Automated unrestricted multigene recombineering for multiprotein complex production

Christoph Bieniossek^{1–3,8}, Yan Nie^{1,2,4,8}, Daniel Frey⁵, Natacha Olieric⁵, Christiane Schaffitzel^{1,2}, Ian Collinson⁶, Christophe Romier⁷, Philipp Berger⁵, Timothy J Richmond³, Michel O Steinmetz⁵ & Imre Berger^{1,2}

Structural and functional studies of many multiprotein complexes depend on recombinant-protein overexpression. Rapid revision of expression experiments and diversification of the complexes are often crucial for success of these projects; therefore, automation is increasingly indispensable. We introduce Acembl, a versatile and automatable system for protein-complex expression in *Escherichia coli* that uses recombineering to facilitate multigene assembly and diversification. We demonstrated protein-complex expression using Acembl, including production of the complete prokaryotic holotranslocon.

Many essential processes in cells are controlled by proteins associating into interlocking molecular machines, often containing ten or more subunits^{1,2}. Functional and structural studies that aim to decipher the physiologically relevant molecular mechanisms of these complexes are becoming increasingly important in biology. The low abundance and frequently heterogeneous nature of many multisubunit complexes, however, often precludes their extraction from a native source.

Recombinant production methods, with *E. coli* as the most common expression host, are thus used for overexpressing proteins for a variety of applications. Successful functional analysis of proteins and elucidation of their molecular architecture often crucially depends on introducing alterations, such as truncations, mutations and extensions with purification tags, or with particular promoter and terminator elements. The ensuing requirements in terms of experimental throughput are already considerable for diversifying single open reading frames. To streamline the process, researchers involved in structural genomics efforts have developed standardized subcloning routines and implemented automated procedures. The exponential increase in workload when many open reading frames have to be rapidly diversified and assembled in the context of a multisubunit complex is daunting and remains an unresolved challenge.

Several systems have been introduced in recent years for expression of multiple genes in both eukaryotic and prokaryotic hosts^{3–7}. Despite considerable improvements of eukaryotic expression methods, in particular baculovirus-based systems³, *E. coli* still remains the dominant workhorse in most laboratories, for many good reasons such as low cost and the availability of many specialized expression strains. Current co-expression systems for *E. coli* rely essentially on serial, mostly conventional (that is, restriction and ligation) subcloning of protein-coding genes either as single expression cassettes^{5,6} or as polycistrons comprising several genes under the control of the same promoter⁴. This approach considerably limits the applicability of these co-expression techniques for the production of protein complexes with many subunits, in particular at the throughput typically required for structural characterization.

A major impediment of such largely serial (one gene at a time) constructions is the inherent inability to rapidly revise an expression experiment once the multiprotein complex has been produced, purified and characterized. However, the ability to make such changes, including variations of the protein subunits, is essential for functional and structural analysis. To address this, we designed a modular multiprotein complex expression system in *E. coli*, called Acembl. Multilevel automation is a priority in protein science, especially in structural genomics efforts⁸. To our knowledge, Acembl is the first fully automatable system for simple and rapid assembly and disassembly of multigene constructs for multiprotein complex expression (**Fig. 1**).

We had previously introduced the concept of acceptor and donor vectors for multigene construction via Cre-*loxP* fusion³. Acembl uses small (2–3 kb) *de novo* designed donor and acceptor vectors that are devoid of surplus DNA (**Fig. 1a**). Donor vectors have a conditional origin of replication depending on the expression of a protein encoded by the phage R6K γ *pir* gene in *trans*⁹. Therefore, donor vectors can not be propagated in cell strains that do not express the *pir* gene, unless they are fused with an acceptor containing a regular *E. coli* origin of replication.

