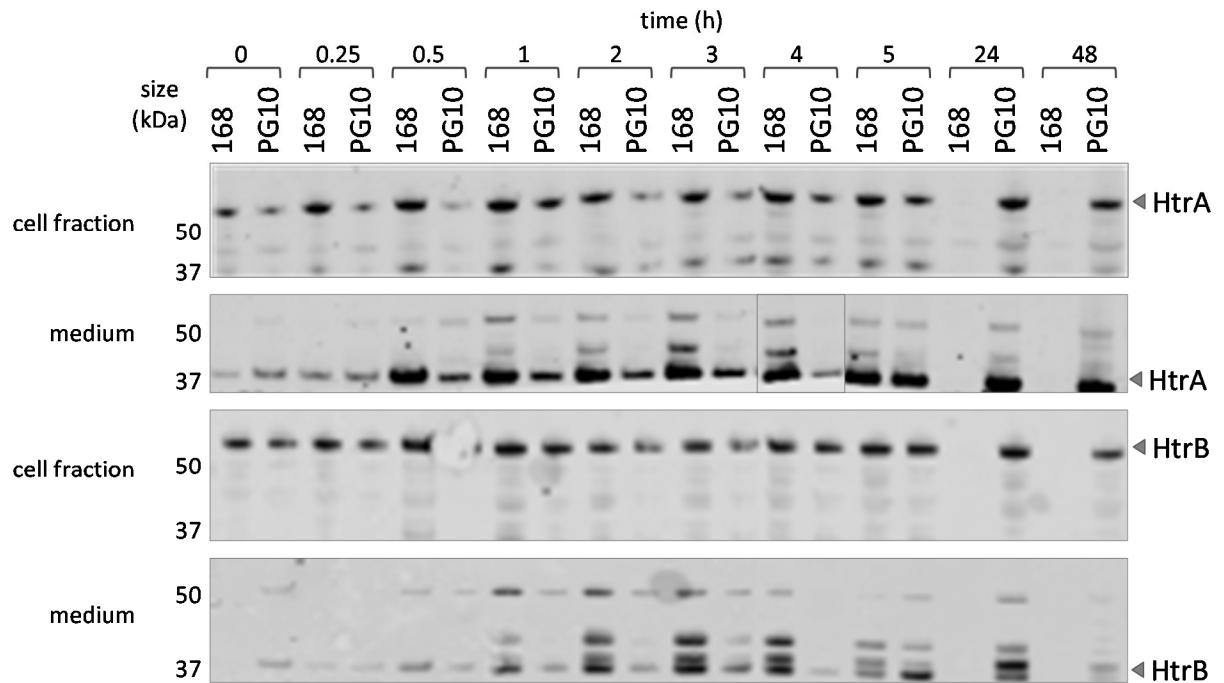


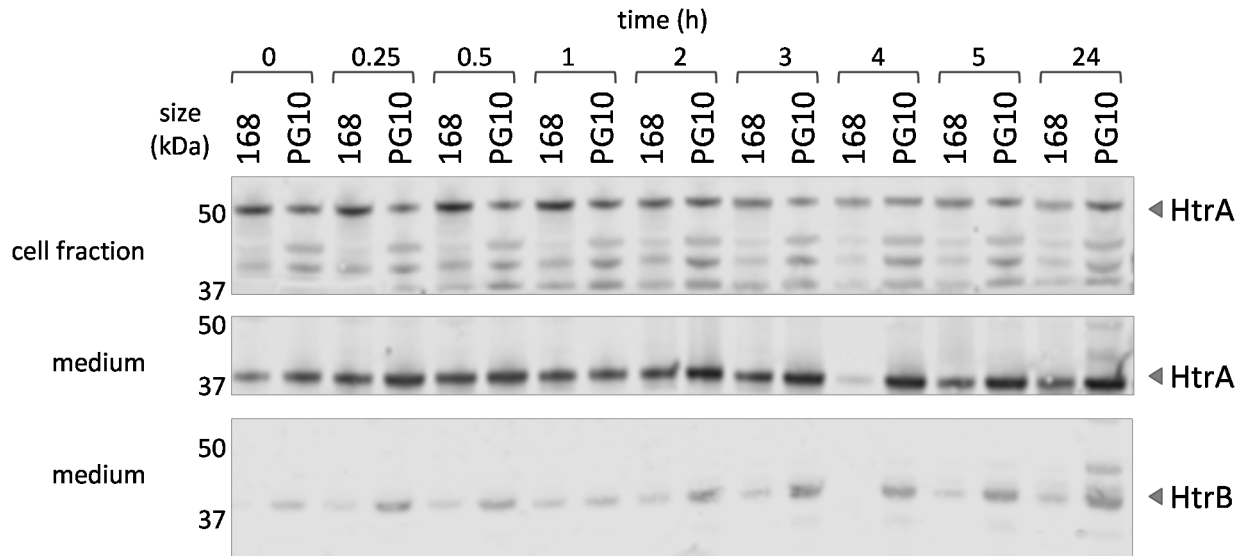
**Less is more: towards a genome-reduced *Bacillus* cell factory for
'difficult proteins'**

