A secretion biosensor for monitoring Sec-dependent protein export in *Corynebacterium glutamicum*

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Abstract

Background

In recent years, the industrial workhorse *Corynebacterium glutamicum* has gained increasing interest as a host organism for the secretory production of heterologous proteins. Generally, the yield of a target protein in the culture supernatant depends on a multitude of interdependent biological and bioprocess parameters which have to be optimized. So far, the monitoring of such optimization processes depends on the availability of a direct assay for the respective target protein that can be handled also in high throughput approaches. Since simple assays, such as standard enzymatic activity assays, are not always at hand, the availability of a general protein secretion biosensor is highly desirable.

Results

High level secretion of proteins via the Sec protein export pathway leads to secretion stress, a phenomenon that is thought to be caused by the accumulation of incompletely or misfolded proteins at the membrane-cell envelope interface. We have analyzed the transcriptional responses of *C. glutamicum* to the secretory production of two different heterologous proteins and found that, in both cases, the expression of the gene encoding a homologue of the extracytosolic HtrA protease was highly upregulated. Based on this finding, a *C. glutamicum* Sec secretion biosensor strain was constructed in which the *htrA* gene on the chromosome was replaced by the *eyfp* gene. The fluorescence of the resulting reporter strain responded to the secretion of different heterologous proteins (cutinase from *Fusarium solani pisi* and alkaline phosphatase PhoA from *Escherichia coli*) in a dose-dependent manner. In addition, three differently efficient signal peptides for the secretory production of the cutinase could be differentiated by the biosensor signal. Furthermore, we have shown that an efficient signal peptide can be separated from a poor signal peptide by using the biosensor signal of the respective cells in fluorescence activated cell sorting experiments.

48

49 **Conclusions**

50 We have succeeded in the construction of a *C. glutamicum* biosensor strain that allows for the
51 monitoring of Sec-dependent secretion of heterologous proteins in a dose-dependent manner,
52 independent of a direct assay for the desired target protein.

53

54 **Keywords**

55 *Corynebacterium glutamicum*, heterologous protein production, Sec pathway, secretion
56 biosensor, signal peptide variation, FACS analysis

57

58 **Background**

59 Biotechnologically or pharmaceutically relevant recombinant proteins represent a steadily
60 increasing multi-billion dollar market, and for their production, various different pro- and
61 eukaryotic expression hosts are currently used. Herein, in many cases, the secretion of the
62 respective target proteins into the culture supernatant of an expression host represents an
63 attractive alternative strategy to intracellular production, since product recovery is greatly
64 simplified and, as a consequence, the production costs can be significantly reduced [1].

65 The diderm Gram-positive bacterium *Corynebacterium glutamicum* is an industrial
66 workhorse that has a long tradition in industry as a producer organism for various amino acids
67 and other low-molecular weight compounds [2, 3]. In recent years however, it became clear
68 that *C. glutamicum* also has a huge potential as a host organism for the secretory production
69 of heterologous proteins [4]. In contrast to many other commonly used bacterial secretory
70 production hosts, such as various *Bacillus* species, *C. glutamicum* secretes only a very limited
71 number of endogenous proteins into its culture supernatant and exhibits very low, if any
72 extracellular proteolytic activity, making this microorganism very attractive for the secretion
73 of even protease-sensitive heterologous proteins. Therefore, the secreted proteins of interest
74 are proteolytically stable and are present in a very high relative purity in the respective
75 fermentation media [5].

76 Like in other bacteria, also in *C. glutamicum* the great majority of extracytosolic proteins
77 are transported out of the cytosol via the general secretion (Sec) protein export system [4, 6].
78 Sec substrates are synthesized as precursor proteins that possess an amino-terminal signal
79 peptide that is responsible for the targeting of the proteins to the Sec translocase located in the
80 cytoplasmic membrane [7]. Subsequently, the proteins destined for export are translocated
81 across the membrane in an unfolded state through a pore formed by the SecYEG complex [8].
82 During or shortly after membrane translocation, the signal peptide is removed by signal