Acceptor and donor vectors (**Fig. 1a**) contain an identical multiple integration element (MIE) derived from a polylinker⁴. One gene (single expression cassette) or several genes (polycistron) can be inserted into the MIE. We inserted genes by recombination

RECEIVED 6 FEBRUARY; ACCEPTED 16 MARCH; PUBLISHED ONLINE 3 MAY 2009; DOI:10.1038/NMETH.1326

¹European Molecular Biology Laboratory, Grenoble Outstation, B.P. 181, Grenoble, France. ²Unit of Virus Host-Cell Interactions, Unités Mixtes de Recherche 5233, Grenoble, France. ³Eidgenössische Technische Hochschule Zürich, Institut für Molekularbiologie und Biophysik, Hönggerberg, Zürich, Switzerland. ⁴Department of Applied Physics, Royal Institute of Technology, Albanova University Center, Stockholm, Sweden. ⁵Biomolecular Research, Structural Biology, Paul Scherrer Institut, Villigen, Switzerland. ⁶Department of Biochemistry, School of Medical Sciences, Bristol, UK. ⁷Department of Biology and Structural Genomics, Institute Génétique et Biologie Moléculaire et Cellulaire, Illkirch, France. ⁸These authors contributed equally to this work. Correspondence should be addressed to M.O.S. (michel.steinmetz@psi.ch) or I.B. (iberger@embl.fr).

BRIEF COMMUNICATIONS



using sequence- and ligation-independent cloning (SLIC) procedures¹⁰ making use of T4 DNA ligase exonuclease activity to generate long single-stranded overhangs that can anneal to each other efficiently (**Fig. 1b**). Tried-and-tested primer sequences are present in the MIE, which can be used as adaptors in PCRs to generate these regions of homology for single or multifragment SLIC (**Supplementary Protocol** online). In the experiments shown here, MIEs are flanked by a T7 (pACE, pACE2 and pDC) or *lac* (pDK and pDS) promoter and terminator sequences. These are the most powerful and widely used promoter systems for *E. coli* expression. Note that all donor and acceptor vectors can be fitted easily with exclusively T7 (or *lac*) promoters if desired, by exchanging the corresponding DNA fragments (**Supplementary Protocol**). In principle, any other promoter and terminator system can be inserted in this way.

Each vector contains a homing endonuclease recognition site (acceptor vectors: I-*Ceu*I; donor vectors: PI-*Sce*I) and a complementary *Bst*XI site (**Fig. 1a**). Homing endonucleases are rare cutters with long (~ 20 –30 bp) recognition sequences that are unique even in very large DNAs. Digestion by homing endonuclease gives rise to a specific overhang that matches a corresponding *Bst*XI site. This can be used to generate multiple expression cassettes iteratively by insertion of expression cassettes liberated by homing endonuclease and *Bst*XI digestion into constructs linearized at the homing endonuclease site (**Supplementary Protocol**).

We fused donor and acceptor vectors carrying genes of choice via Cre-*loxP* plasmid fusion. Cre recombinase–catalyzed plasmid

Figure 1 | Multiprotein complex expression with Acembl. (a) Donor and acceptor vectors contain loxP sequences and identical MIEs. Origins of replication (BR322 and R6K γ ori) are indicated. Promoters (T7, lac), terminators (black squares) and homing endonuclease sites (dark blue, I-CeuI and PI-SceI sites) and matching BstXI sites (small light blue squares) are shown. Antibiotic resistance genes indicate resistance to the following antibiotics: Ap, ampicillin; Cm, chloramphenicol; Kn, kanamycin; and Sp, spectinomycin. (b) Genes of interest (A, B and C) were amplified by PCR and inserted into acceptor or donor vectors by singlegene or multigene SLIC. Ribosome binding sites (RBS) on forward primers are boxed in black. Complementary sequences are colored identically. T4 DNA polymerase-exonuclease-treated DNA fragments (insert and vector) were mixed and transformed into appropriate cells (pir+ for donor vectors). (c) Incubation of acceptor and donor constructs (genes shown as white arrows) with Cre recombinase resulted in all combinations of fusions, including acceptor-donor (AD) and acceptor-donor (ADD). Fusion constructs were readily deconstructed in the reverse approach. (d) In Acembl, genes are integrated by ligation-independent methods (SLIC) followed by combinatorial multigene vector generation using Cre-loxP fusion, protein expression and analysis of purified complex. Deconstruction by Cre recombinase-mediated excision liberates starting vectors for gene modification that are reintegrated into the workflow in an iterative cycle. The reactions were scripted into robotic routines (Supplementary Protocol).