Rocío Aguilar Suárez, Jörg Stülke, Jan Maarten van Dijk

Supplementary Figures and Tables

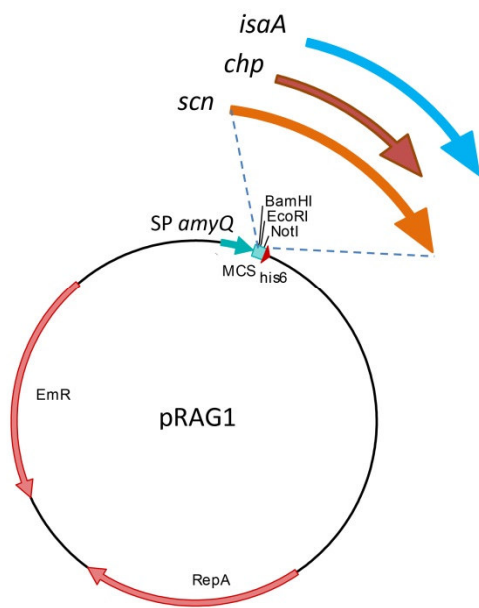
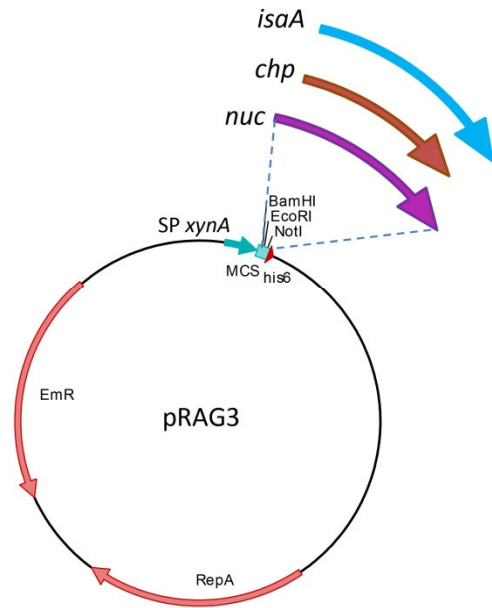


Supplementary Figure 1. Expression of HtrA and HtrB in *B. subtilis* strains 168 and PG10 producing IsaA. Production of IsaA in *B. subtilis* 168 and PG10 was induced with subtilin (t=0) as described for Figure 5. Samples were collected as a function of the time indicated and analyzed by Western blotting with specific antibodies for the secretion stress marker proteins HtrA and HtrB. The positions of HtrA and HtrB are marked with arrow heads.



Supplementary Figure 2. Expression of HtrA and HtrB in *B. subtilis* strains 168 and PG10 producing IsaA in the presence of protease inhibitors.

Production of IsaA in *B. subtilis* 168 and PG10 by cells grown in the presence of the Complete protease inhibitor cocktail from Roche was induced with subtilin (t=0) as described for Figure 6. Samples were collected as a function of the time indicated and analyzed by Western blotting with specific antibodies for the secretion stress marker proteins HtrA and HtrB. The positions of HtrA and HtrB are marked with arrow heads.

a**b**

Supplementary Figure 3. Schematic representation of plasmids pRAG1, pRAG3 and the respective derivatives for expression of *scn*, *nuc*, *chp*, or *isaA*.

Supplementary Table 1. Plasmids and strains used in this study.

