cryoprotectant instead of glycerol greatly improved the diffraction resolution of the LsrK crystal from 3.3 to 2.9 Å. Analysis of the diffraction data showed that the LsrK crystals belonged to the primitive hexagonal space group *P*6₂22 and that the crystal unit cell was very large (unit-cell parameters $a = 102.26$, $b = 102.26$, $c = 343.75$ Å, $\alpha = 90$, $\beta = 90$, $\gamma = 120^\circ$). We also confirmed that the crystals contained 'Protein-X' by SDS-PAGE analysis (data not shown).

4. Discussion

It has recently been reported that the *lsrK* and *lsrR* genes are widespread in various Gram-positive and Gram-negative bacteria, and both the LsrK and LsrR proteins can be targeted to suppress biofilm formation by inhibiting AI-2-mediated QS (Li *et al.*, 2007; Roy, Fernandes *et al.*, 2010). Recently, we have determined three-dimensional structures of the LsrR protein complexed with AI-2-P and various AI-2-P analogues, and have shown the presence of another antagonist binding mode (Ha *et al.*, 2013). Similarly, the three-dimensional structure of LsrK will provide a useful basis for the design of novel drugs targeting LsrK activity. Various structural and *in vitro* biochemical studies on LsrK necessitate high-yield protein production. Here, we showed that the presence of osmotic stress and heat shock increased the soluble expression of GST-LsrK, which was able to bind to a GST-affinity column. It would be more accurate to say that these treatments probably increased the amount of soluble LsrK with proper folding, because SDS-PAGE analysis indicated that the GST-LsrK in the supernatant at 37°C appeared to be comparable to that in

Figure 3
X-ray diffraction of an LsrK crystal. The circles show the resolution limits (labelled in Å) and the diffraction data were processed to 2.9 Å resolution. A photograph of the LsrK crystals is shown in the inset.

the supernatant following osmotic stress and heat shock (Figs. 1a and 1c). Although we do not know the detailed mechanism by which osmotic stress and heat shock increase the soluble expression of GST-LsrK, the increased chaperone activity was likely to support proper folding of GST-LsrK. The increased yield of purified LsrK made it possible for us to perform structural studies on LsrK and also to identify complex formation of LsrK with 'Protein-X'. Large amounts of LsrK will accelerate various *in vitro* studies on the activity of LsrK related to QS signalling.

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