fusion is an equilibrium reaction that favors the excision reaction¹¹. When a mixture of donor vectors and an acceptor vector is incubated with Cre recombinase, single plasmids and all possible plasmid fusion combinations co-existed in the reaction (**Fig. 1c**). These could be conveniently recovered by transforming the mixture into pir^- strains. By challenging aliquots of the transformed cells with the appropriate antibiotic combinations and then counterselecting in a 96-well microtiter plate, all possible donor-acceptor fusions could be recovered for expression of the encoded genes in a combinatorial fashion (**Fig. 2**).

Notably, the reverse applies as well in the disassembly of acceptor-donor multigene fusion constructs (**Fig. 1c,d**). We incubated the tetrameric fusion vector consisting of the acceptor and all three donors (ADDD) shown in **Figure 2** with Cre recombinase and transformed the reaction into a *pir*⁺ strain. Microtiter plate analysis of the resulting transformants efficiently recovered all starting plasmids (\geq 50% efficacy) from the deconstruction reaction (**Supplementary Protocol**). We identified partially deconstructed double and triple fusions in this experiment, implying that donor or acceptor constructs can be selectively liberated from the tetramer. This can be exploited, for example, to modify the gene(s) present in the liberated entity, by mutation, truncation, replacement with isoforms or homologs of the encoded protein(s) and so forth, without having to restart the multigene combination procedure.

We validated Acembl by performing 22 complex expressions, each with 2-6 different subunits (protein and RNA) and with



different protein classes (**Fig. 3** and **Supplementary Results** online) both manually and also with a robotics setup using a Tecan Freedom EvoII 200 liquid-handling workstation (**Supplementary Protocol**). We expressed fusion constructs and isolated the complexes from *E. coli* lysates by Ni²⁺ affinity capture, except in the case of the holotranslocon transmembrane complex, for which we prepared and solubilized membrane vesicles manually. We achieved multigene expression from single gene cassettes, polycistrons or a combination thereof, involving double, triple and quadruple acceptor-donor combinations (**Fig. 3** and **Supplementary Results**).

Figure 3 | Expression of complexes. (a) Denaturing polyacrylamide gel analysis of uninduced and induced whole-cell extracts of cells transformed with pACEMBL-TFIIF, and of hTFIIF purified from these cells with subunits marked. RAP74 contained a C-terminal oligohistidine tag. pACEMBL_TFIIF plasmid diagram is shown below the gel; 30 and 74 mark genes encoding RAP30 and RAP74-His, respectively. T7, T7 promoter; Cm^R, chloramphenicol resistance marker; Ap^R, ampicillin resistance marker. (b) All multigene constructs shown in **Figure 2** were assembled and expressed, and cell lysates were analyzed. The VCB complex was captured by an oligohistidine-thioredoxin fusion tag on the Van Hippel-Lindau subunit⁴

