Deciphering *Sulfolobus* spindle-shaped virus 9.1 toxin B310: Insights in killing phenotype and challenges in recombinant production

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Abstract

Viruses are often associated with pathogenicity in their hosts. However, recent research suggests mutualistic relationships between viruses and their archaeal host. *Sulfolobus* spindle-shaped virus 9.1 encodes a proteinaceous killing factor B310. Supernatant from a *S. islandicus* RJW004 strain overexpressing B310 is toxic to uninfected *S. islandicus* strains, possibly aiding in outcompeting the uninfected strains. To date, the B310 killing phenotype across the domains of life remains unexplored. Currently, using B310 derived from supernatant limits its further research on biochemical, structural and mode of action analysis. Designing a method to heterogeneously produce and purify B310 in high quantities will allow for this further research.

Spot-on-lawn assays with supernatant from *S. islandicus* RJW004 over-expressing B310 was used to assess the killing phenotype on other *Sulfolobus* spp., bacteria, and a eukaryotic organism. Additionally, heterogenous expression of wild-type B310, as well as signalling sequence truncated and codon-optimised variants, in *Escherichia coli* Rosetta™ 2(DE3) and BL21(DE3) were explored. We observed no killing phenotype of B310 in other *Sulfolobus* spp., bacteria, and a eukaryotic organism. This study revealed expression of B310 and its variants failed in *E. coli* Rosetta™ 2 and BL21 expression systems.

We propose that B310 possibly acquires its killing phenotype by interacting with the *Sulfolobus* cell membrane and might have a similar mechanism to other toxin-antitoxin systems. Furthermore, we propose that heterogenous expression and purification of B310 could be performed in *E. coli* Lemo21 expression vector, specialised for expressing toxic proteins, or in an archaeal expression vector of closely-related species *S. acidocaldarius*.

Table of Contents

List of abbreviations

Abbreviation	Definition
B310_F	Full *B310* gene construct amplified from Sulfolobus spindle-shaped Virus 9.1 (SSV9.1) DNA
B310_opt	Codon-optimised full *B310* gene for *Escherichia coli* transcription synthesised by Integrated DNA Technologies, USA
B310_S1	*B310* gene construct without N-terminal signalling peptide and membrane trafficking signal (amino acid positions 2-3) amplified from SSV9.1 DNA
B310_S2	*B310* gene construct without N-terminal signalling peptide (amino acid positions 2-22) amplified from Spindle-Shaped Virus 9.1 DNA
BL-B310_opt	pET-B310_opt transformed into *Escherichia coli* BL21 (DE3)
BL-Empty_v	pET-Empty_v transformed into *Escherichia coli* BL21 (DE3)
IPTG	Isopropyl β-D-1-thiogalactopyranoside, Inducer used in this study
pET-B310_F	*B310_F* cloned into pET-30a (+) plasmid
pET-B310_opt	*B310_opt* cloned into pET-30a (+) plasmid
pET-B310_S1	*B310_S1* cloned into pET-30a (+) plasmid
pET-B310_S2	*B310_S2* cloned into pET-30a (+) plasmid
pET-Empty_v	Empty pET-30a (+) plasmid
pOE-B310	Full *B310* gene amplified from SSV9.1 DNA cloned into *Sulfolobus* over-expression plasmid pSeSd-SsoargD, as described in Dewerff et al. (2022)
pOE-Empty	Empty over-expression plasmid pSeSd-SsoargD, as described in Dewerff et al. (2022)
R.O.I	Region of interest
Rosetta-B310_F	pET-B310_F transformed into *Escherichia coli* Rosetta^{TM}2 (DE3)
Rosetta-B310_S1	pET-B310_S1 transformed into *Escherichia coli* Rosetta^{TM}2 (DE3)
Rosetta-B310_S2	pET-B310_S2 transformed into *Escherichia coli* Rosetta^{TM}2 (DE3)
Rosetta-Empty_v	pET-Empty_v transformed into *Escherichia coli* Rosetta^{TM}2 (DE3)
SSVs	*Sulfolobus* spindle-shaped viruses

Layman's summary

Microbes live in diverse ecosystems. Archaea, such as *Sulfolobus islandicus*, are single-celled organism which often live in extreme conditions. They are often found in hot springs, where they grow at temperatures between 60-91 ºC and a pH of around 2-4 (Reno et al., 2009). Very often, archaeal species are infected with viruses. Over 60% of archaea found in Yellowstone National Park's hot springs contain one or more viruses (Munson-Mcgee et al., 2018). Interaction between archaea and viruses can influence the ecosystem in which they live.

One of these viruses that infect archaea include *Sulfolobus* Spindle-shaped viruses (SSVs). Their genomes typically encode an integrase protein, allowing viral DNA to be integrated into a specific site within the chromosome of their hosts. While viruses are mostly associated with pathogenicity, they can also form a symbiotic relationships with their host. For example, SSV9.1 can chronically infect *S. islandicus* cells. When SSV9.1 infects a host, it integrates a viral gene encoding for the toxic protein, B310. This toxic protein kills uninfected *S. islandicus* strains that do not have the *B310* gene (3). This suggests that infection of SSV9.1 can aid in outcompeting uninfected *S. islandicus* cells.

To date, archaeal toxin systems are not as well characterised as their bacterial counterparts, especially toxin systems derived from viruses of archaea. Previous research demonstrated that B310 obtained from *S. islandicus* cell overproducing B310 exerts inhibitory effects on uninfected *S. islandicus* strains (Dewerff et al., 2022). However, whether B310 also has an inhibitory effect on less closely related *Sulfolobus* species or bacterial and eukaryotic organisms, remains yet to be discovered. Furthermore, a limitation in the research by Dewerff et al. (2022) is that an impure toxin was used, resulting in a lack of specificity in their research, as well as hindering further biochemical and structural assays.

In this research we tested the B310 against various *Sulfolobus* species, as well as on several bacteria and a eukaryotic species. We found that B310 inhibits *S. islandicus* strains lacking the B310 gene, and exerts no inhibitory effect on other *Sulfolobus*, bacterial and a eukaryotic species.

With the current research, B310 production in *S. islandicus* is tedious and lacks specificity (Dewerff et al., 2022). Therefore, we tried designing a method allowing for easy production and purification of B310, subsequently increasing scientific specificity. We attempted B310 production in bacterial *Escherichia coli* protein expression systems, which are often used to produce high amounts of proteins. We used several varieties of B310: the full gene, and two shortened genes missing the cellular localisation signals of the *B310* gene. We additionally used a version of the full *B310* gene that contains the DNA sequence optimised for production in *E. coli*. Production of B310 in *E. coli* proved to be unsuccessful in our specific conditions.

There are several possibilities for why we are unable to produce the B310 and its variants in *E.coli,* For example, difficulties in production, solubility, and toxicity of the protein. Unfortunately, it is difficult to pinpoint the exact reason of the problem. However, we provide several options that could result in successful production of B310 and its variants, like changing production conditions and/or using different *E. coli* strains that are optimised for producing difficult and toxic proteins. Another way could be to improve the production in *S. islandicus* so the protein can be purified, by adding a 6xHis-tag that allows extraction of the protein from an impure protein solution. Or alternatively, by trying to produce B310 in the species *Sulfolobus acidocaldarius*, an organism closely related to *S. islandicus*. This organism is genetically well studied and appeared to be unaffected by B310 inhibition in our research.

Introduction

Microbial ecosystems and dynamics are heavily influenced by virus-host interactions. Viruses drive microbial evolution and diversity through host infection, allow horizontal gene transfer and biogeochemical cycling (French & Holmes, 2020; Rohwer et al., 2009).
Commonly, viruses are considered pathogens to their host, however, recent research has shown that viruses can have symbiotic relationships with their host (Dewerff et al., 2022; Roossinck & Bazán, 2017). Symbiotic relationships of viruses and archaea are not well-studied.

Recent research has shown that archaea-virus interactions are common, over 60% of cells present in different Yellowstone National Park hot springs contain one or more types of viruses within the cell (Munson-Mcgee et al., 2018). Archaeal viruses, like *Sulfolobus* spindle-shaped viruses (SSVs) have a variety of relationships with their host. SSVs are chronic, non-lytic proviruses of *Sulfolobus* spp., encoding integrases allowing integration of viral DNA in their *Sulfolobus* host (Ceballos et al., 2020; Clore & Stedman, 2007; Serre et al., 2002). Recent research demonstrates that a proteinaceous killing factor B310 encoded by SSV9.1 was identified to have a killing phenotype to closely related, uninfected strains (Dewerff et al., 2022). The relationship of SSV 9.1 with *S. islandicus* strains shows symbiotic properties: viral replication can be maintained in the host, while the SSV9.1 chronically infected hosts can outcompete uninfected, virus-resistant or immune strains (Dewerff et al., 2020).

To date, archaeal toxin systems are not as well characterised as their bacterial counterparts, especially toxin systems derived from viruses of archaea. Recent research on B310 has been performed by transforming *S. islandicus* RJW004 with an over-expression plasmid containing the B310 gene and performing inhibition assays on uninfected *S. islandicus* strains (Dewerff et al., 2022). Their research demonstrates that B310 is toxic to uninfected strains of the same species. However, whether B310 can also aid in outcompeting less closely related *Sulfolobus* spp., and has a killing phenotype across the two other domains of life has yet to be revealed.

Moreover, an issue with Dewerff and colleague's (2022) research is a lack in specificity due to assays being performed with impure B310, derived from supernatant. A variety of external factors can influence the killing phenotype that Dewerff and colleagues (2022) observed in their research (Eguchi et al., 2018; Glick, 1995; Özkan et al., 2005). Furthermore, impure B310 limits future biochemical, structural and mode of action assays. Besides this, Dewerff and colleagues (2022) reported that collecting high amounts of B310 from pOE-B310 is tedious (Dewerff et al., 2022). To increase specificity; perform biochemical, and structural analysis; gain an understanding in mode of action; as well as increase production of B310, a method will have to be designed to collect high amounts of purified B310.

In our research, we verify whether B310 derived from *S. islandicus* RJW004 supernatant (hereafter referred to as: pOE-B310) has a killing phenotype on other *Sulfolobus* spp., bacteria and eukaryotes. Additionally, we explore options in heterogeneous expression and purification of B310 structural and codon-optimised variants using *E. coli* Rosetta™ 2(DE3) and BL21(DE3) expression systems.

Materials & Methods

Strains

Sulofolobus islandicus M.16.4, *Sulfolobus islandicus* Y08.82.36, *Sulfolobus acidocaldarius* DSM639 and *Sulfolobus tokodaii* 7 were obtained from Whitaker lab (Carl R. Woese Institute for Genomic Biology, University of Illinois Urbana-Champaign, USA). *Escherichia coli* OneShot™ TOP10 competent cells used for molecular cloning were purchased from Invitrogen™. *Escherichia coli* BL21(DE3) and *Escherichia coli* Rosetta™ 2(DE3) competent cells used for protein expression were purchased from Thermo Scientific™, USA. *Streptomyces griseus* subsp. *Griseus* NRRL F-2227, as described by Ju et al. (2015), was from lab stock. *Pseudomonas aeruginosa* PAO1, as described in Holloway. (1979), was provided by George O'Toole lab, Darthmouth College, USA. *Saccharomyces cerevisiae* S228C was provided by the CABBI lab in Carl R. Woese Institute for Genomic Biology, University of Illinois Urbana-Champaign, USA.

Table 1. Overview of strains used in this study

Strain	Genotype	Source/Reference
Archaea		
Sulfolobus islandicus M.16.4	WT	Whitaker Lab, Carl R. Woese Institute for Genomic Biology, University of Illinois Urbana-Champaign
Sulfolobus islandicus Y08.82.36	WT	Whitaker Lab, Carl R. Woese Institute for Genomic Biology, University of Illinois Urbana-Champaign
Sulfolobus acidocaldarius DSM639	WT	Whitaker Lab, Carl R. Woese Institute for Genomic Biology, University of Illinois, Urbana-Champaign
Sulfolobus tokodaii 7	WT	Lab stock, Suzuki et al., 2002
Bacteria		
Escherichia coli OneShot™ TOP10	F⁻*mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) φ80*lacZ*ΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K λ– *rps*L(Str^R) *end*A1 *nup*G	Invitrogen™, USA
Escherichia coli BL21(DE3)	F– *omp*T *hsdS$_B$* (r_B–, m_B–) *gal dcm* (DE3)	Thermo Scientific™, USA
Escherichia coli Rosetta™ 2(DE3)	F- *ompT hsdS*$_B$(r_B- m_B-) *gal dcm* (DE3) pRARE2 (Cam^R)	Sigma-Aldrich, USA
Streptomyces griseus subsp. *griseus* NRRL F-2227	unknown	Lab stock, Ju et al. 2015
Pseudomonas aeruginosa PAO1	WT	Lab stock, Holloway 1955, 1979
Eukaryotes		
Saccharomyces cerevisiae S228C	*MATα SUC2 gal2 mal2 mel flo1 flo8-1 hap1 ho bio1 bio6*	CABBI, Carl R. Woese Institute for Genomic Biology, University of Illinois Urbana-Champaign

Culturing conditions and storage

Sulfolobus spp.

Sulfolobus spp. frozen stock at -80 ºC are inoculated in DT liquid medium [in 1000 mL MilliQ H_2O: 3 gL^{-1} K_2SO_4, 0.5 gL^{-1} NaH_2PO_4, 0.145 gL^{-1} $MgSO_4$, 0.1 gL^{-1} $CaCl_2 \cdot 2H_2O$, 20 µL trace mineral stock solution (3.0% $FeCl_3$, 0.5% $CoCl_2 \cdot 6H_2O$, 0.5% $MnCl_2 \cdot 4H_2O$, 0.5% $ZnCl_2$, and 0.5% $CuCl_2 \cdot 2H_2O$), 1 gL^{-1} Dextrin, 1 gL^{-1} EZ-mix, final pH=3.5] at 76 ºC until an OD_{600} of approximately 0.2-0.4 is reached. DT liquid medium is 0.22 µm filter sterilised.

Escherichia coli

All transformed *Escherichia coli* genotypes are grown overnight at 37 ºC on plates or at 225 rpm in (Lennox) LB + 30 µg/mL kanamycin sulfate [in 1000 mL MilliQ H_2O: 20gL^{-1} LB Broth Lennox (BD Difco™, Cat: DF0402-17-0), 0.3% v/v Kanamycin Sulfate 100x (Gibco™, Cat: 15160054), opt: 1.5% w/v Difco™ Agar (BD Difco™, Lot: 304509)] and stored at -80 ºC in 50% glycerol (v/v). LB medium is autoclaved at 121 ºC for 30 min.