83 peptidase [9] and the mature part of the protein is released on the *trans*-side of the membrane
84 where the folding of the protein into its native conformation takes place.
85 The final yield of a heterologous target protein in the culture supernatant of the bacterial
86 secretory production host strongly depends on a multitude of interdependent biological and
87 bioprocess paramenters [10]. For instance, the identity of the signal peptide that is used to
88 drive the Sec-dependent membrane translocation of the target protein has been shown to be
89 one of the critical parameters that are decisive whether a production process becomes
90 successful and economically relevant or not [11, 12]. Furthermore, process conditions such as
91 medium composition, inducer concentration, induction time, temperature, and substrate feed
92 rates also strongly influence the amounts of a desired target protein in the culture supernatant
93 of the respective expression host [10, 13, 14]. Since the number of possible parameter
94 combinations exponentially grows with every additional parameter, the testing of a huge
95 number of conditions is desirable to achieve the optimal yields for each individual target
96 protein. At present, the monitoring of such an optimization process relies heavily on the
97 availability of a direct assay for the respective target protein. However, simple assays, such as
98 standard enzymatic activity assays, are not always at hand. For example, the quantification of
99 pharmaceutical proteins often requires elaborate biological activity assays, which limits the
100 number of samples that can be tested during the process optimization procedure. Therefore,
101 process optimization would greatly benefit from the availability of a general secretion
102 biosensor that can be handled in high throughput approaches and that allows for an assay-
103 independent monitoring of target protein secretion in an efficient and dose-dependent manner.
104 The overproduction of exported proteins leads to secretion stress, a phenomenon that is
105 thought to be caused by the accumulation of incompletely or misfolded proteins at the
106 membrane-cell envelope interface [15]. A highly conserved cellular response to this secretion
107 stress is the upregulation of extracytosolic proteases, such as HtrA/DegP, that counteract this
108 stress by degrading the unfolded proteins at the *trans*-side of the membrane [16]. For the

109 Gram-positive model organism *Bacillus subtilis*, it has been shown that the two component
110 system CssRS senses the secretion stress and activates the expression of the *htrA* and *htrB*
111 genes, resulting in increased amounts of the quality control proteases HtrA and HtrB,
112 respectively [17, 18]. Based on the respective sensing system, a reporter strain was
113 constructed in which the *gfp* gene was placed under the control of the promoter of the *htrA*
114 gene and the respective strain was used for the identification of secreted proteins by
115 fluorescence activated cell sorting (FACS) after expressing a partial genomic library from the
116 plant root-colonizing bacterium *Bacillus amyloliquefaciens* [19].
117 In the present manuscript, we have analyzed the transcriptional responses of *C. glutamicum*
118 to the secretory production of two different heterologous proteins, *i.e.* an α-amylase (AmyE)
119 from *B. subtilis*, and a cutinase from the fungus *Fusarium solani pisi*, by microarray
120 experiments. Two major stress responses, *i.e.* (1) the induction of the EsrISR cell envelope
121 stress response system [20] and (2) the upregulation of the gene encoding a homologue of the
122 HtrA protease were observed. For the construction of a *C. glutamicum* Sec secretion
123 biosensor, we replaced the *htrA* gene on the chromosome by the *eyfp* gene and, in fact, the
124 eYFP fluorescence of the resulting *C. glutamicum* reporter strain responded to the secretion of
125 different heterologous proteins in a dose-dependent manner. Next, we fused three different
126 Sec signal peptides to the heterologous model protein cutinase from *F. solani pisi* and
127 produced the corresponding hybrid precursor proteins in *C. glutamicum*. The amounts of
128 cutinase in the respective culture supernatants varied significantly, depending on the signal
129 peptide that was used to drive Sec-dependent membrane transport of the cutinase.
130 Importantly, the fluorescence of the Sec secretion biosensor in the respective cutinase
131 secreting cells reflected the amount of cutinase that was secreted into the culture supernatant
132 of the corresponding cells. Using mixtures of two strains that secreted cutinase with different
133 efficiencies, we subsequently showed that the cells producing the cutinase with the superior
134 signal peptide can be sorted out by FACS, demonstrating the suitability of the Sec secretion

135 biosensor for the high throughput optimization of secretory protein production by *C.*
136 *glutamicum*.
137

138 **Results**

139 **Identification of Sec secretion stress-responsive genes in *C. glutamicum***