BRIEF COMMUNICATIONS

Figure 2 | Acceptor-donor recombineering. Genes encoding for Van Hippel-Lindau ElonginC-ElonginB (VCB) complex⁴, FtsH soluble domain¹⁴, BFP and monomeric GFP (mGFP) with a coiled-coil domain¹⁵ were inserted into pACE, pDC, pDK and pDS, respectively. Cre recombinase-mediated fusion was followed by transformation into *pir*⁻ cells (TOP10). Aliquots were plated on agar with two, three or four antibiotics as indicated by boxes outlining regions of the 96-well plate. Four colonies from each plate were grown in a 96-well microtiter plate. Labels left of the plate image denote antibiotics contained in media aliquots in horizontal rows. Wells in the bottom two rows were charged differently (labels below the plate image). Those inoculated with four colonies each from one agar plate are boxed in black and labeled with antibiotics contained in the agar plate. Four vertical rows in each such 16-well box were inoculated with the same colony. In the bottom two rows, four wells in a row were inoculated with the same colony. Expected vector architecture of the double (AD), triple (ADD) and quadruple (ADDD) fusions is shown left or right (16-well boxes), respectively, or below (bottom two rows) the plate image. Red dye was used as positional marker.

The efficient soluble expression of full-length human general transcription factor II F (TFIIF) (**Fig. 3a**) is noteworthy, as individual expression of the subunits leads to insoluble material. Crystal structure analysis of human TFIIF dimerization domain had necessitated many iterative cycles of limited proteolysis, recloning, insoluble expression of the designed fragments and corefolding¹². Such laborious situations are commonplace when analyzing protein complexes. It is conceivable that the large investment of labor involved can be substantially reduced applying the Acembl approach.

In 24-well deep-well plates, we performed multiprotein expression experiments from all acceptor-donor combination constructs shown in **Figure 2**. Analysis of the lysates by Ni²⁺ affinity capture, denaturing and western blot revealed expression of all recombinant proteins and proper complex assembly (**Fig. 3b** and **Supplementary Fig. 1** online), thus illustrating how, with our approach, multiple genes can be co-expressed in parallel in a combinatorial fashion.

Using Acembl, we also produced a large multiprotein complex, the YidC-SecYEGDF holotranslocon, which contains 33 transmembrane helices. This machinery is used to transport unfolded polypeptides into the cell membrane or for translocation into the periplasm of bacteria¹³. We isolated the complex from



(His-TRX-VHL). FtsH contains an oligohistidine tag at its C terminus¹⁴. Fluorescent proteins were identified in lysates by western blot with a mouse antibody to GFP and a secondary goat antibody to the mouse antibody coupled to alkaline phosphatase. Full-length western blots are presented in **Supplementary Figure 1**. (c) Production of the entire prokaryotic transmembrane holotranslocon YidC-SecYEGDF. A breakdown product of SecY is marked with an asterisk. Marker, Biorad Precision Plus broad range marker. pACEMBL-HTL plasmid diagram is shown below the gel. Y, E, G, D and E mark genes encoding SecYEGDF. Ara, arabinose promoter; and trc, trp-*lac* promoter.

BRIEF COMMUNICATIONS

detergent-solubilized membrane vesicles (**Fig. 3c**). We anticipate that factorial approaches for detergent solubilization will mature into formats that eventually can be incorporated into our robotic process to allow expression and detergent-mediated solubilization of many other membrane protein complexes. Moreover, proteins such as YajC and SecA associate with the translocon¹³. Using pDK and pDS for Cre recombinase–mediated integration of genes encoding SecA and YajC, our modular setup should allow us to assemble an even larger functional translocon complex.

Arrays of genes, encoding subunits of a particular multiprotein complex, and potentially also accessory proteins such as chaperones, specific kinases or phosphatases, can be assembled, disassembled and exchanged using the Acembl system. This offers intriguing avenues for combinatorial analyses of protein-protein interactions or of interactions between protein complexes and modifiers. Interactions between several multiprotein complexes may also be studied in this way. We showed that the steps involved in multigene assembly, construct analysis, small scale expression and complex purification can be scripted into a robotics routine. We anticipate that automated recombineering will be extended to investigating reciprocal functional relationships between entire arrays of protein complexes and their variants, in a rapid and flexible systems approach, by using *E. coli* as a convenient and robust expression host.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturemethods/.

Note: Supplementary information is available on the Nature Methods website.