Streptomyces griseus

Streptomyces griseus subsp. *griseus* NRRL F-2227 is grown for 1-2 days at 30 ºC in (Lennox) LB plates or in roller drum at maximum speed in (Lennox) LB and stored at -80 ºC in 50% glycerol (v/v).

Pseudomonas aeruginosa

Pseudomonas aeruginosa PAO1 is grown for 1 day at 37 ºC in (Lennox) LB plates or in roller drum at maximum speed in (Lennox) LB and stored at -80 ºC in 50% glycerol (v/v).

Saccharomyces cerevisiae

Saccharomyces cerevisiae S228C is grown in YPD [in 1000 mL MilliQ H_2O: 10 gL^{-1} Bacto™ yeast extract (BD Difco™, Lot: 9109987), 20 gL^{-1} Bacto™ peptone (BD Difco™, Lot: 7100982) 20 gL^{-1} dextrose (Sigma-Aldrich, Cat: G5767), opt: 1.5% w/v Difco™ Agar] for 1 day at 30 ºC on plates or roller drum at maximum speed for 1 day and stored at -80 ºC in 50% glycerol (v/v). YPD is autoclaved at 121 ºC for 30 min.

Spot-on-lawn assay: Killing phenotypes of archaeal viral toxin against strains across three domains of life

Supernatant collected from *S. islandicus* RJW004 cells harbouring either an pOE-B310 or empty vector (pOE-Empty), as described in Dewerff & Zhang et al. (2022), was used to assess the toxicity of B310 against various strains.

Sulfolobus spp.

Starting cultures of WT *Sulfolobus* spp. (*Sulofolobus islandicus* M.16.4, *Sulfolobus islandicus* Y08.82.36, *Sulfolobus acidocaldarius* DSM639, *Sulfolobus tokodaii 7,* see Table 1) are grown at 76 ºC in DT liquid medium (pH=3.5) until an OD_{600} of approximately 0.2-0.4 is reached. Starting cultures are then inoculated and diluted to an OD_{600} of 0.02 and grown at 76 ºC in fresh DT liquid medium (pH=3.5) for 48 hours.

Cells are concentrated to an OD_{600} of 1.5 by centrifugation at 4000 rpm for 20 min at room temperature. Subsequently, 500 µL of concentrated cells are added to 5 ml of pre-warmed SY medium-Gelrite (0.6%; w/v) mixture in 1:1 ratio. The recipe of SY medium is as follows: 3 gL^{-1} K_2SO_4, 0.5 gL^{-1} NaH_2PO_4, 0.14025 gL^{-1} $MgSO_4$, 0.48 gL^{-1} $CaCl_2 \cdot 2H_2O$, 20 µL trace mineral stock solution (3.0% $FeCl_3$, 0.5% $CoCl_2 \cdot 6H_2O$, 0.5% $MnCl_2 \cdot 4H_2O$, 0.5% $ZnCl_2$, and 0.5% $CuCl_2 \cdot 2H_2O$), 2 gL^{-1} sucrose, 1 gL^{-1} yeast extract. Cells are vortexed and poured immediately on SY plates. Plates are allowed to cool for approximately 15 minutes.

10 µL of supernatant from the toxin-overexpression construct (pOE-B310), the empty vector construct (pOE-Empty) as well as DT medium is spotted on lawns of *Sulfolobus* cells. After drying,

plates are inverted and double bagged in zip-lock bags with two empty SY-Gelrite plates at the top and bottom to prevent dehydration. The plates are then incubated at 76 ºC. The diameter of the inhibition zones is measured at 72h, 96h and 120h of incubation. Experiments are performed in triplicate.

Bacteria and Saccharomyces cerevisiae

Starting cultures of *Escherichia coli* OneShot™ TOP10 (Cat#: C404010, Invitrogen) and *Pseudomonas aeruginosa* PAO1 cells are grown overnight at 37 ºC, in roller drum at maximum speed in (Lennox) LB medium, *Streptomyces griseus* subsp. *Griseus* NRRL F-2227 is grown for 1-2 days at 30 ºC in roller drum at maximum speed in (Lennox) LB medium and *Saccharomyces cerevisiae* S228C is grown for 1 day at 30 ºC in roller drum at maximum speed in YPD medium. Starting cultures are inoculated in the appropriate fresh medium and grown at their respective growing conditions until an OD600 is reached that corresponds to their respective mid-log phase (Olivares-Marin et al., 2018; Scheffler et al., 2021; Sezonov et al., 2007; Shepherd et al., 2010). 200 µL of cells are added to their respective luke-warm molten medium + 0.5% agar, vortexed and poured immediately on their respective medium plates. Plates are allowed to cool down for approximately 15 minutes.

10 µL of supernatant from pOE-B310 and pOE-Empty, and their respective medium control is spotted on the top layer containing the cells. After drying, plates are inverted and stored at the respective culturing conditions. Presence of inhibition is assessed after full coverage of the plates. Experiments are performed in triplicate.

Plasmid construction

The plasmids used in this study are shown in

Gene **and plasmid construction**

Using SignalP6.0, we defined *B310_F* as containing the full *B310* gene (310 total amino acid residues), *B310_S1* as the *B310* gene without N-terminal signalling peptide and membrane trafficking signal (amino acids 2-22 removed) and *B310_S2* as the *B310* gene without the N-terminal signal peptide (amino acids 2-3 removed).These three different gene constructs were obtained from the viral genome of SSV9.1 through PCR amplification, with restriction sites XhoI and NdeI introduced at the 5’ end and 3’ end respectively. PCR amplification was performed with primers described in Supplemental table 1. Samples were loaded into 0.5 mL microcentrifuge tubes containing 2 µL viral DNA template, 10 µL 5x Buffer HF (Phusion® High-Fidelity PCR Kit: E0553S, New England Biolabs), 200 µM dNTPs, 0.5 µM forward primer, 0.5 µM reverse primer, 0.25 µL Phusion High-Fidelity [0.02 U/µL] and 35 µL Milli-Q leading to a total end-volume of 50 µL. PCR amplification was performed by initially denaturing at 98 ºC for 30 seconds before entering 30 cycles of 7 seconds denaturation at 98 ºC, 30 seconds annealing at 55 ºC, 10 seconds extension at 72 ºC. Final extension was performed for 600 seconds at 72 ºC. The final product is cooled at 4 ºC to be used in downstream processing.

B310_opt was synthesised by IDT technologies, USA.

the PCR primers used are shown in Supplemental table 1 and full sequences as well as Sanger sequencing results are given in Supplemental file 1.

Gene and plasmid construction

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Supplemental table 1. Samples were loaded into 0.5 mL microcentrifuge tubes containing 2 µL viral DNA template, 10 µL 5x Buffer HF (Phusion® High-Fidelity PCR Kit: E0553S, New England Biolabs), 200 µM dNTPs, 0.5 µM forward primer, 0.5 µM reverse primer, 0.25 µL Phusion High-Fidelity [0.02 U/µL] and 35 µL Milli-Q leading to a total end-volume of 50 µL. PCR amplification was performed by initially denaturing at 98 ºC for 30 seconds before entering 30 cycles of 7 seconds denaturation at 98 ºC, 30 seconds annealing at 55 ºC, 10 seconds extension at 72 ºC. Final extension was performed for 600 seconds at 72 ºC. The final product is cooled at 4 ºC to be used in downstream processing.

B310_opt was synthesised by IDT technologies, USA.

The gene constructs *B310_F, B310_S1, B310_S2* and *B310_opt* (insert) as well as pET-30a(+) (vector) are digested by 1U of XhoI (XhoI kit 5.000U, Cat#: R0146S, New England Biolabs) and 1U NdeI (NdeI kit 4.000U, Cat#: R0111S, New England Biolabs) in 1x CutSmart Buffer (New England Biolabs) for 4 hours at 37 ºC and stored at -20 ºC for downstream processing. Digested constructs were cleaned up by PCR clean-up kit (Qiagen®) according to manufacturer protocol and the amount of DNA is assessed by Nanodrop. Digested constructs were subsequently ligated with 20U of T4 DNA ligase (T4 DNA Ligase 400.000 U. Cat#: M0202S, New England Biolabs) in 1x T4 DNA Ligase Reaction Buffer (New England Biolabs) in a vector:insert of 1:8 for 4 hours at room temperature. *Escherichia coli* OneShot™ TOP10 cells were transformed with 10 ng of the ligated constructs mentioned above following manufacturer protocol for chemically competent cells. Transformed cells were plated on (Lennox) LB +30 µg/mL kanamycin sulphate and grown overnight at 37 ºC.

Table 2. Overview of plasmids used including the gene of interest. kan=kanamycin, G.O.I=gene of interest.

Construct name	Description	Resistance	Source/Reference
pET-B310_F	pET-30a (+) plasmid; T7 promotor; T7 terminator; full size B310 gene amplified from Δ*cas6*:SSV9.1 (Dewerff et al., 2022)) with 6x His-tag at the C-terminal of the G.O.I	kan	This work
pET-B310_S1	pET-30a (+) plasmid; T7 promotor; T7 terminator; PCR amplified from Δ*cas6*:SSV9.1 with N-terminal signalling peptide and membrane trafficking signal (amino acid 2-22) region removed. 6x His-tag was introduced at the C-terminal of the G.O.I	kan	This work
pET-B310_S2	pET-30a(+) plasmid; T7 promotor; T7 terminator; truncated version of B310 gene PCR amplified from Δ*cas6*:SSV9.1 with suspected membrane trafficking signal (amino acid 2-3) removed. 6x His-tag was introduced at C-terminal of the G.O.I	kan	This work
pET-B310_opt	pET-30a(+) plasmid; T7 promotor; T7 terminator; full size B310 gene codon optimised for BL21(DE3) *Escherichia coli* with 6x His-tag at C-terminal of the GOI	kan	This work
pET-30a (+)	Protein expression plasmid; T7 promotor; T7 terminator; lacI promotor; lacI gene; lac operator; 6x His-tag; thrombin site; enterokinase site	kan	Millipore, Sigma, USA

Random colonies from transformants mentioned above were inoculated in fresh liquid

(Lennox) LB + 30 µg/mL kanamycin sulphate and grown overnight at 37 ºC. To assess correct insert of the gene constructs in pET-30a(+), plasmids were obtained by QIAprep Spin Miniprep Kit (Cat#: 27104, Qiagen) and digested for 2 hours according to protocol mentioned above. Digested plasmids were analysed through DNA gel electrophoresis for 60 minutes at 90V with 1.5% agarose gel and visualised with 1x SYBR™ Safe DNA Gel Stain (Cat#: S33102, Invitrogen™) and referenced to Quick-Load® Purple 1kb DNA ladder, no SDS (Cat#:N0552S, New England Biolabs).

Plasmids that showed the correct insert size were sequenced by Sanger sequencing with standard primers for the T7 promotor and T7 terminator. The pET-30a(+) plasmids with correct inserts sequences and the highest sequencing depth were chosen for downstream processing (Supplemental file 1).

The plasmids containing the correct insert of *B310_F, B310_S1, B310_S2* and an empty pET-30a (+) plasmid were transformed in *Escherichia coli* Rosetta™ 2(DE3) (Cat#: 71405-3, Sigma-Aldrich, Novagen), hereafter referred to as: Rosetta-B310_F, Rosetta-B310_S1, Rosetta-B310_S2 and Rosetta-Empty_v, following manufacturer protocol for chemically competent cells - as described for *Escherichia coli* OneShot™ TOP10 cells - using 10 ng template.

10 ng of plasmid containing *B310_opt* and an empty pET-30a (+) plasmid were transformed in *Escherichia coli* BL21(DE3) (Cat#: EC0114, Thermo Scientific™), hereafter referred to as: BL-B310_F and BL-Empty_v, following the manufacturer protocol for chemically competent cells as described for *Escherichia coli* OneShot™ TOP10 cells.

Heterogenous expression of archaeal toxin B310 in *Escherichia coli* expression systems

Induction of expression systems

Overnight cultures of *E. coli* BL21(DE3) and *E. coli* Rosetta™ 2(DE3) are inoculated in fresh (Lennox) LB + 30 µg/mL kanamycin sulphate medium and grown at 37 ºC and 225 rpm until an OD_{600} of 0.6-1.0 nm is reached. Either IPTG or MilliQ H_2O, induced group and control group respectively, is added to the medium. Cultures are induced overnight at 16 ºC and 225 rpm. Terminal OD_{600} is measured after induction.

Cell and supernatant collection

After induction, the cell suspension is centrifuged at 4.000g for 30 minutes at 4 ºC. The supernatant is transferred to fresh flasks, the cell pellet is resuspended in MilliQ H_2O and again centrifuged at 4.000g for 30 minutes at 4 ºC after which the supernatant is discarded. The cell pellet is stored at -20 ºC for future use.

Sample processing

The collected cell pellet is resuspended in filter sterilised equilibration buffer [in 1000 mL MilliQ H_2O: 1.78 gL^{-1} $Na_2HPO_4 \cdot H_2O$, 1.38 gL^{-1} $NaH_2PO_4 \cdot 2H_2O$, 29.22 gL^{-1} NaCl] and lysed through sonication (Fisherbrand™ 505 Sonicator, Thermo Scientific) with a 3 mm probe at 40% amplitude and 30 seconds on 60 seconds off on ice for experiments in *E. coli* Rosetta™ 2(DE3). *E. coli* BL21(DE3) cells were lysed by homogenisation (EmulsiFlex-C3, Avestin) according to manufacturer protocol for 4 rounds at ~11.000 psi, while kept on ice inbetween rounds. After lysing, the cell debris suspension is centrifuged at 12.000g for 30 minutes at 4 ºC for both groups. Cell debris is kept at -20 ºC.

The cell supernatant, after collection, is processed immediately by centrifuging at 11.000g for 20 minutes. The supernatant acquired by this step is transferred to fresh tubes and the pellet is discarded. The supernatant was then either analysed directly on SDS-PAGE or concentrated using Pierce™ Protein Concentrator PES 10k MWCO (Molecular Weight Cut-Off) (Lot#: VK313883, Thermo Scientific). When concentrated, 20 mL of supernatant was added to the concentrator and subsequently centrifuged for 30 minutes at 3.900g, the flowthrough was discarded and the retentate

was kept. This step is performed 3 times until a total of 60 mL supernatant is processed. The amount of retentate per sample differed greatly and was diluted with equilibration buffer to a total volume of 1 mL per sample. The protein concentrate was either diluted 10x or 100x with equilibration buffer.