140 For the identification of Sec secretion stress-responsive genes in *C. glutamicum*, two
141 heterologous secretory model precursor proteins, *i.e.* a cutinase from the fungus *F. solani pisi*
142 fused to the *B. subtilis* NprE signal peptide [10] and the α-amylase AmyE from *B. subtilis*
143 containing its authentic signal peptide [21] and a carboxyl-terminal His_6-tag were used. The
144 corresponding genes were cloned into the expression vector pEKEx2 under the regulatory
145 control of the *tac* promoter, allowing an IPTG-inducible expression in *C. glutamicum*. After
146 transformation of *C. glutamicum* with the respective plasmids, the transcriptome of cells that
147 were fully induced by the addition of 1 mM IPTG was compared with the transcriptome of
148 uninduced cells that only show a basal expression of the corresponding genes and a low level
149 secretion of the two heterologous model proteins (Additional file 1: Figure S1). To
150 differentiate the cellular responses that are caused by the expression of a heterologous protein
151 *per se* (such as the upregulation of the genes encoding the general chaperone systems GroELS
152 and DnaK/DnaJ/GrpE) from those that are caused by the secretion of the protein across the
153 cytoplasmic membrane, we also included *C. glutamicum* cells that expressed a signal peptide-
154 less and therefore cytosolic variant (Additional file 1: Figure S1) of the His_6-tagged AmyE
155 protein (ΔSP-AmyE) in our microarray analyses.

156 In Table 1, a selection of several genes is shown that were specifically upregulated more
157 than two-fold in the *C. glutamicum* strain overproducing and secreting AmyE, but not in the
158 strain producing the signal peptide-less AmyE variant. Three of these genes, *esrI* (cg0706),
159 cg1325, and *rsmP* (cg3264), belong to envelope stress response (Esr) regulon that is regulated

160 by the EsrISR three component system. The Esr regulon is induced under conditions that
161 threaten the integrity of the cell envelope and it can be activated, among others, by antibiotics
162 that inhibit the lipid II cycle, such as bacitracin and vancomycin [20]. Another strong
163 secretion-specific upregulation upon AmyE overproduction (*i.e.* 4.22 fold) was observed for
164 the *htrA* (cg0998) gene. A similar upregulation (4.40 fold) of the *htrA* gene, but not of the
165 genes belonging to the Esr regulon, was observed when the *F. solani pisi* cutinase was
166 secreted via the Sec pathway using the NprE signal peptide (Table 1). Taken together, these
167 results indicate that, like in other microorganisms [22-24], the upregulation of the
168 extracytosolic protease HtrA is a common stress response that also in *C. glutamicum*
169 counteracts secretion stress by degrading accumulated misfolded proteins at the outer surface
170 of the cytoplasmic membrane.
171

172 **Construction of a Sec secretion stress-responsive *C. glutamicum* biosensor strain**

173 Since *htrA* gene expression was increased upon secretory production of two different
174 heterologous proteins, we reasoned that placing the gene encoding eYFP under the respective
175 regulatory control should result in a fluorescent biosensor that is able to monitor the extent of
176 protein secretion in *C. glutamicum*. As shown in Fig. 1A, *htrA* is the third gene in an operon
177 consisting of the genes *cgtR2* – *cgtS2* – *htrA* – *moa* – cg1000. *cgtR2* and *cgtS2* code for the
178 response regulator and the sensor kinase of a two-component system which, similar to the
179 situation in *Mycobacterium tuberculosis*, might be involved in the stress-responsive regulation
180 of the adjacent *htrA* gene [23]. Downstream of *htrA*, a gene (*moa*) encoding a predicted
181 molybdopterin biosynthetic protein and a small gene (cg1000) for a hypothetical gene product
182 of unknown function are located in the operon. According to the RNAseq analysis of *C.*
183 *glutamicum* transcriptomes [25], a polycistronic mRNA encompassing all five genes of the
184 operon is transcribed from one or possibly two promoters upstream of the *cgtR2* gene. In
185 addition, an mRNA encompassing *htrA*-*moa*-cg1000 is transcribed from a promoter located

186 directly upstream the *htrA* gene. A third transcript starts from a promoter located in front of
187 *moa* and encompasses the last two genes (*moa*-cg1000) from the operon. The detailed
188 mechanism of the stress-responsive upregulation of *htrA* expression in *C. glutamicum* is
189 unknown so far.