ACKNOWLEDGMENTS

We thank R. Jaussi and D. Hart for helpful suggestions, the members of the Berger and Schaffitzel laboratories for discussions and technical assistance, S. Trowitzsch (Max Planck Institute, Göttingen) and the scientists at the Partnership for Structural Biology in Grenoble for providing cDNAs and advice. M.O.S. and T.J.R. are supported by the Swiss National Science Foundation. C.R. and I.B. are supported by the European commission projects Structural Proteomics In Europe 2Complexes (SPINE2C) (European Commission (EC) FP6) and European Infrastructure for Structural Biology INSTRUCT (EC FP7). I.B. is also supported by the Centre National de la Recherche Scientifique (CNRS) and the European commission projects 3D-Repertoire (EC FP6) and Protein Production Platform Pcube (EC-FP7).

COMPETING INTERESTS STATEMENT

The authors declare competing financial interests: details accompany the full-text HTML version of the paper at http://www.nature.com/naturemethods/.

Published online at http://www.nature.com/naturemethods/ Reprints and permissions information is available online at http://npg.nature.com/reprintsandpermissions/

- 1. Rual, J.F. et al. Nature 437, 1173-1178 (2005).
- 2. Charbonnier, S., Gallego, O. & Gavin, A.C. Biotechnol. Annu. Rev. 14, 1-28 (2008).
- 3. Fitzgerald, D.J. et al. Nat. Methods 3, 1021–1032 (2006).
- 4. Tan, S., Kern, R.C. & Selleck, W. Protein Expr. Purif. 40, 385-395 (2005).
- 5. Tolia, N.H. & Joshua-Tor, L. Nat. Methods **3**, 55–64 (2006).
- 6. Chanda, P.K., Edris, W.A. & Kennedy, J.D. Protein Expr. Purif. 47, 217-224 (2006).
- Scheich, C., Kümmel, D., Soumailakakis, D., Heinemann, U. & Büssow, K. Nucleic Acids Res. 35, e43 (2007).
- 8. Kambach, C. Curr. Protein Pept. Sci. 8, 205–217 (2007).
- 9. Penfold, R.J. & Pemberton, J.M. Gene 118, 145-146 (1992).
- 10. Li, M.Z. & Elledge, S.J. Nat. Methods 4, 251-256 (2007).
- 11. Abremski, K., Hoess, R. & Sternberg, N. Cell 32, 1301-1311 (1983).
- 12. Gaiser, F., Tan, S. & Richmond, T.J. J. Mol. Biol. 302, 1119-1127 (2000).
- 13. Duong, F. & Wickner, W. EMBO J. 16, 2757-2768 (1997).
- 14. Bieniossek, C. et al. Proc. Natl. Acad. Sci. USA 103, 3066-3071 (2006).
- Berger, P., Schaffitzel, C., Berger, I., Ban, N. & Suter, U. Proc. Natl. Acad. Sci. USA 100, 12177–12182 (2003).

ONLINE METHODS

System design and vector preparation. Acembl vectors were created from the respective fragments (origin of replication, resistance marker gene, *loxP*) by standard methods including SLIC methods¹⁰ as well as restriction and ligation. An AlwNI site (asymmetric recognition sequence) was incorporated in every vector backbone between the antibiotic resistance marker and the origin of replication, to render these elements easily exchangeable. The MIE including homing endonuclease sites and complementary BstXI sites were synthesized by a commercial supplier (GenScript Corporation). All vectors were verified by DNA sequencing (Macrogen Inc.). Vector sequences were compiled by using the program VectorNTI (Invitrogen) and plasmid maps were generated by using the program DNAMAN version 4.0 (Lynnon Corporation). Sequences and maps are provided in the Supplementary Protocol. Requests for Acembl reagents should be addressed to I.B. (iberger@embl.fr).