Whole cell lysate analysis was performed by centrifuging 1 mL of culture (OD_{600}=2.0) for 3 minutes at 12.000g, discarding the supernatant and resuspending the pellet in 50 µL 1x Laemmli sample buffer. The sample was then boiled at 95 ºC for 10 minutes and subsequently centrifuged at 12.000g for 10 minutes, 4 µL of sample was used for SDS-PAGE analysis.

Metal-affinity chromatography

Metal-affinity chromatography was performed on the soluble fraction of the B310_F lysate using His GraviTrap columns (Cat#: 11003399, Cytiva) according to manufacturer protocol. 6 fractions were eluted, containing 5 droplets each. All fractions were analysed separately and together in one vial. For binding and elution, standard binding buffer [in 1000 mL MilliQ H_2O: 1.78 gL^{-1} $Na_2HPO_4 \cdot H_2O$, 1.38 gL^{-1} $NaH_2PO_4 \cdot 2H_2O$, 29.22 gL^{-1} NaCl, 20 mM imidazole] and elution buffer [in 1000 mL MilliQ H_2O: 1.78 gL^{-1} $Na_2HPO_4 \cdot H_2O$, 1.38 gL^{-1} $NaH_2PO_4 \cdot 2H_2O$, 29.22 gL^{-1} NaCl, 500 mM imidazole] were used as described in the manufacturer protocol.

Sample analysis on SDS-PAGE

Samples were analysed using SDS-PAGE. Apart from B310_F, B310_S1, B310_S2 and concentrated protein samples of B310_opt, all sample amounts used for analysis were normalised to the terminal OD_{600} after induction. Samples are added to microcentrifuge tubes and diluted with MilliQ H_2O and 4x Laemmli sample buffer to yield a concentration of 1x Laemmli sample buffer. Samples are then boiled at 95 ºC for 5 minutes, quickly vortexed, centrifuged, and resuspended. Samples were loaded on 4-20% polyacrylamide gel (4–20% Mini-PROTEAN® TGX™ Precast Protein Gels, 10-well, 30 µl, Cat#:4561093, Bio-Rad) and referenced to Precision Plus Protein™ All Blue ladder (Precision Plus Protein™ All Blue Prestained Protein Standards, Cat#: 1610373, BioRad®). 1xTGS (10x Tris/Glycine/SDS, Cat#: 1610732, BioRad®) was used as running buffer. Samples were run on gel for ~50 minutes at 170V. Gel was subsequently stained with SimplyBlue™ SafeStain (Cat#: LC6060, Invitrogen™) according to manufacturer protocol.

Results

Killing phenotypes of archaeal viral toxin against strains across three domains of life

Previous research has shown that the B310 protein has an inhibitory effect on *S. islandicus* strains lacking the *B310* gene (Dewerff et al., 2022). In this research, we test whether this viral toxin has a broad killing spectrum against various strains across three domains of life.

Our results showed that B310 exhibits inhibitory effects on *S. islandicus* M.16.4 and *S. islandicus* Y08.82.36, both lacking the *B310* gene, and no inhibitory effects on *S. acidocaldarius* and *S. tokodaii,* which also lack the *B310* gene. There was no significant difference observed in the diameter of the inhibition zone between *S. islandicus* M.16.4 and *S. islandicus* Y08.82.36. *S. islandicus* M.16.4 was significantly different from *S. acidocaldarius* DSM639 and *S. tokodaii* 7 ($Z=-4.38, p<0.05$; $Z=4.382, p<0.05$) (Figure 1). *S. islandicus* Y08.82.36 was significantly different from *S. acidocaldarius* DSM639 and *S. tokodaii* 7 ($Z=-3.71, p<0.05$; $Z=3.71, p<0.05$). Summarized results are shown in Figure 1, individual results are shown in Supplemental figure 1-4.

We also found that B310 had no inhibitory effect on *Escherichia coli* OneShot™ TOP10, *Pseudomonas aeruginosa* PAO1, and *Streptomyces griseus* subsp. *griseus* NRRL-F-2227 and *Saccharomyces cerevisiae* S228C. Individual results are shown in Supplemental figure 5-6.

Heterogenous expression of archaeal toxin B310 in *Escherichia coli* expression systems

Expression of B310, N-terminal signal peptide and membrane trafficking signal truncated variants in *E. coli* Rosetta™ 2(DE3)

Production of B310 in *S. islandicus* is tedious and lacks specificity for further research in structural and biochemical analysis. Here, we aimed to develop a method that allows high production

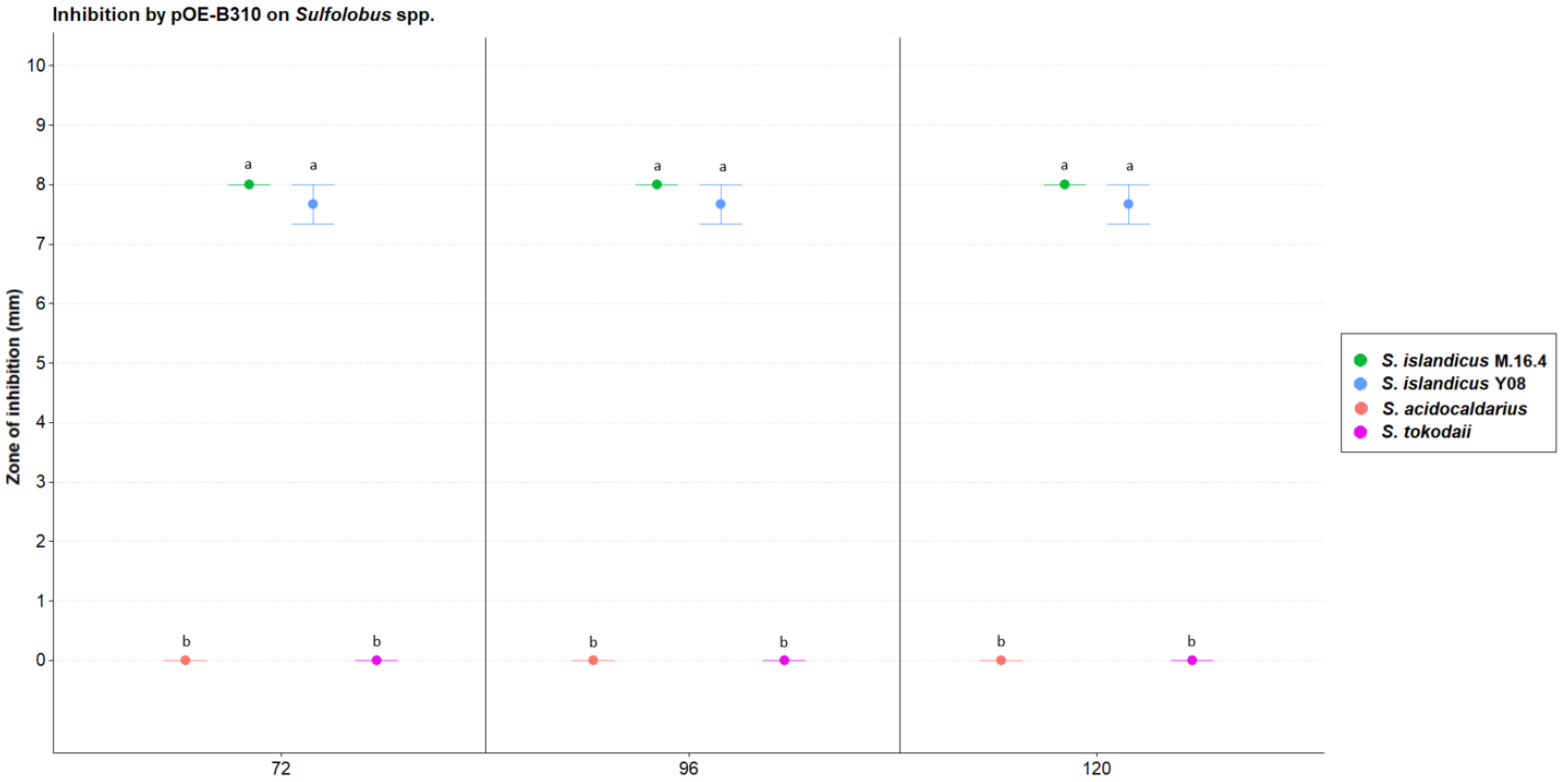


Figure 1. Summary of spot-on-lawn assay on growth inhibition of *Sulfolobus* spp. by B310 derived from supernatant of RJW004 over-producing B310. Dot plot shows average (n=3) of zone of inhibition on all samples, measured at 72h, 96h and 120h. **Error bars**: CI95. a and b depict significant differences. *S. islandicus* M.16.4 vs *S. acidocaldarius* and *S. tokodaii* (Z=-4.38,p<0.05; Z=4.382,p<0.05), *S. islandicus* Y08 vs *S. acidocaldarius* and *S. tokodaii* (Z=-3.71,p<0.05; Z=3.71,p<0.05).

and purification of B310 in a bacterial expression system. We used SignalP6.0 to verify the N-terminal signal peptide and membrane trafficking signal as described by Dewerff and colleagues (2022). We found a N-terminal signalling peptide at amino acid position 2-3 and a membrane trafficking signalling peptide at amino acid position 2-22 (Teufel et al., 2022).

We initially observed no obvious expression levels nor differences in the soluble fraction of induced (0.75 mM IPTG) and uninduced lysates between -B310_F, Rosetta-B310_S1 and Rosetta-B310_S2 (Figure 2).
Attempts to purify B310_F from the soluble fraction of the lysate did not result in our protein of interest (Figure 3).

We theorised that the membrane trafficking signal could hinder solubility of our target protein and result in a lack of expression levels within the soluble fraction of the lysate. However, when assessing expression levels in the supernatant and soluble fraction between induced (0.75 mM IPTG) and uninduced Rosetta-B310_S1 and Rosetta-Empty_v no differences in presence/absence of bands

were observed in the region of interest (~33 kDa), nor at suggested dimer/trimer molecular weight (Figure 4).

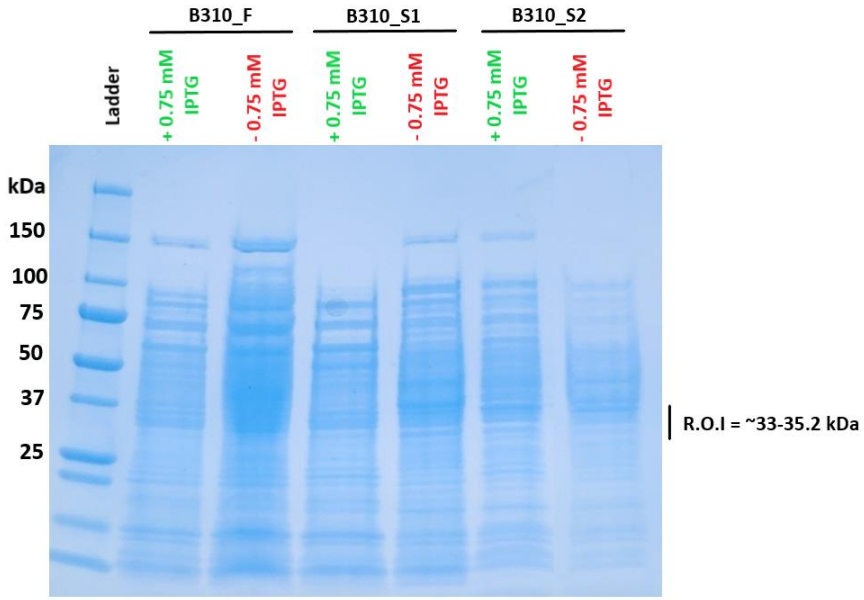


Figure 2. SDS-PAGE results on soluble fraction of the lysate of induced (green, 0.75 mM IPTG) or uninduced (red) *Escherichia coli* Rosetta™ 2(DE3) carrying pET-B310_F, pET-B310_S1 or pET-B310_S2. **Ladder** = Precision Plus Protein™ All Blue Prestained Protein Standards (BioRad®). **R.O.I** = Region of interest.

Figure 3. SDS-PAGE results on unpurified soluble fraction of the lysate, Metal affinity chromatography purified eluted fractions, and combined purified elute fractions of induced (0.75 mM IPTG) *Escherichia coli* RosettaTM 2(DE3) carrying pET-B310_F. **Ladder:** Precision Plus Protein™ All Blue Prestained Protein Standards (BioRad®). **R.O.I:** Region of interest.

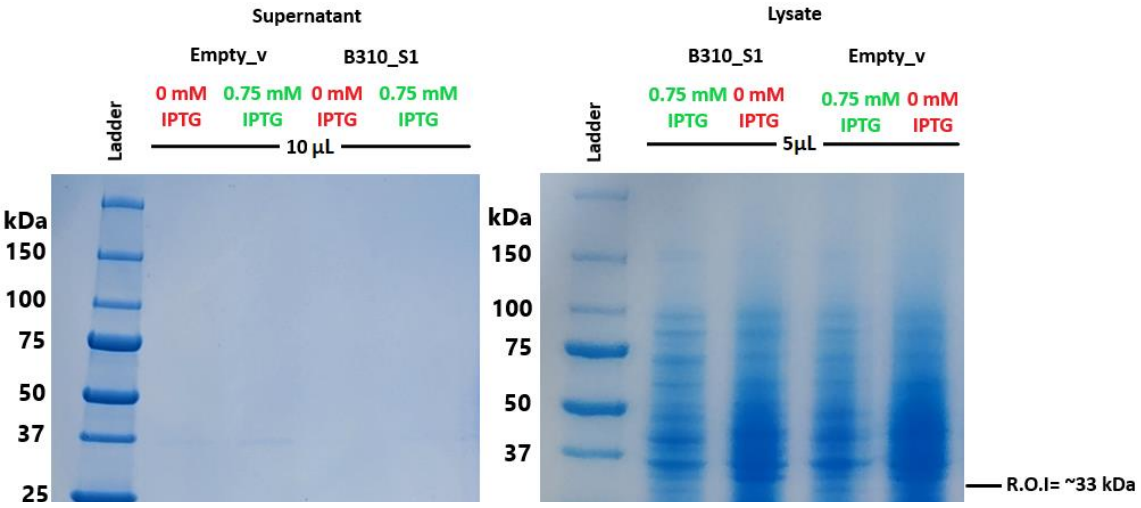
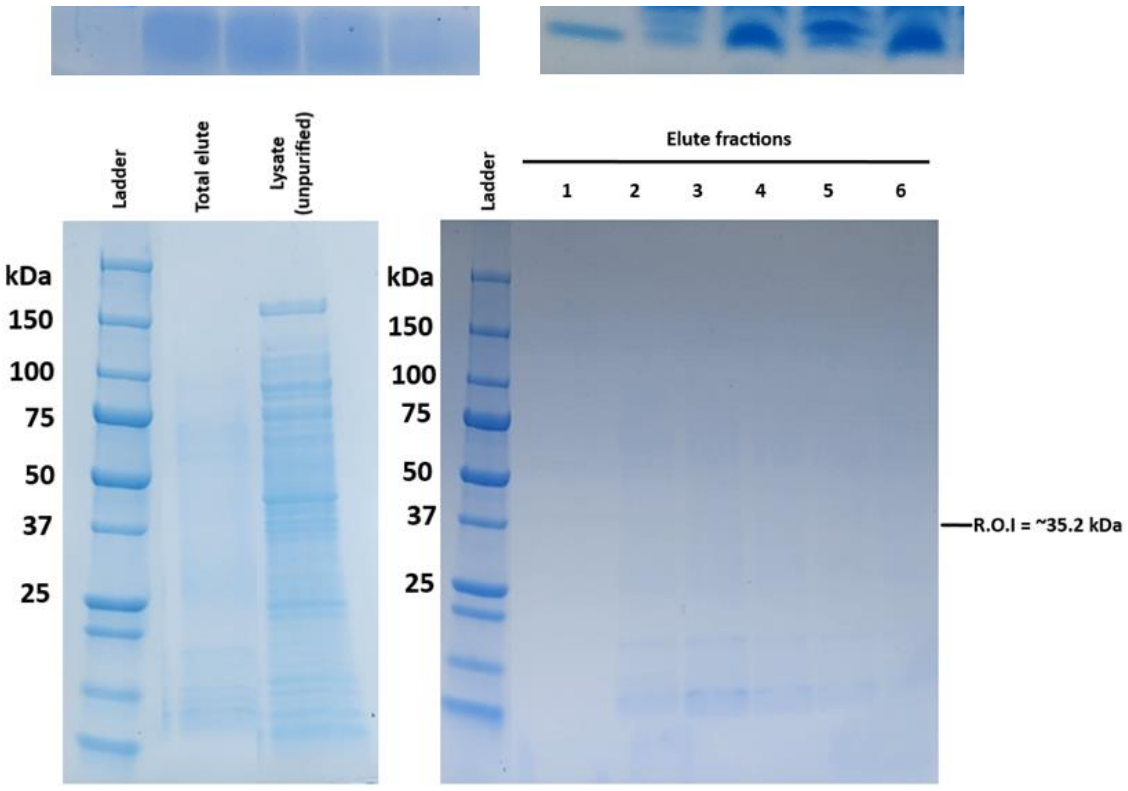