190 For the construction of a Sec secretion stress-responsive biosensor strain, we replaced the
191 *htrA* gene on the chromosome of *C. glutamicum* ATCC13032 by a DNA fragment
192 encompassing the first 51 nucleotides of the *htrA* structural gene followed by a TAG stop
193 codon, a 16 bp spacer sequence containing a ribosome binding site, and the *eyfp* gene (Fig.
194 1B). The resulting *C. glutamicum* strain K9 was subsequently transformed with pEKEx2-
195 NprE-cutinase and cultivated in a BioLector device that allows the online monitoring of cell
196 growth and fluoresence [26] in the absence or presence of different concentrations of IPTG
197 for the induction of NprE-cutinase expression. As a control, *C. glutamicum* K9 containing the
198 pEKEx2 empty vector was also included in the analysis. As shown in Fig. 2A, the growth of
199 the various strains was similar, although a slight growth defect, the extent of which depended
200 on the IPTG concentrations used, could be observed in *C. glutamicum* K9 (pEKEx2-NprE-
201 cutinase) compared with *C. glutamicum* K9 containing the pEKEx2 empty vector. This is
202 fully in line with the known fact that recombinant protein production and secretion causes a
203 metabolic burden to bacterial cells which results in growth reduction [27, 28]. Remarkably,
204 the control strain K9 containing the pEKEx2 empty vector already shows a significant
205 specific fluorescence (i.e. 0.35 AU at 24 h of cultivation) with no apparent effect of IPTG
206 addition (Fig. 2B). In contrast, *C. glutamicum* per se does not possess any significant intrinsic
207 fluoresence (Additional file 1: Figure S2). Since the *eyfp* gene in the control strain is under the
208 control of the native regulatory elements of the *htrA* gene whose expression responds to the
209 existing secretion stress, the fluorescence of the control strain likely reflects the basal
210 secretion stress level that is exerted by the Sec-dependent export of host-derived proteins
211 proteins across the cytoplasmic membrane. Importantly, the additional expression and

212 secretion of NprE-cutinase resulted in a significant increase of the specific fluorescence
213 values above the basal level, whereby increasing amounts of IPTG resulted in
214 correspondingly increasing specific fluorescence values (Fig. 2B). Since with increasing
215 IPTG concentrations, increasing cutinase activities (Fig. 2C) and increasing amounts of
216 cutinase protein (Fig. 2D) are observed in the respective culture supernatants, our results
217 indicate that the K9 secretion biosensor fluorescence signal reflects the amount of cutinase
218 that is secreted across the cytoplasmic membrane into the culture supernatant. Indeed, there is
219 a very good correlation between the cutinase activity in the supernatant determined at the end
220 of the BioLector cultivation (at 24 h) and the specific fluorescence measured at the same time
221 point (Fig. 2C).

222 We next analyzed the behaviour of the K9 Sec secretion biosensor in response to the
223 secretion of another, completely unrelated heterologous model protein, *i.e.* the alkaline
224 phosphatase PhoA of *E. coli*. For the channeling of PhoA into the *C. glutamicum* Sec protein
225 export pathway, we used the signal peptide (Pre^{Lip}) of the *Staphylococcus hyicus* lipase [29].
226 Previous experiments with *B. subtilis* [30] and also with *C. glutamicum* (our observations)
227 have indicated that PhoA is secreted only very poorly with its native Sec signal peptide in the
228 respective foreign Gram-positive hosts and that efficient secretion of PhoA can be achieved
229 when the mature PhoA protein is fused to the lipase-derived signal peptide. *C. glutamicum* K9
230 was transformed with plasmid pEKEx2-Pre^{Lip}-PhoA and the respective strain was cultivated
231 in the BioLector device in the absence or presence of different IPTG concentrations. Similar
232 to the situtation with NprE-cutinase, increasing amounts of IPTG resulted in a concomitant
233 slight decrease in growth (Fig. 3A), reflecting the metabolic burden due to increasing
234 recombinant protein production and secretion. Furthermore, increasing concentrations of
235 IPTG resulted in a corresponding increase in the specific fluorescence of the respective
236 cultures (Fig. 3B), in increasing alkaline phosphatase activities (Fig. 3C), and in increasing
237 amounts of secreted PhoA protein in the culture supernatant (Fig. 3D). Based on these