	Relevant properties	Reference
Plasmids		
pNZ8910	SURE expression vector; <i>PspaS</i> ^a	1
pNG4210:: <i>chp</i>	pNG4210 encoding CHIPS with a C-terminal his6	2
pNG4210:: <i>SCIN</i>	pNG4210 encoding SCIN with C-terminal his6	2
pNG4210:: <i>isaA</i>	pNG4210 encoding <i>IsaA</i> with C-terminal his6	Laboratory collection
pNG400:: <i>nuc</i>	pNG400 encoding Nuc with C-terminal his6	3
pNZ8900	<i>amyE</i> :: <i>spaRK</i> ^b	1
pRAG1	pNZ8910 backbone; <i>SP_{amyQ}</i> , C-terminal his6	This study
pRAG1:: <i>chp</i>	pRAG1 encoding CHIPS	This study
pRAG1:: <i>isaA</i>	pRAG1 encoding <i>IsaA</i>	This study
pRAG1:: <i>scn</i>	pRAG1 encoding SCIN	This study
pRAG3:: <i>chp</i>	pRAG1 derivative with <i>SP_{xynA}</i> encoding CHIPS with a C-terminal his6	This study
pRAG3:: <i>nuc</i>	pRAG1 derivative with <i>SP_{xynA}</i> encoding Nuc with a C-terminal his6	This study
pRAG3:: <i>isaA</i>	pRAG1 derivative with <i>SP_{xynA}</i> encoding <i>isaA</i> with a C-terminal his6	This study
pKTH10	UB110 derivative encoding the <i>amyQ</i> gene of <i>B. amyloliquefaciens</i> ^b	4
pRMC-5' <i>bmrC</i> -gfp	pMRC carrying the upstream region of <i>bmrB</i> the 5' <i>bmrC</i> -gfp	
Strains		
<i>L. lactis</i>		
MG1363	Plasmid-free derivative of SH4109	5
PA1001	MG1363 <i>pepN</i> :: <i>nisRK</i> , allows nisin-inducible expression, Δ <i>acmA</i> Δ <i>htrA</i>	Mucosis culture collection, Groningen, The Netherlands
NZ9700	Nisin producer	6
<i>B. subtilis</i>		
168	<i>trpc2</i>	7
NZ8900	168, <i>amyE</i> :: <i>spaRK</i> ^b	1
ATCC6633	Subtilin producer	American Type Culture Collection
BRB02	<i>trpc2</i> , Δ <i>nprB</i> , Δ <i>aprE</i>	8
BRB03	<i>trpc2</i> , Δ <i>nprB</i> , Δ <i>aprE</i> , Δ <i>epr</i>	8
BRB04	<i>trpc2</i> , Δ <i>nprB</i> , Δ <i>aprE</i> , Δ <i>epr</i> , Δ <i>bpr</i>	8
BRB05	<i>trpc2</i> , Δ <i>nprB</i> , Δ <i>aprE</i> , Δ <i>epr</i> , Δ <i>bpr</i> , Δ <i>nprE</i>	8
BRB06	<i>trpc2</i> , Δ <i>nprB</i> , Δ <i>aprE</i> , Δ <i>epr</i> , Δ <i>bpr</i> , Δ <i>nprE</i> , Δ <i>mpr</i>	8
BRB07	<i>trpc2</i> , Δ <i>nprB</i> , Δ <i>aprE</i> , Δ <i>epr</i> , Δ <i>bpr</i> , Δ <i>nprE</i> , Δ <i>mpr</i> , Δ <i>vp</i>	8
BRB08	<i>trpc2</i> , Δ <i>nprB</i> , Δ <i>aprE</i> , Δ <i>epr</i> , Δ <i>bpr</i> , Δ <i>nprE</i> , Δ <i>mpr</i> , Δ <i>vp</i> , Δ <i>wprA</i>	8
BRB09	<i>trpc2</i> , Δ <i>nprB</i> , Δ <i>aprE</i> , Δ <i>epr</i> , Δ <i>bpr</i> , Δ <i>nprE</i> , Δ <i>mpr</i> , Δ <i>vp</i> , Δ <i>htrA</i>	8
BRB10	<i>trpc2</i> , Δ <i>nprB</i> , Δ <i>aprE</i> , Δ <i>epr</i> , Δ <i>bpr</i> , Δ <i>nprE</i> , Δ <i>mpr</i> , Δ <i>vp</i> , Δ <i>htrB</i>	8
BRB11	<i>trpc2</i> , Δ <i>nprB</i> , Δ <i>aprE</i> , Δ <i>epr</i> , Δ <i>bpr</i> , Δ <i>nprE</i> , Δ <i>mpr</i> , Δ <i>vp</i> , Δ <i>wprA</i> , Δ <i>htrA</i>	8
BRB12	<i>trpc2</i> , Δ <i>nprB</i> , Δ <i>aprE</i> , Δ <i>epr</i> , Δ <i>bpr</i> , Δ <i>nprE</i> , Δ <i>mpr</i> , Δ <i>vp</i> , Δ <i>wprA</i> , Δ <i>htrB</i>	8
BRB13	<i>trpc2</i> , Δ <i>nprB</i> , Δ <i>aprE</i> , Δ <i>epr</i> , Δ <i>bpr</i> , Δ <i>nprE</i> , Δ <i>mpr</i> , Δ <i>vp</i> , Δ <i>htrA</i> , Δ <i>htrB</i>	8
BRB14	<i>trpc2</i> , Δ <i>nprB</i> , Δ <i>aprE</i> , Δ <i>epr</i> , Δ <i>bpr</i> , Δ <i>nprE</i> , Δ <i>mpr</i> , Δ <i>vp</i> , Δ <i>wprA</i> , Δ <i>htrA</i> , Δ <i>htrB</i>	8
PG10	Large-scale reduction of the <i>B. subtilis</i> genome	9
PS38	Large-scale reduction of the <i>B. subtilis</i> genome	9
PG10 <i>spaRK</i>	PG10 transformed with pNZ8900	This study
BSBII-5' <i>bmrC</i> gfp	Chromosomally integrated copy of pBaSysBioII with a region of ~600 bp region upstream of <i>bmrC</i>	10
<i>S. aureus</i>		
...N315	Hospital-acquired MRSA, Restriction-deficient	11