Figure 4. SDS-PAGE results on supernatant (supernatant) and whole cell lysate (lysate) of induced (green, 0.75 mM IPTG) and uninduced (red) *Escherichia coli* Rosetta^{TM} 2(DE3) carrying pET-B310_S1 or pET_Empty_v control. **Ladder**: Precision Plus Protein™ All Blue Prestained Protein Standards (BioRad®). **R.O.I:** Region of interest.



Expression of codon-optimised B310 in *Escherichia coli* BL21(DE3)

Previous research has shown that rare codons, even when using an expression vector suitable for reading these rare codons, lead to lower expression levels than standard *E. coli* BL21(DE3) expressing codon-optimised genes (Lipinszki et al., 2018). Therefore, we chose to codon-optimise the full *B310* gene clone it into *E. coli* BL21 (DE3) (Hereafter referred to as: BL-B310_opt).

The soluble fraction of the lysate as well as supernatant and concentrated supernatant of induced (0.75mM IPTG) or uninduced BL-B310_opt and BL-Empty_v showed no difference in

expression level around the region of interest o(~35.2 kDa) nor at 2x or 3x this molecular weight (dimer/trimer) Figure 5A-C.
Intense bands can be seen around the 10 kDa ladder mark for Empty_v groups in the soluble fraction of the lysate (Figure 5A). This can be explained by the expression of the region between NdeI and XhoI in pET-Empty_v and is estimated to yield an ~8 kDa protein.

To verify whether B310 might be present in amounts invisible on SDS-PAGE, spot-on-lawn assays were performed. B310 has been suggested to have very high inhibition levels on *S. islandicus* strains, even at low concentrations (Dewerff et al., 2022). Their research has additionally shown that suspected B310 bands on SDS-PAGE were only visible after 20x of pOE-B310 (Dewerff et al., 2022). However, the spot-on-lawn assay did not show any inhibition on *Sulfolobus* spp. for the soluble fraction of the lysate, supernatant and concentrated supernatant of induced (0.75mM IPTG) or uninduced BL-B310_opt or BL-Empty_v (Supplemental figure 7).

To decrease possible B310 toxicity from within *E. coli* BL21(DE3) cells, we lowered the amount of IPTG to 0.075 mM. Overnight induction of B310_opt with 0.075 mM showed an average increase in OD_{600} of 0.59 (n=2) as compared to 0.11 (n=1) with 0.75 mM IPTG. However, the soluble fraction of the lysate as well as supernatant and concentrated supernatant of induced (0.075mM IPTG) or uninduced BL-B310_opt or BL-Empty_v showed no difference in expression level around the region of interest (~35.2 kDa) nor at 2x or 3x this molecular weight (dimer/trimer) (Figure 6). Lysate sample amount was standardised to OD600 value of induced B310_opt. For the groups induced with 0.075mM IPTG, we additionally performed a spot-on-lawn assay. The spot-on-lawn assay did not show any inhibition on *Sulfolobus* strains for the soluble fraction of the lysate as well as supernatant and concentrated supernatant. (Supplemental figure 8).

To rule out whether B310 might be expressed in the insoluble fraction of the lysate or inclusion bodies, we performed a whole cell lysate analysis of induced (0.075 mM IPTG) and uninduced BL-B310_opt and BL-Empty_v (Supplemental figure 10). Smearing of the samples within the gel resulted in difficult assessment (Supplemental figure 10). However, induced BL-B310_F and BL-Empty_v samples were easy to distinguish, especially around the R.O.I (Figure 7B-C). Between

these groups, there seems to be no apparent difference in expression levels around the R.O.I that point to the production of B310_opt in the insoluble fraction of the lysate, nor inclusion bodies.

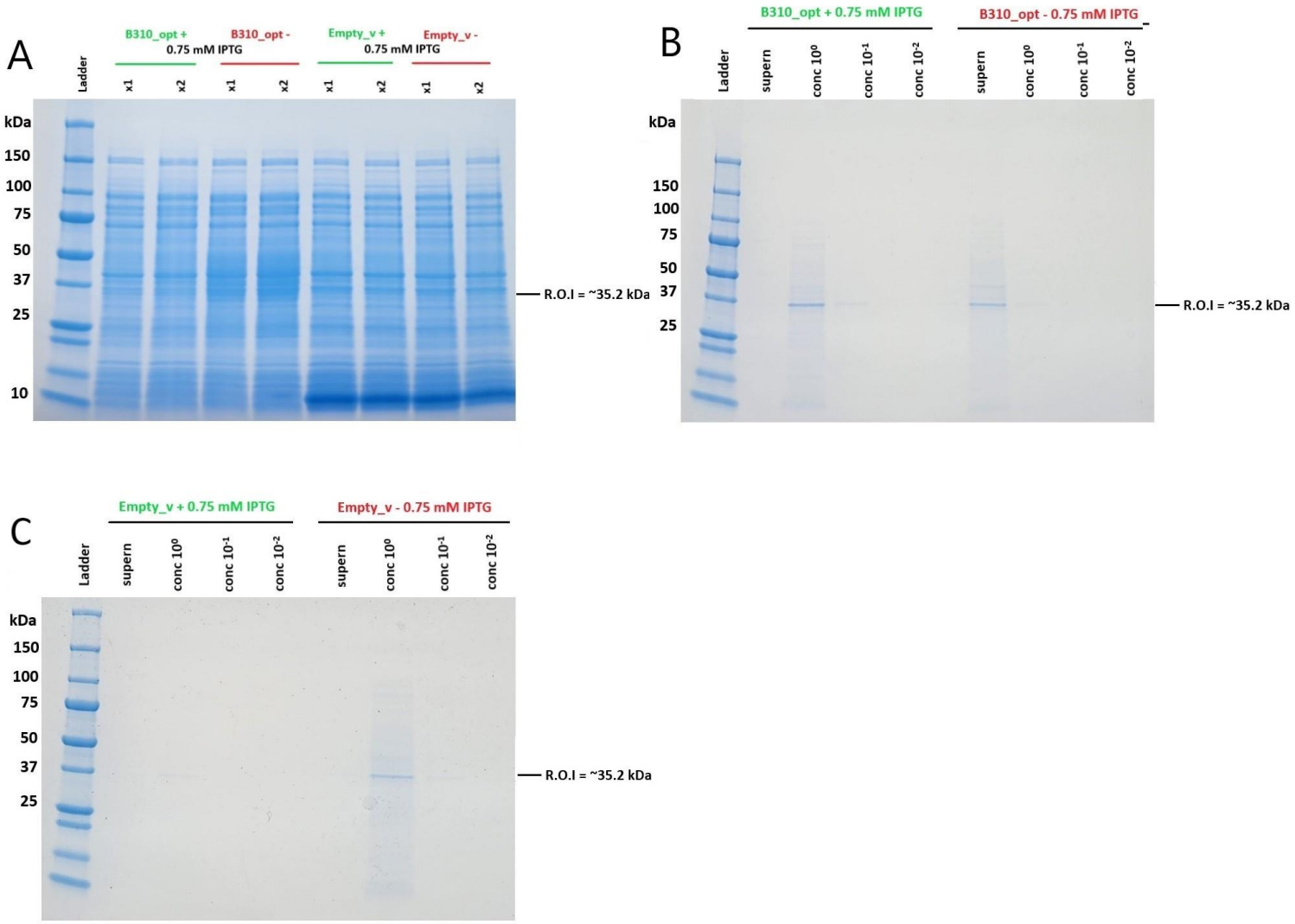


Figure 5. SDS-PAGE results on soluble fraction of the lysate and (concentrated supernatant) of induced (green, 0.75 mM IPTG) or uninduced (red) *Escherichia coli* BL21 (DE3) carrying pET-B310_opt, or pET-B310_Empty_v empty control. **(A)** Soluble fraction of the lysate SDS-PAGE analysis **(B)** (Concentrated) supernatant SDS-PAGE analysis of pET-B310_opt. **(C)** (Concentrated) supernatant SDS-PAGE analysis of pET-Empty_v control. **Ladder**: Precision Plus Protein™ All Blue Prestained Protein Standards (BioRad®). **R.O.I**: Region of interest. **1x**: 1 time sample amount. **2x**: 2 times sample amount. **Supern**: unconcentrated supernatant. **Conc 10^0:** concentrated supernatant. **Conc 10^{-1}:** 10 times diluted supernatant. **Conc 10^{-2}:** 100 times diluted supernatant.

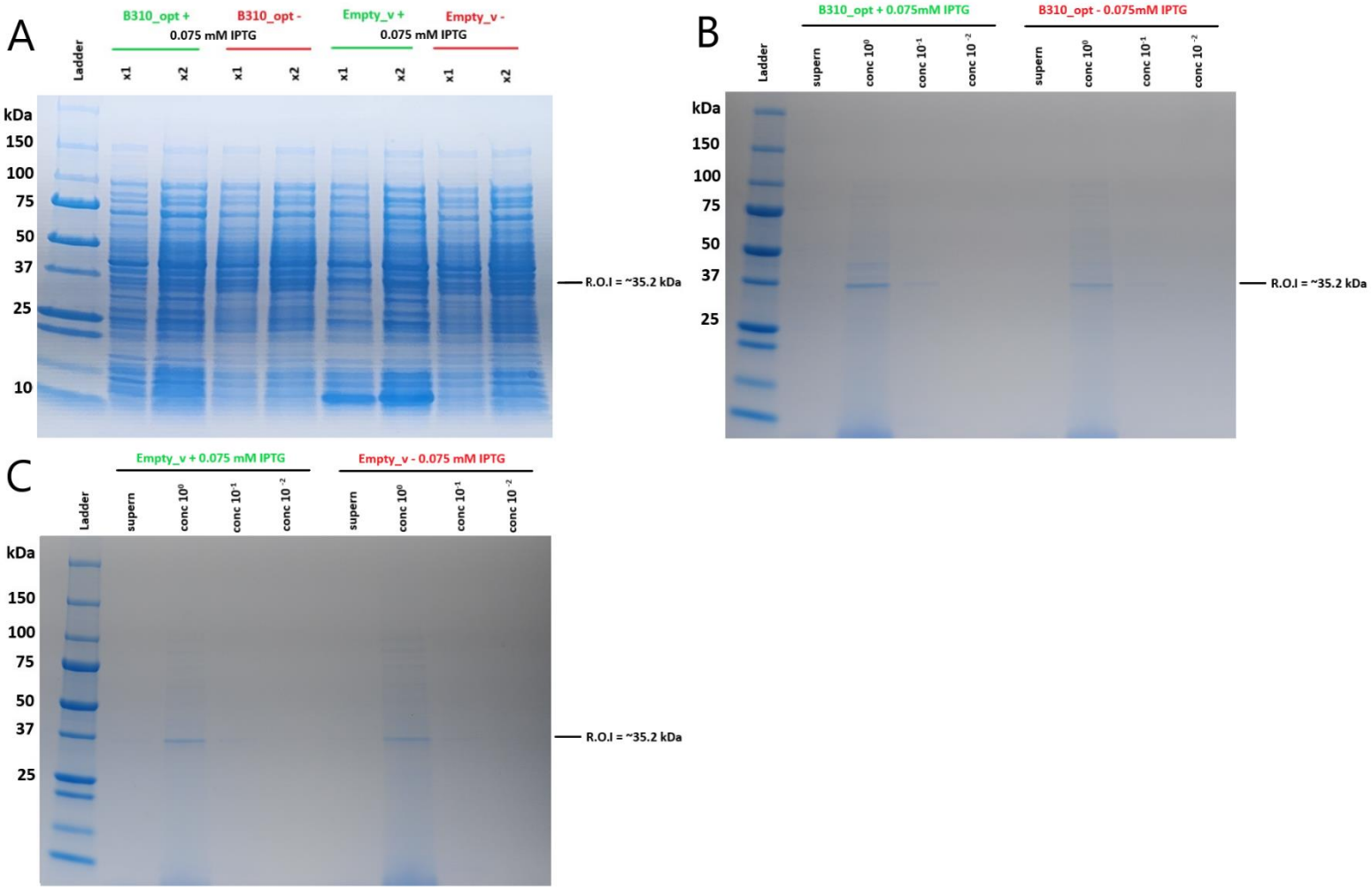


Figure 6. SDS-PAGE results on soluble fraction of the lysate and (concentrated supernatant) of induced (green, 0.075 mM IPTG) or uninduced (red) *Escherichia coli* BL21 (DE3) carrying pET-B310_opt, or pET-B310_Empty_v empty control. **(A)** Soluble fraction of the lysate SDS-PAGE analysis **(B)** (Concentrated) supernatant SDS-PAGE analysis of pET-B310_opt. **(C)** (Concentrated) supernatant SDS-PAGE analysis of pET-Empty_v control. **Ladder**: Precision Plus Protein™ All Blue Prestained Protein Standards (BioRad®). **R.O.I**: Region of interest. **1x**: 1 time sample amount. **2x**: 2 times sample amount. **Supern**: unconcentrated supernatant. **Conc 10^0:** concentrated supernatant. **Conc 10^{-1}:** 10 times diluted supernatant. **Conc 10^{-2}:** 100 times diluted supernatant.