238 findings, we conclude that we have succeeded in the construction of a functional Sec secretion
239 biosensor that is able to monitor the extent of secretion of different heterologous proteins in
240 *C. glutamicum*.
241
242 **Differently efficient signal peptides for cutinase secretion in *C. glutamicum* can be**
243 **distinguished by the K9 secretion biosensor signal**
244 In recent years, it has become increasingly clear that the choice of the signal peptide that is
245 used to direct a desired heterologous protein into the Sec export pathway is one of the most
246 critical steps on the way to an efficient secretory production process. Since so far it is not
247 possible to predict which signal peptide will perform best in the context of a given
248 heterologous target protein, the most promising way to find the optimal signal peptide is to
249 screen a large diversity of signal peptides, either generated by signal peptide variation using
250 signal peptide libraries or, alternatively, by optimization of a selected signal peptide by
251 random mutagenesis approaches [12]. Since the K9 secretion biosensor responded to cutinase
252 and PhoA secretion in a dose-dependent manner, we next investigated if the K9 secretion
253 biosensor can be used to distinguish between differently efficient signal peptides with respect
254 to secretory protein production by *C. glutamicum*. For this purpose, a set of three cutinase
255 precursor proteins possessing three selected signal peptides from *B. subtilis*, *i.e.* those derived
256 from the secreted proteins NprE, YpjP, and YwmC [10], was chosen and the corresponding
257 plasmids encoding the different precursor proteins were electroporated into the *C. glutamicum*
258 K9 secretion biosensor strain. The respective strains were cultivated in the BioLector device
259 in the presence of 250 µM IPTG and analyzed with respect to growth, biosensor
260 flurorescence, cutinase protein production, and cutinase activity in the culture supernatant. As
261 shown in Fig. 4, the three signal peptides mediated the secretion of different amounts of
262 cutinase protein into the culture supernatant of the respective *C. glutamicum* K9 strains (Fig.
263 4D), with corresponding different cutinase activities (Fig. 4C). Herein, the relative efficiency

264 of the signal peptides with respect to cutinase secretion followed the order of NprE > YpjP >
265 YwmC. The relative secretion efficiency mediated by the different signal peptides was also
266 reflected in the growth behaviour of the corresponding recombinant strains, with *C.*
267 *glutamicum* K9 expressing the NprE-cutinase showing the strongest and *C. glutamicum* K9
268 expressing the YwmC-cutinase showing the weakest growth defect compared to the *C.*
269 *glutamicum* K9 control strain harboring the pEKEx2 empty vector (Fig. 4A). Importantly, the
270 relative secretion performance governed by the different signal peptides was also reflected by
271 the specific fluorescence of the corresponding *C. glutamicum* K9 secretion biosensor strains
272 (Fig. 4B and C). From these results, we conclude that signal peptides that facilitate secretion
273 with different efficacies can be distinguished by their respective K9 secretion biosensor
274 signals.
275

276 **Biosensor-based signal peptide screening using fluorescence-activated cell sorting**
277 **(FACS)**

278 Next, we investigated whether the secretion biosensor can be exploited in conjunction with
279 fluoresence-activated cell sorting (FACS) to separate and sort cells with different secretion
280 performances, *i.e.* to discriminate between a potent signal peptide (*e.g.* NprE) and a poor
281 signal peptide (*e.g.* YwmC). A graphical workflow of the general setup for the FACS analysis
282 and sorting experiments is shown in the Supplementary information (Additional file 1: Figure
283 S3). First, the *C. glutamicum* K9 secretion biosensor strains containing either pEKEx2-NprE-
284 cutinase or pEKEx2-YwmC-cutinase were cultivated separately and analyzed by FACS. In a
285 typical experiment, first 10^5 cells from cultures of the respective strains were analyzed,
286 followed by a preselection of cells to exclude cell doublets and debris by electronic gating
287 using FSC-W against FSC-H (Table 2, gate 0). The respective strains exhibited rather small
288 differences in fluorescence output, as illustrated by the overlay plots shown in Fig. 5A and B.
289 Cells secreting NprE-cutinase exhibited a median fluorescene of 124, which is 12.1% higher

290 than that of cells secreting YwmC-cutinase. These results indicate that the secretion biosensor
291 strain, in principle, can be used to distinguish between between NprE-cutinase- and YwmC-
292 cutinase-secreting cells.