DNA manipulation. Genes of interest were inserted into the MIE of the Acembl system by using SLIC and, in select cases, also restriction and ligation (**Supplementary Results**). Primers contain the sequences necessary for insertion (SLIC homology region or restriction sites) and optionally the sequences encoding ribosome binding sites, tags or stop codons. DNA sequences used to design the primers are listed in the **Supplementary Protocol**. Step-by-step instructions to insert genes, both by SLIC (manually and with a robot) as well as by restriction and ligation (manually) are provided in the **Supplementary Protocol**. If only domains rather than full-length proteins were used in the complex expression experiments, the exact amino acid residue boundaries are listed in **Supplementary Results**.

Reactions using Cre recombinase enzyme (fusion and deconstruction) were carried out according to the recommendations of commercial suppliers of the Cre enzyme. In the experiments, commercial Cre recombinase (New England Biolabs) was used, as well as Cre recombinase supplied by the European Molecular Biology Laboratory (EMBL) core facility (EMBL Heidelberg).

All DNA manipulation, including expression cassette multiplication by using homing endonucleases, is detailed, both for manual and robotic applications, in the **Supplementary Protocol**.

Multiprotein expression and purification. hTFIIF and VCB-BFPmGFP-FtsH series: fusion plasmids encoding for hTFIIF, or the VCB-BFP-mGFP-FtsH series, respectively, were expressed overnight in BL21(DE3) cells in 24 well deep-well plates in small scale using Studier autoinduction media. Ampicillin was added to the growth media (to 100 μ g ml⁻¹). Proteins were purified by Ni²⁺ capture as described in the **Supplementary Protocol**.

Holotranslocon YidC-SecYEGDF: subunits SecY, SecE and SecG were present as a polycistron in pDC^{trc}, a derivative of pDC containing a trc promoter instead of T7. Subunits YidC, SecD and SecF are present as a polycistron in pACE^{ara}, a derivative of pACE with an arabinose promoter instead of T7 (**Supplementary Results**). Owing to the presence of two separately inducible promoters, expression of the respective polycistrons is regulated separately by addition of isopropyl β -D-1-thiogalactopyranoside (IPTG) and L-arabinose, respectively. Holotranslocon was expressed in BL21 cells in Terrific Broth (TB) media in the presence of ampicillin (100 µg ml⁻¹) and chloramphenicol

(25 μg ml⁻¹). Overexpressed holotranslocon components were identified by specific immunological staining of the subunits in a western blot (data not shown). Membrane vesicles were prepared manually using standard buffers and procedures¹³. Detergent solubilised holotranslocon was purified by our standard Ni²⁺ capture as described in the **Supplementary Protocol**. For purification by size exclusion chromatography using a S300 gel filtration column (GE Healthcare), expression was scaled up to 1-l volume, and Ni²⁺ capture was carried out by using nickel-NTA agarose (Qiagen GmbH) packed in a 5 ml column (GE Healthcare).

Complexes S1–S12: complexes S1–S12 (**Supplementary Results**) were expressed using the standard protocols provided in the **Supplementary Protocol**. Exceptions with respect to expression strains used, as well as special buffer conditions, necessary owing to the particular nature of the complexes, are listed in the **Supplementary Results**. All expressions were scaled up to 1 l of culture volume for purifying the protein complexes by size exclusion chromatography (SEC). All preparations were carried out by applying the following standard protocol.

Cell pellets from 1 l cultures were obtained by centrifugation at 6,891g (6,000 r.p.m. using a Beckman Coulter Avanti J20 centrifuge with a Beckman JLA rotor) at 4 °C. Pellets were resuspended in Buffer A (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM DTT). Cells were lysed by sonication on ice, with a Bioblock Scientific Ultrasonic Processor Vibracell 75115. A broad tip was used, with a total sonication time of 7 min, 10-s pulse at 15-s intervals, at an amplitude of 80%. Lysates were cleared by centrifugation at 15,366g (14,000 r.p.m. in a Beckman Coulter Avanti J-20 XP centrifuge with a Beckman JA20 rotor) for 30 min at 4 °C. Lysates then were passed to fresh tubes and centrifugation repeated with identical equipment settings.