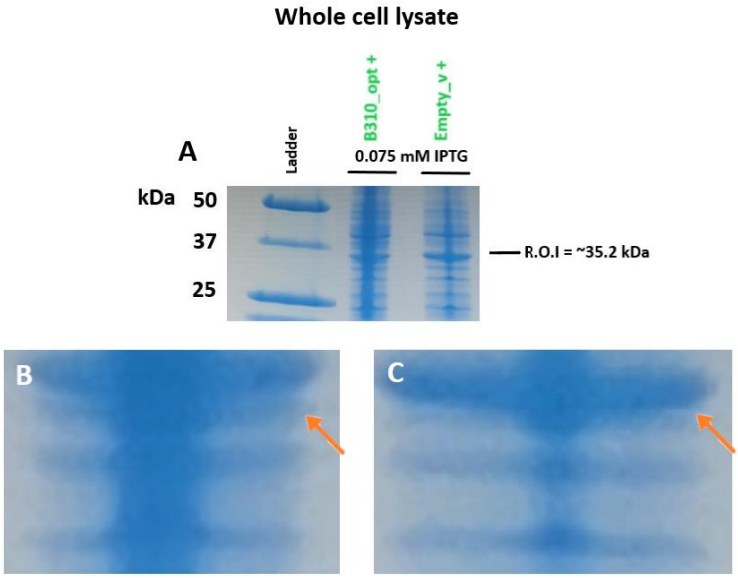


Figure 7. Whole cell lysate SDS-PAGE analysis of induced (0.075 mM IPTG) *Escherichia coli* BL21 carrying pET-B310_opt or pET-Empty_v. **(A)** Comparison of B310_opt and Empty_v. **(B)** Zoomed in view of R.O.I. of B310_opt. **(C)** zoomed in view of R.O.I. of Empty_v. **Orange arrows:** depict lack of difference in expression levels in R.O.I between B310_opt and Empty_v. **Ladder:** Precision Plus Protein™ All Blue Prestained Protein Standards (BioRad®). **R.O.I**: region of interest.

Discussion

It has been demonstrated that the viral toxin B310, encoded in SSV9.1, has a killing phenotype on *S. islandicus* lacking the *B310* gene, suggesting a symbiotic relationship between virus and host (Dewerff et al., 2022). However, the killing phenotype of B310 on organisms outside of *S. islandicus* had yet to be elucidated. Furthermore, B310 production as described by Dewerff and colleagues (2022) is tedious and lacks specificity for future biochemical, structural and mode of action analysis. In our research, we assessed whether B310, derived from the supernatant of B310 overexpression archaeal host, has a killing phenotype across three different domains of life. We additionally attempted to design a method to heterogeneously express B310 and its variants in *E. coli* Rosetta™ 2(DE3) and BL21(DE3). Our data suggests that B310 is toxic specifically for *S. islandicus* strains lacking *B310* and not to other *Sulfolobus* spp., nor other domains of life. Furthermore, the results indicate that the heterogenous expression of B310, structural variants and codon optimised *E. coli* Rosetta™ 2(DE3) and BL21(DE3) under our conditions was unsuccessful.

Previous research suggests that *S. islandicus* strains lacking *B310* are subject to toxic effects of B310 (Dewerff et al., 2022). We strengthened this discovery by demonstrating a killing phenotype of B310 on other *S. islandicus* strains: M.16.4 and Y08.82.36. Targeting closely related strains, as well as offering own protection from the toxin, can be seen as an evolutionary benefit by means of outcompeting. In turn, SSV9.1 can propagate in *S. islandicus*. Therefore, one could suggest that SSV9.1 chronically infected *S. islandicus* strains harbour a mutualistic relationship through B310.

Interestingly, exposure to B310 treatment did not seem to affect *S. acidocaldarius* DSM 639 and *S. tokodaii* 7, despite the absence of a homologous B310 in both species. Previous research showed that both *S. acidocaldarius* and *S. tokodaii* produce an antimicrobial peptide, known as sulfolobicin (Ellen et al., 2011). Sulfolobicin appears to be similar to bacteriocins (Ellen et al., 2011; O'Connor & Shand, 2002). While research groups suggest that sulfolobicin is also produced by *S. islandicus*, this has now been shown to be a miss-annotation (NCBI, BLAST). Ellen and colleagues (2011) showed that sulfolobicin is toxic to closely related *Sulfolobus* spp. and having the genes encoding for sulfolobicin protects from its toxicity. This suggests some similarity to B310. Nonetheless, the presence of sulfolobicin in *S. acidocaldarius* and *S. tokodaii* does not explain why B310 has no effect on these organisms. Further research must elucidate whether sulfolobicin protects against B310 and vice versa.

One might argue that using B310-containing supernatant is not sufficient to demonstrate that B310 is the inhibitory factor affecting the growth of *S. islandicus* M.16.4 and *S. islandicus* Y08.82.36. Previous research in *E. coli* has elucidated that the use of an overexpression plasmid can influence expression of other genes through metabolic load (Glick, 1995; Özkan et al., 2005). It is conceivable that *S. islandicus* RJW004 likewise experiences metabolic load upon on the introduction of pOE-B310 expression vector. Therefore, the supernatant could contain other compounds that influence the growth of *S. islandicus* M.16.4 and *S. islandicus* Y08.82.36. However, Dewerff and colleagues (2022) showed that SDS-PAGE gel lane slices of B310 exclusively caused zones of inhibition on uninfected *S. islandicus* RJW002, suggesting that the inhibition is unlikely to be caused by a factor outside of B310. Nonetheless, these limitations further underline the importance of devising a method to produce a pure toxin for conducting the experiment.

We used several organisms with significantly different outer cell structures to provide an initial idea into the mode of action of B310 and whether it has a killing phenotype outside of archaeal *Sulfolobus* species. We theorise that B310 could interact with the lipids in the cell membrane, the S-layer or cell specific membrane proteins of *Sulfolobus* species. *Sulfolobus* spp. have a different cell structure than bacterial and eukaryotic organisms. Their S-layer is different from bacteria and eukaryotes lack an S-layer (Fagan & Fairweather, 2014; König, 1988; Sleytr et al., 2014). Additionally, *Sulfolobus* spp. lipid cell membranes are arranged in a monolayer as opposed to a bilayer and do not contain peptidoglycan (Palmieri et al., 2013; Villanueva et al., 2014).

In our research we have tried to produce B310 and its structural variants in *E. coli* Rosetta™ 2(DE3) and BL21(DE3). B310_S1 does not contain an N-terminal peptide nor a membrane trafficking signal. Therefore we theorised that B310_S1 could have increased solubility due to a reduction of embedding the protein in the membrane (Freudl, 2018). However, our data suggests no production of B310 in the supernatant. The whole cell lysate data indicates that B310_S1 is not present in inclusion bodies, thus indicating absence in the (in)soluble fraction of the lysate.

For the creation of pET-B310_F, pET-B310_S1 and pET-B310_S2, we directly amplified the constructs from the SSV9.1 genome. The SSV9.1 genome contains codons that are rarely used by standard *E. coli* BL21(DE3). To enhance expression of rare codons, we used *E. coli* Rosetta™ 2(DE3). However, *E. coli* Rosetta™ 2(DE3) shows a lower basal expression level than standard BL21(DE3) (Lipinszki et al., 2018). Nonetheless, *E. coli* Rosetta™ 2(DE3) has been used to successfully produce archaeal proteins before (He et al., 2018; Sugii et al., 2014). To rule out the negative effects of codon bias on the production of B310_F, we codon optimised B310_F.

Using concentrated supernatant for B310_opt instead of metal affinity chromatography allows us to cost-effectively increase the amount of protein per sample. However, according to manufacturer protocol (Pierce™ Protein Concentrator, PES, Thermo Scientific), a 10k MW cut-off only reliably concentrates proteins 2-fold the cut-off. This could mean that if B310_opt is broken down into smaller fragments, it would not be visible on SDS-PAGE. We used SignalP6.0 to predict cleavage sites in B310_opt. It predicted a cleave site between position 27 and 28 with a probability of 0.584, this would result in a protein/peptide with a theoretical MW of 32.3 kDa (Teufel et al., 2022). If B310_opt was still produced normally, but cleaved, you would expect to see a difference in expression levels of B310_opt and controls on SDS-PAGE gel around 32.3 kDa. This lack of difference in expression levels at 32.3 kDa suggests that B310 is not cleaved.

It could be that B310_opt production after induction by 0.75 mM IPTG increases the metabolic load in BL21 to a critical amount and therefore causes the cells to die (Glick, 1995). Lowering the IPTG concentration has been shown to increase cell viability in some studies (James et al., 2021). Besides having an effect on the metabolic load, lowering the IPTG could also increase solubility of proteins (Francis & Page, 2010; Jhamb & Sahoo, 2012). However, other studies have shown that lowering IPTG does not necessarily result in correct protein production due to the fact that the proteins and *Lac* permeases are heterogeneously expressed (Sina et al., 2015). We still chose to decrease the amount of IPTG with 10-fold to a total concentration of 0.075 mM to possibly enhance expression. Our data suggests that B310_opt production is not enhanced when B310_opt is induced by 0.075 mM IPTG.

One could argue that due to the fact that there is incomplete coverage of the top-layer in our spot-on-lawn assay – for 0.075 mM for *S. islandicus* M.16.4 spotted with concentrated supernatant of B310_opt – is inconclusive. However, because *S. islandicus* Y08.82.36 is susceptible to B310, and no inhibition can be seen here, it can be assumed that concentrated supernatant of B310_opt would not result in inhibition of M.16.4. Besides, the B310_opt $conc^{-1}$ lane shows a significant band at the region of interest, suggesting that there is enough protein present to verify toxicity of B310. We can therefore assume that there is no B310_opt present in the supernatant of (un)induced pET-B310_opt.

The Empty_v + 0.075 mM IPTG control groups of both M.16.4 and Y08.82.36 also show incomplete coverage at $conc^{0}$ lane. However, the SDS-PAGE gel shows a significant band at ~37 kDa for all samples in the $conc^{-1}$ lane, suggesting high amount of protein in the retentate and allowing for control comparison between samples.

Even though B310_opt is predicted to be highly soluble (Hebditch et al., 2017), it could be insoluble when present in higher concentrations of the cell. We therefore performed a whole cell lysate on BL-B310_opt or BL-Empty_v, induced with 0.075 mM IPTG (results from uninduced control were inconclusive) and found no difference in expression levels at the region of interest, suggesting no production of B310.

Even if the protein is produced and soluble, many problems can still arise from over-expression of recombinant membrane-bound proteins (Kim et al., 2017; Mathieu et al., 2019).

Recombinant membrane-bound proteins are often toxic to BL21(DE3) cells. Over-expression can cause deleterious events due to oversaturation of membrane protein biogenesis and secretion pathways, ultimately resulting in cell death. Even though we observed no apparent toxicity from B310 on *E. coli* OneShot™ TOP 10 cells administered on the outside of the cell, it does not rule out that the protein can be toxic from within the cell. We did observe an increase of cell viability in 0.075 mM IPTG induced samples as compared to 0.75 mM IPTG, this could suggest that less cells are producing B310_opt, limiting B310_opt toxicity and therefore surviving. However, this did not result in obvious B310_opt expression.

Besides toxicity, improper folding could also result in difficulties during protein expression. *E. coli* has different systems to properly fold proteins. *E. coli* uses GroEL and GroES as compared to e.g. TF55 in *Sulfolobus shibatae* (Knapp et al., 1994; Zolghadr et al., 2011). Furthermore, TF55α and TF55β are additionally present in *S. islandicus* (BLASTp, NCBI). Both are not present in *E. coli*. Furthermore, normally B310 is folded at a temperature of 76 ºC instead of 16 ºC, in which *E.coli* BL21 and Rosetta2 are induced. This difference in chaperonins and folding temperature could result in an unfolded protein. However, if the unfolded protein was still produced and cells still function properly, a band at the R.O.I would be expected. Or if the unfolded protein aggregates, a band would be expected at a higher molecular weight. Nonetheless, improper folding and aggregation could also result in toxicity and eventually death of the induced cells (Francis & Page, 2010; Glick, 1995; Kim et al., 2017).

Future prospects

It is difficult to pinpoint what the exact reason is behind the lack of B310 (and variants) expression. There are several reasons that could explain this: a lack of production, insolubility, improper folding, and toxicity of the protein. These problems are most prominent with overproduction of membrane proteins (Kim et al., 2017).

To verify whether the mRNA is transcribed at all, western blot analysis, using a general antibody that binds to 6xHis-tags can be used (Strandmann et al., 1995).

Increasing the solubility as well as lowering the protein production could reduce the possibility of inclusion body formation (Singh et al., 2015). To increase the solubility of the protein, maltose-binding protein (MBP-tag) could be used instead of a standard 6xHis-tag (Bhatwa et al., 2021; Chen et al., 2023; Francis & Page, 2010).

Decreased protein production can additionally improve solubility. This can be performed by methods that allow titratable induction through the use of a different expression vector. As previously described, IPTG induction can lead to leaky expression. Furthermore, many T7 promotor systems additionally suffer from leaky expression. pBAD as plasmid vector, which uses L-arabinose as an inducer can be used as an alternative to IPTG induction. Furthermore, addition of glucose can further regulate gene expression through catabolic repression. Using L-arabinose and glucose allow for very tightly controlled expression (Guzman et al., 1997).

As mentioned before, membrane bound proteins are difficult to express proteins (Kim et al., 2017; Mathieu et al., 2019). Another advantage of using different *E. coli* vectors that allow for tightly controlled expression, is that it may reduce toxicity of (membrane bound) proteins. Vectors characterised by low T7 lysozyme activity can prevent leaky expression, subsequently preventing cell death from over-expressing toxic proteins, especially for membrane bound proteins (Chen et al., 2023; Haichao et al., 1989; Kim et al., 2017; Mathieu et al., 2019). Lemo21(DE3) allows for tuneable expression of the T7 lysozyme by addition of L-rhamnose (Wagner et al., 2008). Lemo21(DE3) cloned with pBAD-B310_opt+MBP-tag and glucose addition could be a viable option for successful heterologous expressing B310_opt.