^a Erythromycin resistance cassette; ^b Kanamycin resistance cassette.

Supplementary Table 2. Primers used in this study

Primer name	5'->3' nucleotide sequence
SPxynA_F	ACGACTGATATCATGTTTAAGTTAAAAAGAATTTCTTAGTTGGATTATCGGCAGC
SPxynA_R	AGTAAAGGATCCTGCAGAGGCGGTTGCCGAAAACAA
PNG42ins_F	TTACGCTGACACAAACTCCATG
PNG42ins_R	TTAATGGTGATGGTGATGGTGT
NucN315_F	CTACTAGGGATCCATCAGTAATGTTTCGAAAGGGCAATACGCAAAG
NucN315_F	AGATGTGCGGCCGCTTATTGACCTGAATCAGCGTTGTC
SPamyQ_F	TGACCATCATGATTCAAAAACGAAAGCGGACAG
SPamyQ_R	GGATCCCATGGAGTTTGTGTCATTTACGGCTGATGTTTTTGTA
MCS4210_F	TACAAAAACATCAGCCGTAATGACACAAACTCCATGGGATCC
MCS4210_R	CAAAGAAAGCTTTTAATGGTGATGGTGATGGTGTGCG
PRAGwoSP_F	ATGACACAAACTCCATGGGATCC
PRAGwoSP_R	ATCGATGATATCCACCTCCTTTCAATACCTAAATAG

Supplementary Table 3. Comparison of the relative copy number of plasmid pRAG3::*isaA* in *B. subtilis* 168 and PG10. The relative copy number of the plasmid pRAG3::*isaA* was calculated as the ratio between the average coverage of plasmid and chromosomal reads. The sequencing data of the two *Bacillus* strains are available in the European Nucleotide Archive (ENA) repository.

	Reference sequence	Reference length	Consensus length	Total read count	Average coverage	Relative copy number: Average coverage (plasmid/genome)	Study accession	Sample accession
PG10 + pRAG3:: <i>isaA</i>	pRAG3:: <i>isaA</i>	4135	4135	815061	20685	107	PRJEB29683	ERS2891774
	CP016788 (PG10)	2759298	2758290	4693536	194			
168 + pRAG3:: <i>isaA</i>	pRAG3:: <i>isaA</i>	4135	4135	383669	9329	80	PRJEB29683	ERS2891775
	NC_000964.3 (168)	4215606	4215233	4655558	117			

Supplementary Table 4. Part ID's of the plasmids constructed in this study as available from the ACS Synthetic Biology registry.

Name	Description	Part ID
pRAG1	Expression and secretion of heterologous proteins in <i>Bacillus</i> , SPamyQ	ACS_000757
pRAG1:: <i>chp</i>	Expression and secretion of the staphylococcal protein CHIPS in <i>Bacillus</i> , SPamyQ	ACS_000760
pRAG1:: <i>isaA</i>	Expression and secretion of the staphylococcal protein IsaA in <i>Bacillus</i> , SPamyQ	ACS_000761
pRAG1:: <i>scn</i>	Expression and secretion of the staphylococcal protein SCIN in <i>Bacillus</i> , SPamyQ	ACS_000762
pRAG3:: <i>chp</i>	Expression and secretion of the staphylococcal protein SCIN in <i>Bacillus</i> , SPxynA	ACS_000763
pRAG3:: <i>isaA</i>	Expression and secretion of the staphylococcal protein IsaA in <i>Bacillus</i> , SPxynA	ACS_000758
pRAG3:: <i>nuc</i>	Expression and secretion of the staphylococcal protein Nuc in <i>Bacillus</i> , SPxynA	ACS_000764