293 For the FACS sorting, we then selected a gate such that it includes as many of the better
294 perfoming cells (*i.e.* 309 cells containing pEKEx2-NprE-cutinase; Table 2A and Additional
295 file 1: Figure S4A) and excludes as many of the less productive cells (*i.e.* 7 cells containing
296 pEKEx2-YwmC-cutinase; Table 2A and Additional file 1: Figure S4A) as possible (Fig. 5C,
297 gate 1). Next, the two strains were mixed in a ratio of 1:1 and the mixture was analyzed by
298 FACS. In this case, 87 cells now fell into the previously defined sorting gate 1 (Table 2A and
299 Additional file 1: Figure S4A). Hereafter, we performed a single round of enrichment by
300 collecting $5x10^4$ cells from gate 1 that were subsequently cultivated in a BioLector for 24
301 hours and then again subjected to FACS analysis. After the enrichment step, FACS analysis
302 indicated that gate 1 now contained 148 cells (Table 2A and Additional file 1: Figure S4A),
303 46 clones of which were sorted out onto a BHI agar plate containing kanamycin for plasmid
304 identification. DNA sequencing of 44 obtained plasmids revealed that 43 clones (*i.e.* 97.7%)
305 in fact contained the pEKEx2-NprE-cutinase plasmid (Table 2A). Thus, starting from a 1:1
306 mixture, a single round of enrichment was sufficient to obtain nearly exclusively cells
307 harboring the plasmid encoding the cutinase precursor protein with the superior NprE signal
308 peptide.

309 Finally, we asked whether cells secreting the cutinase via the superior NprE signal peptide
310 can also be enriched when the mixture of cells is significantly in favour of those cells which
311 secrete the cutinase via the less effective YwmC signal peptide. Also in this case, the two *C.*
312 *glutamicum* K9 strains containing pEKEx2-NprE-cutinase or pEKEx2-YwmC-cutinase,
313 respectively, were first cultivated separately and analyzed by FACS (Table 2B). Again, a
314 sorting gate 1 was selected such that it includes as many of the better perfoming cells (*i.e.* 458
315 cells containing pEKEx2-NprE-cutinase) and excludes as many of the less productive cells

316 (*i.e.* 46 cells containing pEKEx2-YwmC-cutinase) as possible (Table 2B and Additional file
317 1: Figure S4B). The two strains were then mixed in a ratio of 1:100 (NprE:YwmC).
318 Subsequently, the mixture was again analyzed by FACS and, from this mixture, 50 cells now
319 fell into the previously selected gate 1 (Table 2B and Additional file 1: Figure S4B). A single
320 round of enrichment was then performed by collecting $2x10^4$ cells from gate 1. The pooled
321 cells were then cultivated in a BioLector for 24 hours and subsequently analyzed by FACS
322 (Table 2B and Additional file 1: Figure S4B). After the enrichment step, now 286 cells
323 analyzed fell into gate 1. Finally, 46 clones were sorted out of the selected gate onto a BHI
324 agar plate containing kanamycin for further analysis. The identity of the signal peptide fused
325 to cutinase in the respective clones was identified by DNA sequencing of the corresponding
326 plasmids. DNA sequencing of 43 obtained plasmids revealed that 16 clones (*i.e.* 37%)
327 contained the pEKEx2-NprE-cutinase plasmid and 27 clones contained the pEKEx2-YwmC-
328 cutinase plasmid, respectively. These results show that the cells expressing the cutinase with
329 the better performing NprE signal peptide could be significantly enriched from a ratio of
330 1:100 to a ratio of 1:1.7 (Table 2B), indicating that the K9 secretion biosensor can be used for
331 a FACS-based signal peptide screening, independent of an assay for the desired target protein.
332

333 **Discussion**

334 In this study, we have constructed a *C. glutamicum* biosensor strain that allows for the
335 monitoring of Sec-dependent secretion of heterologous proteins in a dose-dependent manner,
336 independent of a direct assay for the desired target protein. By analyzing the cellular
337 transcriptional responses of *C. glutamicum* to the expression and secretion of two
338 heterologous model proteins, the *htrA* gene was found to be upregulated upon high-level
339 secretion of both model proteins. Based on these findings, the *eyfp* gene was integrated into
340 the respective *htrA*-containing five gene operon on the chromosome such that it allows the
341 regulation of *eyfp* expression in a secretion stress-responsive manner. The newly constructed

342 secretion biosensor was able to differentiate between differently efficient signal peptides with
343 respect to the secretion of a cutinase. In addition, we have shown that an efficient signal
344 peptide can be separated from a poor signal peptide by using the biosensor signal of the
345 respective cells in FACS sorting experiments.