Even though we provide options to reduce problems associated with expressing B310 in *E. coli,* it remains that *E. coli* and *S. islandicus* are two very different organisms. Expressing proteins from one domain of life into the other has been historically difficult. We have found various possible challenges for heterogeneous expression of B310 in *E. coli* expression vectors, therefore it might be a

more viable option to express B310 in conditions as close as possible to its native host.

Previous research, by Dewerff et al. (2022) showed successful over-expression of B310 by transforming *S. islandicus* RJW004 with pOE-B310. Adding a 6xHis-tag to B310 in pOE-B310 could allow for B310 to be purified. However, low producibility of B310 in *S. islandicus* remains. A possible solution could be to produce B310 in *S. acidocaldarius*. Expressing B310, again, outside of its native organism, could result in difficulties mentioned before. However, expression systems in *S. acidocaldarius* are well studied and available (Berkers et al., 2018; Schocke et al., 2019). *S. acidocaldarius* has a stable genome, which is beneficial for gene expression. and additionally shows similar cell machinery to *S. islandicus* (Quehenberger et al., 2017). Furthermore, in our research we found that B310 does not appear to be toxic to *S. acidocaldarius*. However, it must be noted that supernatant containing B310 was administered to the outside of the cell. This does not necessarily translate to a lack of toxicity from within cell. Besides, B310 production in *S. islandicus* has been shown to come with a grow cost (Bautista et al., 2015). Future research can elucidate whether B310 expression in *S. acidocaldarius* results in toxicity and the same growth cost as for *S. islandicus*. If B310 expression does not result in toxicity nor a growth cost for *S. acidocaldarius*, the time consuming production and lack of purification possibilities of B310 could be reduced significantly by expressing B310+6x-His-tag in *S. acidocaldarius*.

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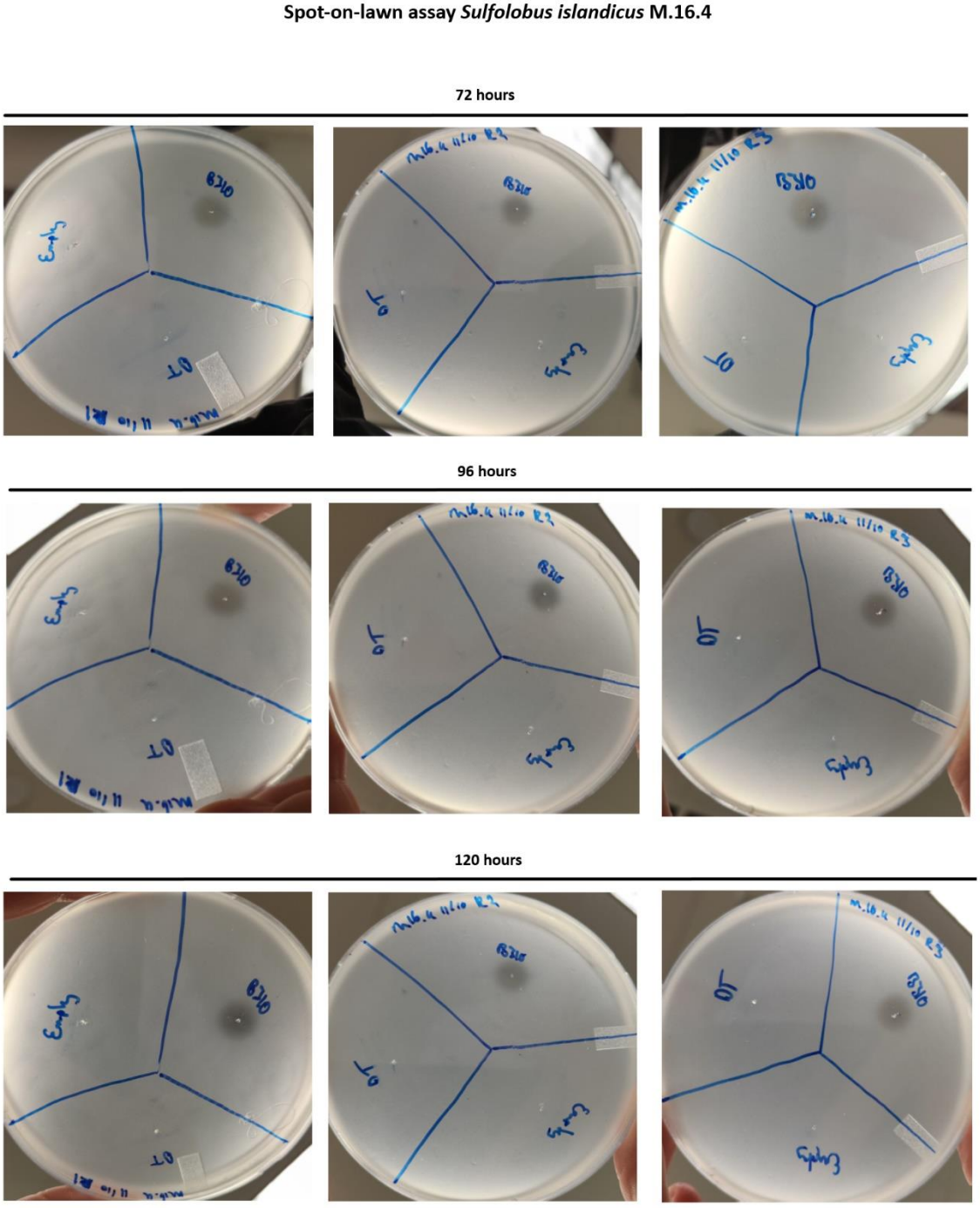
Supplement

Supplemental table 1. Overview of primers used in this study. Restriction enzyme sites are underlined.

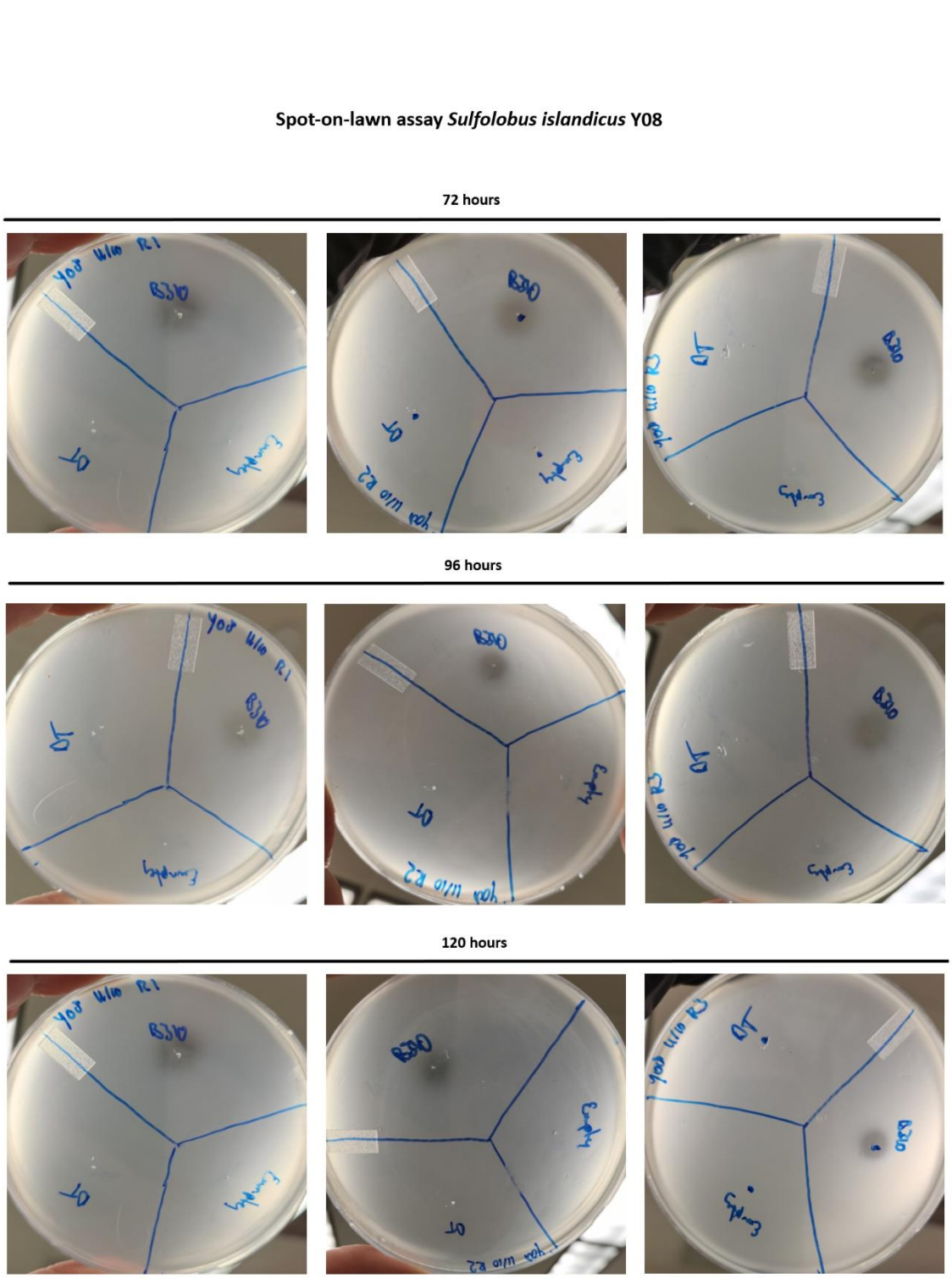
Primer name	Sequence	Restriction enzyme sites
FW_B310_F	5' – CGAGTCGAACCATATGAGGAAGGGTCTACTTTCC – 3'	NdeI
RV_B310_F	5'– ACCGCTCGAGTGGTGAGATCCAATACAAAGC – 3'	XhoI
FW_B310_S1	5'– CGAGTCGAACCATATGTCATCACAAACATACCCAAAC – 3'	NdeI
RV_B310_S1	5'– ACCGCTCGAGTGGTGAGATCCAATACAAAGC – 3'	XhoI
FW_B310_S2	5' – CGAGTCGAACCATATGGGTCTACTTTCCCTAACCTTTC – 3'	NdeI
RV_B310_S2	5' – ACCGCTCGAGTGGTGAGATCCAATACAAAGC – 3'	XhoI

Supplemental file 1. Zip file with full DNA sequences of plasmid + gene of interest and Sanger sequencing data