The collection is available in: <https://acs-registry.jbei.org/folders/33>

REFERENCES

- (1) Bongers, R. S., Veening, J.-W., Van Wieringen, M., Kuipers, O. P., Kleerebezem, M., Van, M., and Wieringen, M. Van. (2005) Development and characterization of a Subtilin-Regulated Expression System in *Bacillus subtilis*: strict control of gene expression by addition of subtilin. *Appl. Environ. Microbiol.* *71*, 8818–24.
- (2) Hoekstra, H., Romero Pastrana, F., Bonarius, H. P. J., van Kessel, K. P. M., Elsinga, G. S., Kooi, N., Groen, H., van Dijk, J. M., and Buist, G. (2017) A human monoclonal antibody that specifically binds and inhibits the staphylococcal complement inhibitor protein SCIN. *Virulence* *0*, 1–13.
- (3) Neef, J., Koedijk, D. G. A. M., Bosma, T., van Dijk, J. M., and Buist, G. (2014) Efficient production of secreted staphylococcal antigens in a non-lysing and proteolytically reduced *Lactococcus lactis* strain. *Appl. Microbiol. Biotechnol.* *98*, 10131–10141.
- (4) Palva, I. (1982) Molecular cloning of alfa-amylase gene from *Bacillus amyloliquefaciens* and its expression in *B. subtilis*. *Gene* *19*, 81–87.
- (5) Gasson, M. J. (1983) Plasmid complements of *Streptococcus lactis* NCDO 712 and other lactic streptococci after protoplast-induced curing. *J. Bacteriol.* *154*, 1–9.
- (6) Antelmann, H., Darmon, E., Noone, D., Veening, J. W., Westers, H., Bron, S., Kuipers, O. P., Devine, K. M., Hecker, M., and Van Dijk, J. M. (2003) The extracellular proteome of *Bacillus subtilis* under secretion stress conditions. *Mol. Microbiol.* *49*, 143–156.
- (7) Kunst, F., Ogasawara, N., Moszer, I., Albertini, A. M., Alloni, G., Azevedo, V., Bertero, M. G., Bessières, P., Bolotin, A., Borchert, S., Borriss, R., Boursier, L., Brans, A., Braun, M., Brignell, S. C., Bron, S., Brouillet, S., Bruschi, C. V., Caldwell, B., Capuano, V., Carter, N. M., Choi, S. K., Codani, J. J., Connerton, I. F., and Danchin, A. (1997) The complete genome sequence of the gram-positive bacterium *Bacillus subtilis*. *Nature* *390*, 249–256.
- (8) Pohl, S., Bhavsar, G., Hulme, J., Bloor, A. E., Misirli, G., Leckenby, M. W., Radford, D. S., Smith, W., Wipat, A., Williamson, E. D., Harwood, C. R., and Cranenburgh, R. M. (2013) Proteomic analysis of *Bacillus subtilis* strains engineered for improved production of heterologous proteins. *Proteomics* *13*, 3298–3308.
- (9) Reuß, D. R., Altenbuchner, J., Mäder, U., Rath, H., Ischebeck, T., Sappa, P. K., Thürmer, A., Guérin, C., Nicolas, P., Steil, L., Zhu, B., Feussner, I., Klumpp, S., Daniel, R., Commichau, F. M., Völker, U., and Stülke, J. (2017) Large-scale reduction of the *Bacillus subtilis* genome : consequences for the transcriptional network , resource allocation , and metabolism. *Genome Res.* *27*, 289–299.
- (10) Reilman, E., Mars, R. A. T. T., Van Dijk, J. M., and Denham, E. L. (2014) The multidrug ABC transporter BmrC/BmrD of *Bacillus subtilis* is regulated via a ribosome-mediated transcriptional attenuation mechanism. *Nucleic Acids Res.* *42*, 11393–11407.
- (11) Kuroda, M., Ohta, T., Uchiyama, I., Baba, T., Yuzawa, H., Kobayashi, I., Cui, L., Oguchi, A., Aoki, K., Nagai, Y., Lian, J., Ito, T., Kanamori, M., Matsumaru, H., Maruyama, A., Murakami, H., Hosoyama, A., Mizutani-Ui, Y., Takahashi, N. K., Sawano, T., Inoue, R., Kaito, C., Sekimizu, K., Hirakawa, H., Kuhara, S., Goto, S., Yabuzaki, J., Kanehisa, M., Yamashita, A., Oshima, K., Furuya, K., Yoshino, C., Shiba, T., Hattori, M., Ogasawara, N., Hayashi, H., and Hiramatsu, K. (2001) Whole genome sequencing of meticillin-resistant *Staphylococcus aureus*. *Lancet* *357*, 1225–40.