Cleared lysates were passed over a nickel-NTA HighTrap column with 1-ml volume (Qiagen) by using an Aekta Prime FPLC (GE Healthcare). Complexes were washed with 10 column volumes Buffer A and eluted by applying a linear gradient to 100% Buffer B (50 mM TrisHCl pH 7.5, 150 mM NaCl, 1 mM DTT, 500 mM imidazole). In certain cases, Buffers A and B contained additives that were required for complex formation (**Supplementary Results**).

Eluates from Ni-NTA affinity capture were pooled and concentrated by using Millipore concentrators with 3 kDa molecular weight cutoff. Concentrates were then purified by using an Aekta Explorer FPLC or Aekta Purifier FPLC (GE Healthcare) by SEC using the columns listed in **Supplementary Results**. The columns used for SEC were pre-equilibrated by passing at least ten column volumes of Buffer A over the columns, optionally supplemented by specific reagents as listed in the **Supplementary Results**.

Gel electrophoresis. Samples (10–12 μ l) from peak fractions of SEC or from Ni-NTA plate elutions, respectively, were loaded manually on 12% or 15% denaturing gels using a Biorad Minigel system, pre-run at 135 V for 25 min, and then run for 65–70 min at 185 V. Gels were stained with Coomassie Brilliant Blue according to standard procedures. Gel images were prepared by scanning with a HP Scanjet 7650 photo scanner using software HPScanning version 4.5 with default settings (highlights, 15; shadows, –69; and midtones, 0) at 300 d.p.i., or, alternatively by photography using a Vilber-Lourmat Bioprint 6.21 photo documentation system with softwareBioCapt version 11.02 (Vilber-Lourmat). The obtained

TIF files were integrated into images of the SEC traces by using Adobe Illustrator CS3 version 13.0.0.

Agarose gels were stained with ethidium bromide and gel images recorded by using the Vilber-Lourmat documentation system in conjunction with a LKB 2011 MacroVue transilluminator (LKB-Produkter AB).

Western blot. Fluorescent proteins mGFP and BFP in the VCBmGFP-mBFP-FtsH expression series were detected by western blotting. The pellet of a 1.5 ml bacterial culture was resuspended in 500 μ l of 1× Lämmli buffer and the cells were lysed with 5 pulses of a Branson sonifier (Cell Disruptor B15, output control on level 4, 40% duty cycle). The disrupted cells were centrifuged for 5 min at 10,000g, and the supernatant transferred to a new tube. The supernatants separated by 12% SDS–polyacrylamide gel electrophoresis (BioRad Mini Protean II, 1 mm thick, 10 slots per gel). Three gels with different amounts of lysate were run in parallel (**Supplementary Fig. 1**) for 1 h at 25 mA with All Blue Precision Plus Protein standards (BioRad) as marker.

Proteins were transferred on PVDF membrane (Immobilon-P, Millipore IPV00010) with a Biometra semidry blotter according to manufacturer's recommendations. Fluorescent proteins were identified by western blotting with a mouse antibody to GFP (Roche; 1814460, 1:1000 in Tris-buffered (pH. 7.5) saline Tween-20 (TBST) with 3% BSA. A goat antibody to mouse antibody coupled to alkaline phosphatase (Sigma), diluted 1:10,000 in TBST with 3% milk powder was used as the secondary antibody. Blots were developed with the ECL Plus Western Blotting System (GE Healthcare), exposed for 5 s on Hyperfilm ECL X-ray film (GE Healthcare) and the X-ray film was then developed with an Agfa Curix 60 machine. The positions of the visible marker lanes were assigned with a pen. The film was scanned in the grayscale mode with 8 bit depth on an Epson Perfection 4870 Photo scanner and then saved as a TIF file. The three full length blots are shown in **Supplementary Figure 1**.