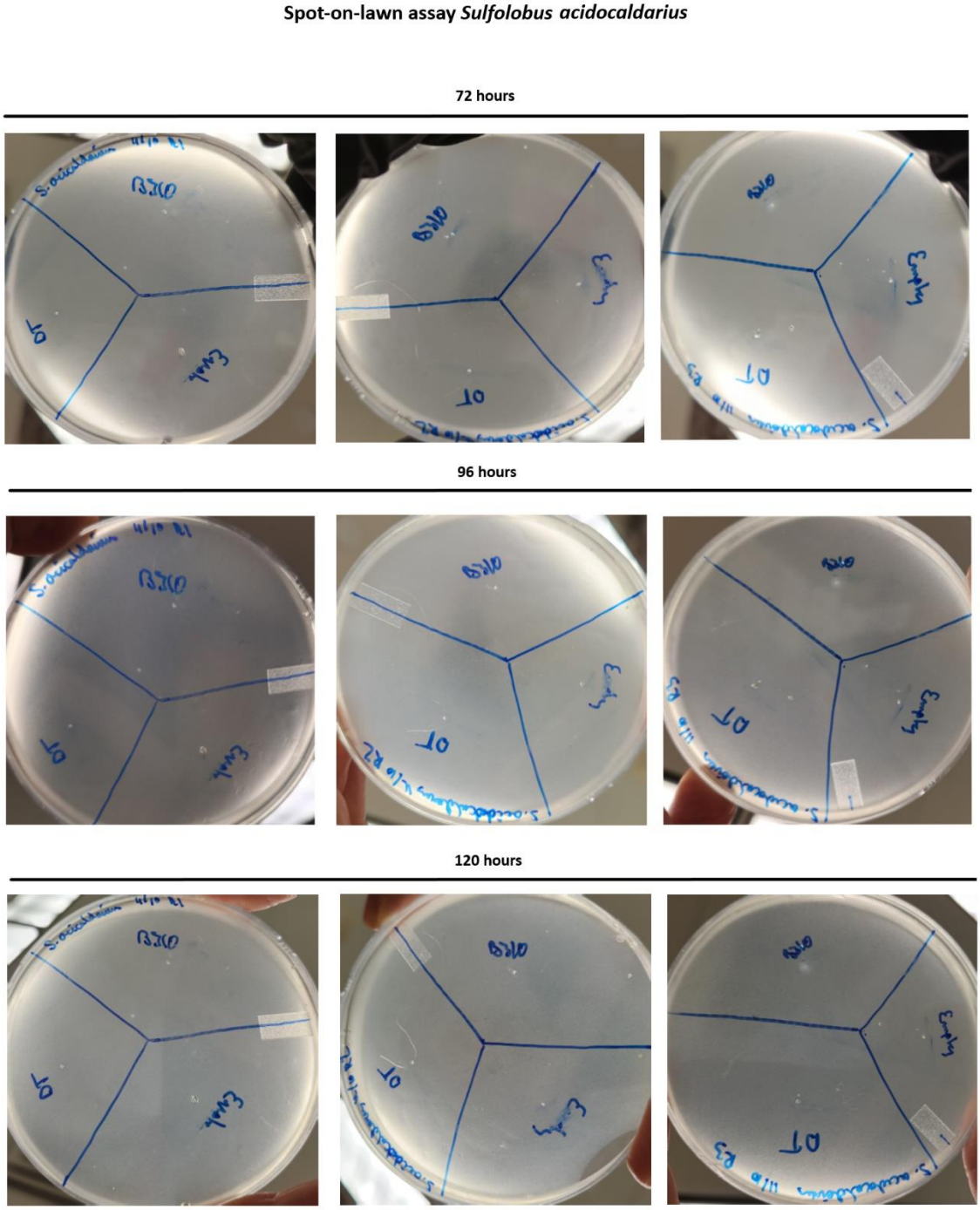
DNA sequences and Sanger data.zip



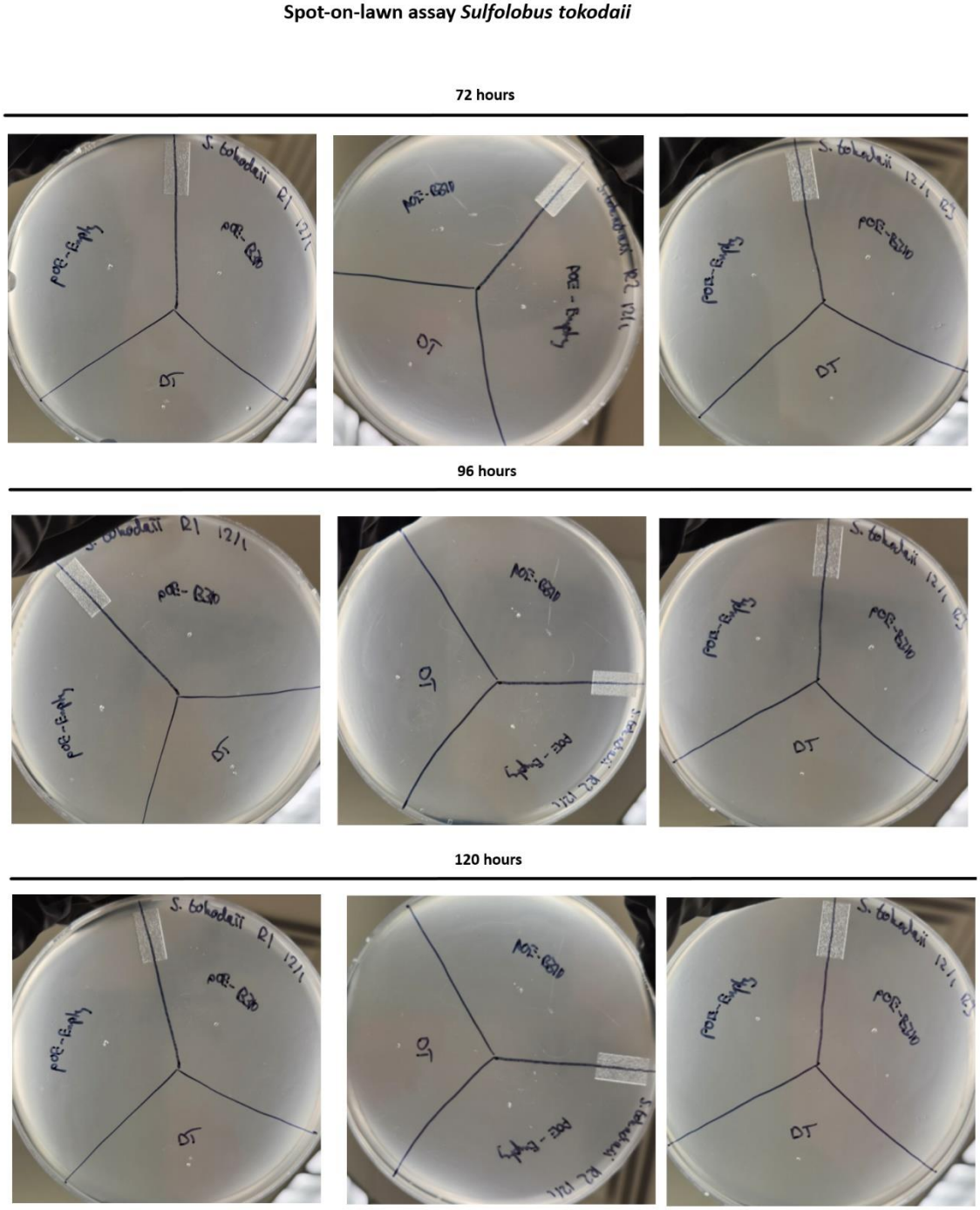
Supplemental figure 1. Individual results of spot-on-lawn assay on growth inhibition of *Sulfolobus islandicus* M.16.4 by B310 derived from supernatant of RJW004 over-producing B310. Zone of inhibition was assessed at 72h, 96h, and 120h (n=3). **B310**: supernatant from RJW004 transformed with over-expression plasmid pOE-B310. **Empty**: supernatant from RJW004 transformed with empty over-expression plasmid pOE-Empty. **DT**: DT medium control.



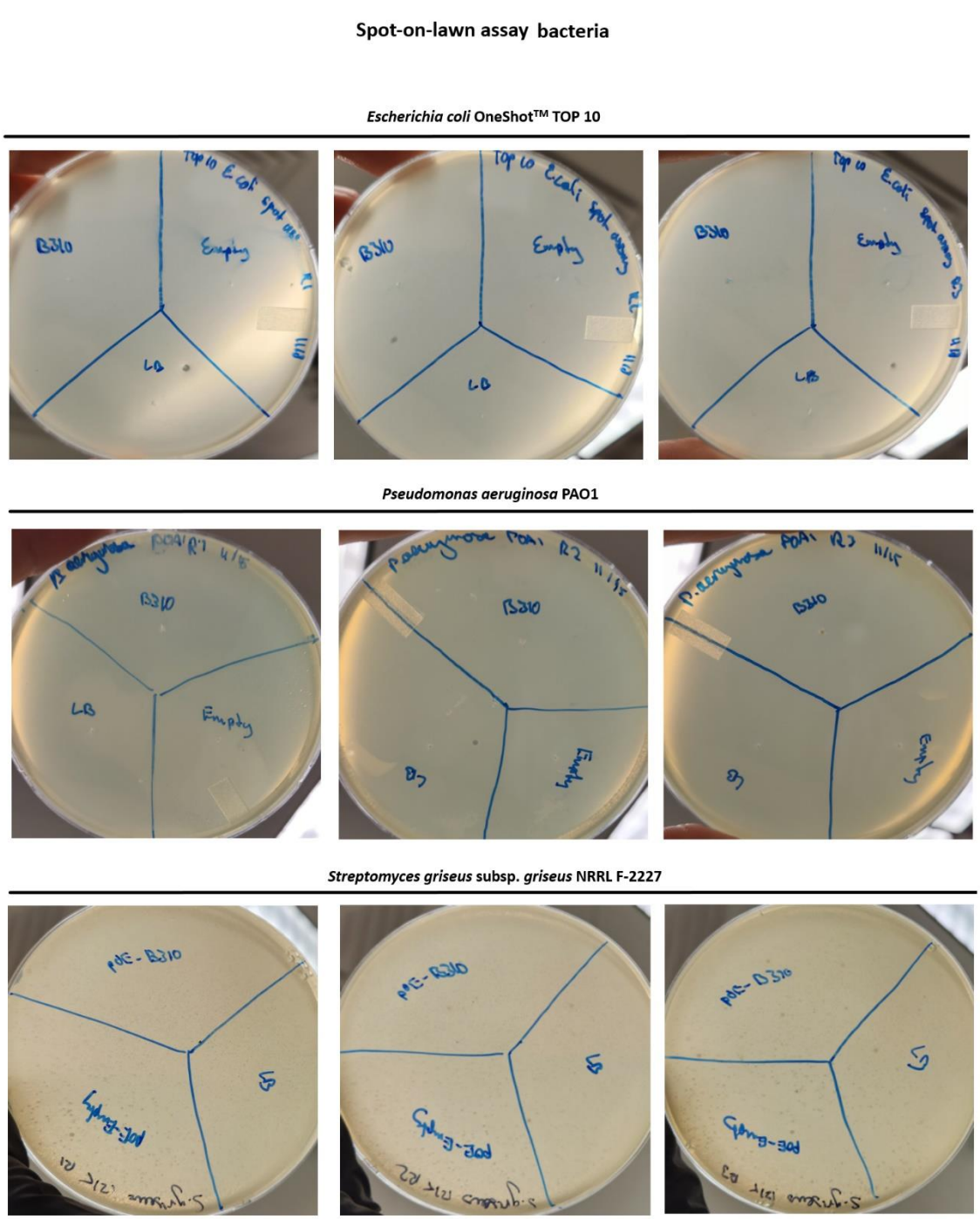
Supplemental figure 2. Individual results of spot-on-lawn assay on growth inhibition of *Sulfolobus islandicus* Y08 by B310 derived from supernatant of RJW004 over-producing B310. Zone of inhibition was assessed at 72h, 96h, and 120h (n=3). **B310**: supernatant from RJW004 transformed with over-expression plasmid pOE-B310. **Empty**: supernatant from RJW004 transformed with empty over-expression plasmid pOE-Empty. **DT**: DT medium control.



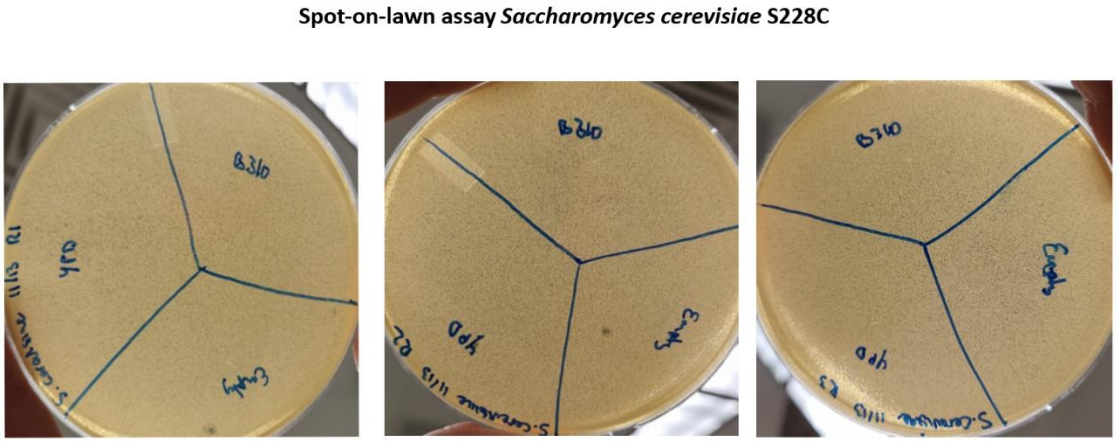
Supplemental figure 3. Individual results of spot-on-lawn assay on growth inhibition of *Sulfolobus acidocaldarius* by B310 derived from supernatant of RJW004 over-producing B310. Zone of inhibition was assessed at 72h, 96h, and 120h (n=3). **B310**: supernatant from RJW004 transformed with over-expression plasmid pOE-B310. **Empty**: supernatant from RJW004 transformed with empty over-expression plasmid **pOE-Empty**. DT: DT medium control.



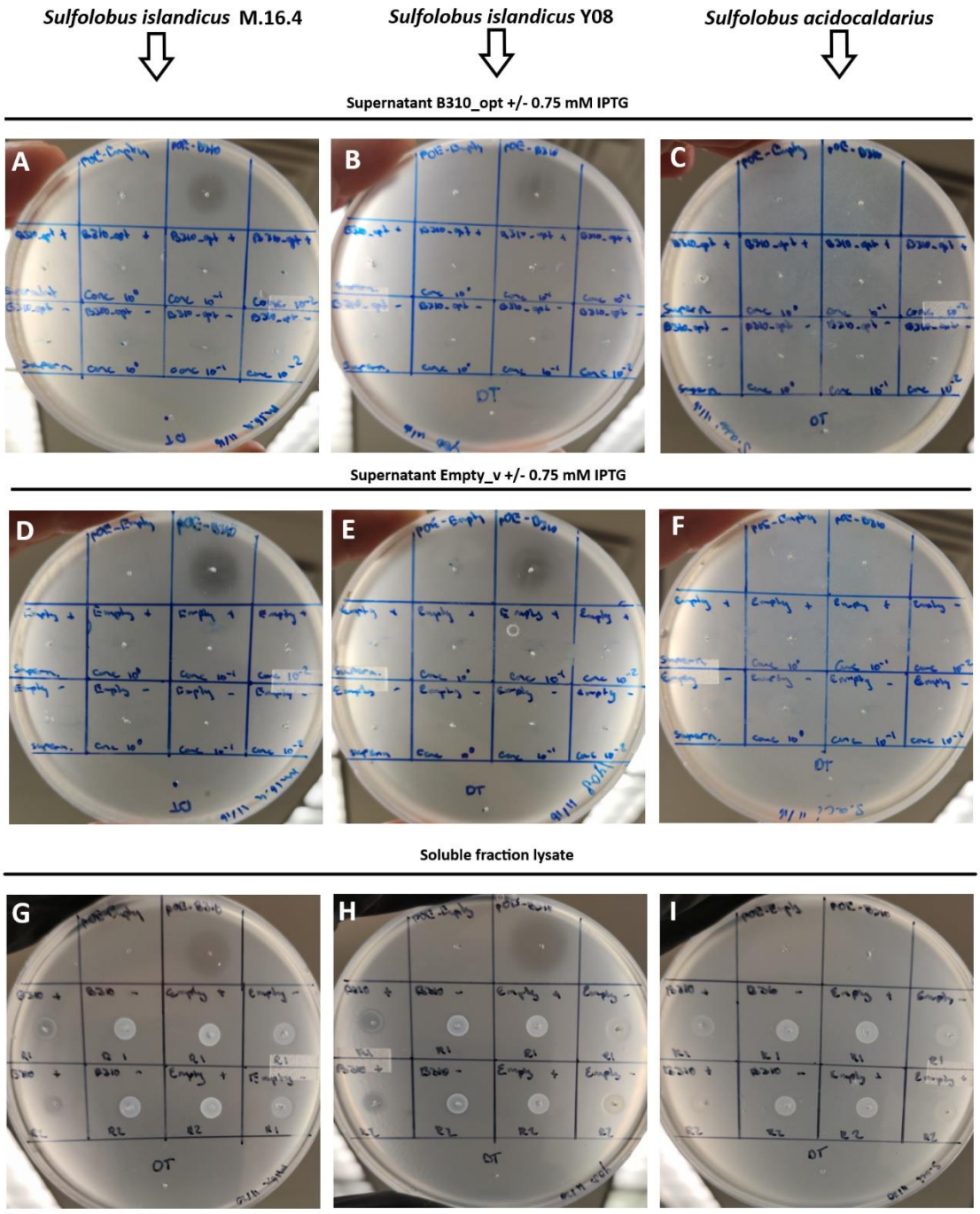
Supplemental figure 4. Individual results of spot-on-lawn assay on growth inhibition of *Sulfolobus tokodaii* by B310 derived from supernatant of RJW004 over-producing B310. Zone of inhibition was assessed at 72h, 96h, and 120h (n=3). **pOE-B310**: supernatant from RJW004 transformed with over-expression plasmid pOE-B310. pOE-**Empty**: supernatant from RJW004 transformed with empty over-expression plasmid pOE-Empty. **DT**: DT medium control.