346 Our finding that *htrA* gene expression was upregulated in response to the secretion of
347 different heterologous proteins indicates that, also in *C. glutamicum*, the extracytosolic
348 protease HtrA seems to be a major factor that combats secretion stress by degrading unfolded
349 proteins that accumulate at the membrane-cell envelope interface. Since the amount of
350 misfolded proteins at the *trans*-side of the membrane increases upon increased secretion of
351 proteins via the Sec pathway, the *htrA* gene is upregulated in response to this secretion stress
352 in a dose-dependent manner. It is however completely unknown how this regulation occurs in
353 *C. glutamicum*. For *B. subtilis,* it has been shown that the secretion stress-responsive
354 upregulation of *htrA* (and *htrB*) is mediated by the CssRS two component system [22]. In *M.*
355 *tuberculosis*, the regulation is more complex and it has been shown that both the
356 extracyoplasmic function (ECF) sigma factor SigE and the two-component system MprAB
357 are involved in the regulation of *htrA* (*pepD*) gene expression in response to various cell
358 envelope stresses [23]. In *M. tuberculosis*, the *htrA* (*pepD*) gene is located in an operon that
359 consists of the genes *mprA-mprB-pepD-moaB2*, a gene organization which is highly similar to
360 that found in *C. glutamicum*, where the *htrA* gene is likewise located in an operon and
361 sandwiched between the genes encoding a two-component system and a *moa* gene (see Fig.
362 1A). Although not experimentally addressed so far, it therefore seems a likely possibility that
363 the two component system CgtSR2 might be at least one of the factors that are involved in the
364 regulation of *htrA* gene expression in *C. glutamicum*.

365 In the biosensor strain *C. glutamicum* K9, the *htrA* gene has been replaced by *eyfp* gene
366 and, therefore, the HtrA quality control protease is missing. At a first glance, this might be
367 considered as a disadvantage with respect to the secretory production of heterologous

368 proteins, since accumulated misfolded secreted protein at the *trans*-side of the cytoplasmic
369 membrane can no longer be removed by HtrA. In line with this view, the deletion of *htrA* can
370 have severe negative effects on growth in various microorganisms, especially under protein
371 folding stress conditions such as high temperature [31] and it has also been reported to have
372 negative effects on the secretion of heterologous proteins [24]. However, a direct comparison
373 between *C. glutamicum* wild-type and *C. glutamicum* K9 clearly showed that the K9 strains
374 producing NprE-cutinase (Additional file 1: Figure S5A) or Pre^{Lip}-PhoA (Additional file 1:
375 Figure S5B) exhibited even a slightly better growth than the corresponding wild-type strains
376 under standard growth conditions (Additional file 1: Figure S5A and B). This indicates that
377 the secretion stress exerted by these two respective heterologous proteins seems to be rather
378 low under these conditions and that the amounts of accumulated misfolded proteins at the
379 *trans*-side of the membrane do not significantly affect the viability of the cells in a negative
380 way. Furthermore, at least for these two heterologous proteins tested, the yields of active
381 protein obtained with the *C. glutamicum* K9 biosensor strain were equal or even slightly
382 higher than the yields obtained with the *C. glutamicum* wild-type strain (Additional file 1:
383 Figure S5C, D, and E). Similar findings have been reported for *Lactococcus lactis,* which
384 demonstrated that the inactivation of the *htrA* gene can have a beneficial effect on the
385 secretory production of heterologous proteins [32-34]. Nevertheless, the situation may turn in
386 the adverse direction when the secretory production of other heterologous proteins is
387 attempted, especially if those proteins show a poor ability to fold after their membrane
388 translocation, leading to larger amounts of accumulated un/misfolded protein at the
389 membrane-cell wall interface.