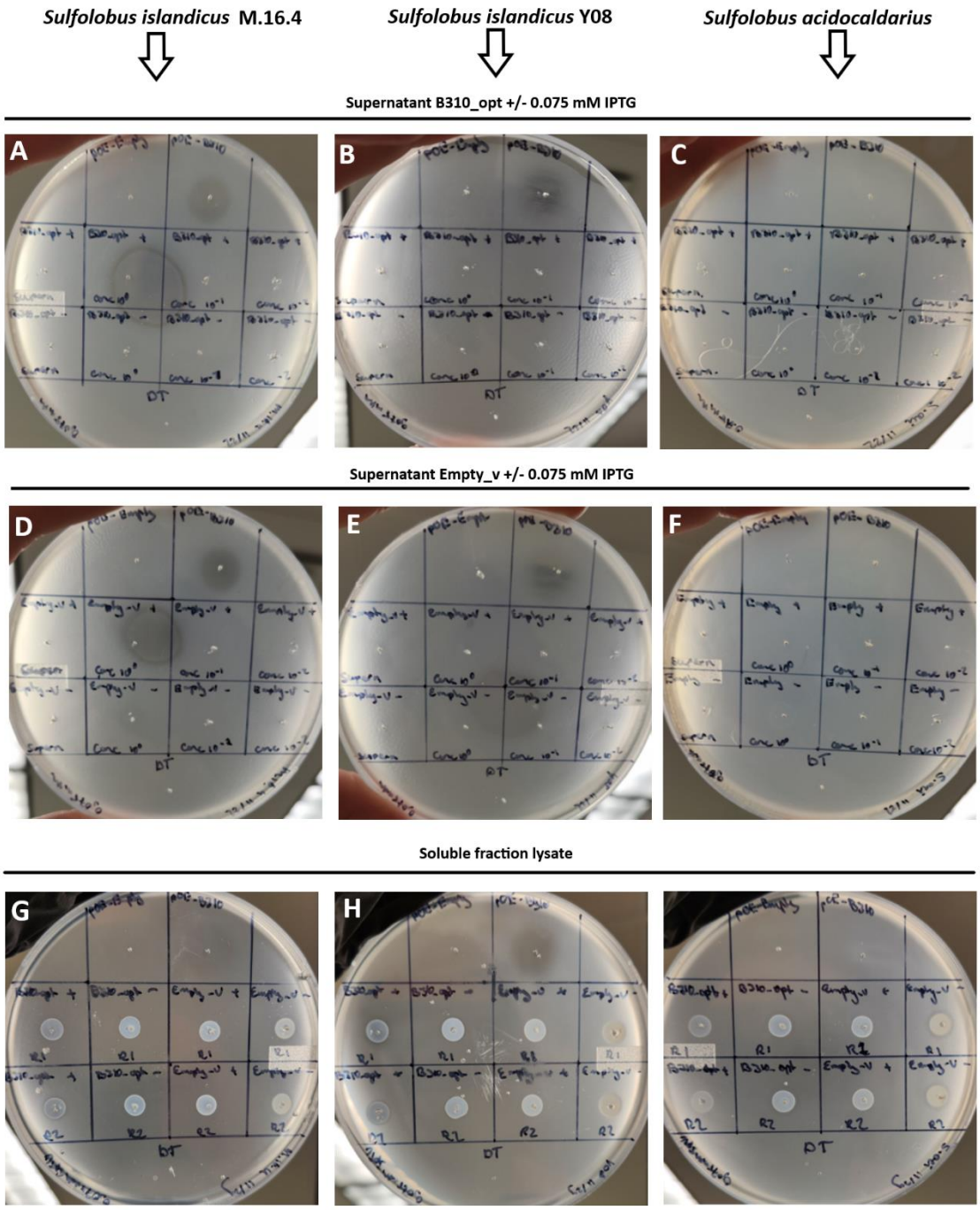
Supplemental figure 5. Individual results of spot-on-lawn assay on growth inhibition of *Escherichia coli* OneShot™ TOP10, *Pseudomonas aeruginosa* PAO1 and *Streptomyces griseus* subsp. *griseus* NRRl F-2227 by B310 derived from supernatant of RJW004 over-producing B310 (n=3). **B310/pOE-B310**: supernatant from RJW004 transformed with over-expression plasmid pOE-B310. **Empty/pOE-Empty**: supernatant from RJW004 transformed with empty over-expression plasmid pOE-Empty. **LB**: LB medium control.



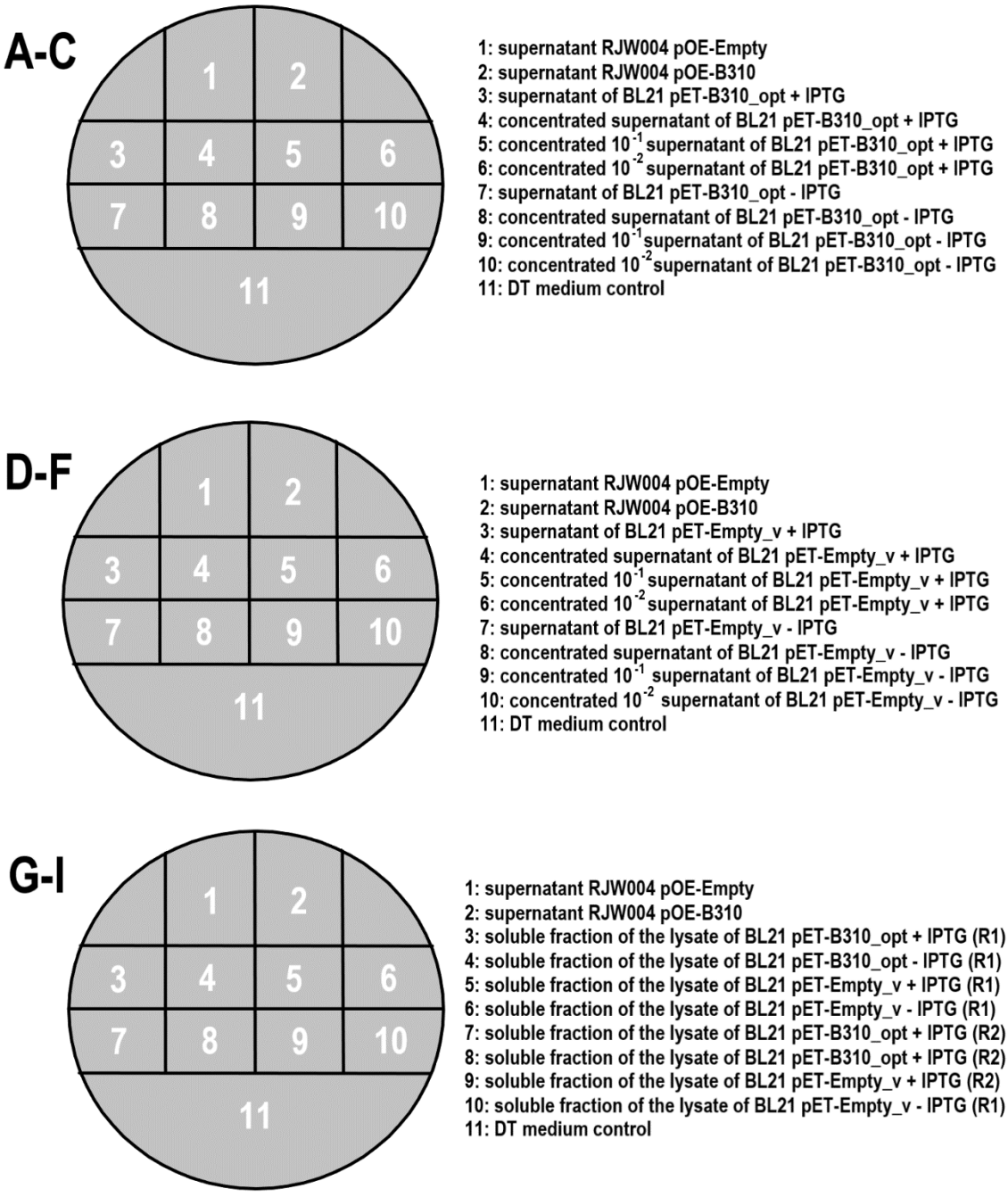
Supplemental figure 6. Individual results of spot-on-lawn assay on growth inhibition of *Saccharomyces cerevisiae* S228C by B310 derived from supernatant of RJW004 over-producing B310 (n=3). **B310**: supernatant from RJW004 transformed with over-expression plasmid pOE-B310. **Empty**: supernatant from RJW004 transformed with empty over-expression plasmid pOE-Empty. **YPD**: YPD medium control



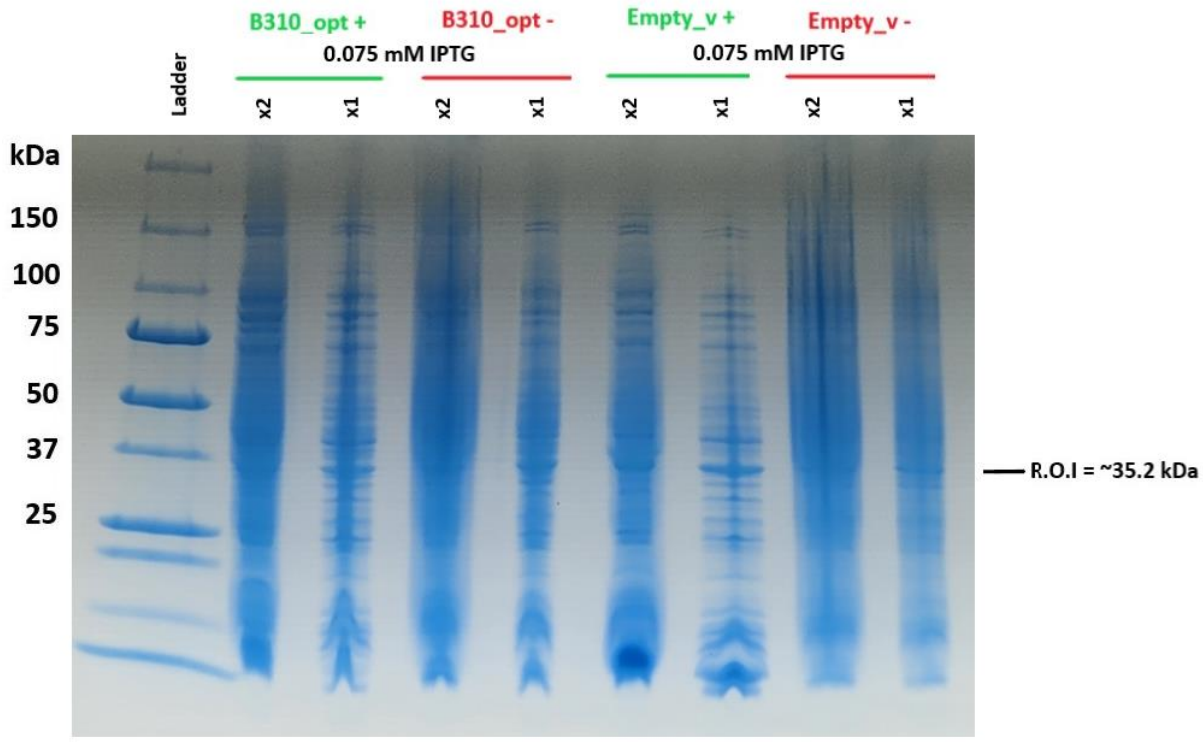
Supplemental figure 7. Spot-on-lawn assay results on the soluble fraction of the lysate and (concentrated) supernatant of induced (+,0.75 mM IPTG) or uninduced (-) *Escherichia coli* BL21 (DE3) carrying pET-B310_opt, or pET-B310_Empty_v empty control. Supernatant of *Sulfolobus islandicus* RJW004 carrying pOE-B310 or pOE-Empty as control. Summary of grid given in Supplemental figure 9 **(A-C)** (concentrated) supernatant of (un)induced BL21 carrying pET-B310 spotted on *S. islandicus* M.16.4, *S. islandicus* Y08 and *S. acidocaldarius* respectively. **(D-F)** (concentrated) supernatant of (un)induced BL21 carrying pET-Empty_v spotted on *S. islandicus* M.16.4, *S. islandicus* Y08 and *S. acidocaldarius* respectively. **(G-I)** Soluble fraction of the lysate of (un)induced BL21 carrying pET-B310 or pET-Empty_v spotted on *S. islandicus* M.16.4, *S. islandicus* Y08 and *S. acidocaldarius* respectively.



Supplemental figure 8. Spot-on-lawn assay results on the soluble fraction of the lysate and (concentrated) supernatant of induced (+,0.075 mM IPTG) or uninduced (-) *Escherichia coli* BL21 (DE3) carrying pET-B310_opt, or pET-B310_Empty_v empty control. Supernatant of *Sulfolobus islandicus* RJW004 carrying pOE-B310 or pOE-Empty as control. Summary of grid given in Supplemental figure 9 **(A-C)** (concentrated) supernatant of (un)induced BL21 carrying pET-B310 spotted on *S. islandicus* M.16.4, *S. islandicus* Y08 and *S. acidocaldarius* respectively. **(D-F)** (concentrated) supernatant of (un)induced BL21 carrying pET-Empty_v spotted on *S. islandicus* M.16.4, *S. islandicus* Y08 and *S. acidocaldarius* respectively. **(G-I)** Soluble fraction of the lysate of (un)induced BL21 carrying pET-B310 or pET-Empty_v spotted on *S. islandicus* M.16.4, *S. islandicus* Y08 and *S. acidocaldarius* respectively.



Supplemental figure 9. Overview of grid describing what sample corresponds to position on grid for spot-on-lawn assay as described in **Supplemental figure 7** & **Supplemental figure 8**.
(A-C) (concentrated) supernatant of (un)induced BL21 carrying pET- **(D-F)** (concentrated) supernatant of (un)induced BL21 carrying pET-Empty_v spotted on *S. islandicus* M.16.4, *S. islandicus* Y08 and *S. acidocaldarius* respectively. **(G-I)** Soluble fraction of the lysate of (un)induced BL21 carrying pET-B310 or pET-Empty_v spotted on *S. islandicus* M.16.4, *S. islandicus* Y08 and *S. acidocaldarius* respectively.



Supplemental figure 10. Whole cell lysate of induced (0.075 mM IPTG) or uninduced *Escherichia coli* BL21 (DE3) carrying pET-B310_opt, or pET-B310_Empty_v empty control. **Ladder:** Precision Plus Protein™ All Blue Prestained Protein Standards (BioRad®). **R.O.I:** region of interest. **1x:** 1 times sample volume. **2x:** 2 times sample volume.