390 The K9 biosensor signal clearly responds to increasing amounts of proteins that are
391 secreted by the Sec pathway across the cytoplasmic membrane in a dose-dependent manner.
392 When NprE-cutinase expression is gradually increased by the addition of increasing amounts
393 of IPTG, the corresponding *C. glutamicum* K9 biosensor cells showed a concomitant increase

394 of both the cutinase activity in the culture supernatant and the specific fluorescence, which
395 holds true also when the IPTG concentration is increased from 50 µM to 250 µM (Fig. 2C). A
396 slightly different behaviour is observed for *C. glutamicum* K9 expressing Pre^{Lip}-PhoA. Here,
397 the upper limit for the formation of active PhoA seems to be reached already at the expression
398 level that is obtained by the addition of 20 µM IPTG, since further increases in IPTG
399 concentration resulted only in minor increases in PhoA activity, although the specific
400 fluorescence of the corresponding cells continuously increased, even at the final step from 50
401 µM to 250 µM IPTG (Fig. 3C). In that respect, it is important to note that the secretion
402 biosensor responds to the extent of Sec-dependent protein secretion, but not to correctly
403 folded and therefore biologically active forms of the secreted target proteins. Therefore, the
404 results obtained with Pre^{Lip}-PhoA suggest that in the presence of more than 20 µM IPTG,
405 more protein molecules are synthesized and secreted across the plasma membrane (Fig. 3D),
406 but that the cellular capacity to correctly fold and release active PhoA has already reached an
407 upper limit. Since PhoA possesses two disulfide bonds that are required for its activity and
408 stability [35] and which must be formed correctly after its membrane passage, one might
409 speculate that a possible bottleneck for the formation of active PhoA could be that the thiol-
410 disulfide oxidoreductases (TDORs) that are responsible for disulfide bond formation and
411 isomerization in *C. glutamicum* [36, 37] are saturated already at the PhoA expression level
412 that is obtained by 20 µM IPTG. If so, then a further increase of PhoA expression and
413 membrane translocation would result in the accumulation and possibly also release of
414 increasing amounts of misfolded and thereby inactive PhoA protein. Interestingly, the
415 cutinase likewise possesses two disulfide bonds that must be formed after its membrane
416 passage [38]. Nevertheless, as mentioned above, the cutinase activity in the culture
417 supernatant steadily increases with higher IPTG concentrations, even at the final step from 50
418 µM to 250 µM IPTG. This might indicate that, in contrast to PhoA secretion, folding after
419 export might be a less severe bottleneck for cutinase secretion. However, an interesting

420 observation in this respect is the finding that the enzymatic activities in the culture
421 supernatants of the NprE-cutinase and YwmC-cutinase expressing strains differ by a factor of
422 two (Fig. 4C), whereas the difference in the amount of secreted cutinase protein seems to be
423 significantly higher (approximately 10-fold or even more, Fig. 4D). These findings suggest
424 that a higher secretion level mediated by the more efficient signal peptide (NprE) results in an
425 overproportionate concomitant production of enzymatically inactive protein, indicating that,
426 also for cutinase, an upper limit of the folding capacity seems to exist within the cell. Besides
427 protein folding, also other steps in the secretion pathway, such as the mechanistically to date
428 completely unknown transport of proteins across the mycolic acid outer membrane layer [4],
429 might restrict the amounts of heterologous proteins that can be secreted into the *C.*
430 *glutamicum* culture supernatant. Furthermore, it is very likely that the bottlenecks in the *C.*
431 *glutamicum* secretion pathway might differ for different target proteins, depending on their
432 intrinsic folding properties, their requirement for folding factors (such as TDORs), or other,
433 positive (*i.e.* required for their secretion) or negative interactions between the target proteins
434 and components of the cell envelope.

435 The identification of an optimal signal peptide for a desired target protein is one of the most
436 critical steps on the way to an efficient secretory production process [12]. Thus far,
437 diffentiation between different efficient signal peptides required the availability of an assay
438 for the respective target protein. However, for many biotechnolgically or pharmaceutically
439 relevant target proteins, a simple assay that also can be handled for a large number of samples
440 in a high-throughput screening approach is not available. In these cases, the availability of a
441 secretion biosensor that can differentiate between different efficient signal peptides via its
442 fluorescence signal will be of great advantage. As shown in this work, three differently
443 efficient signal peptides for cutinase secretion could clearly be distinguished by the specific
444 fluorescence of the corresponding recombinant *C. glutamicum* K9 biosensor strains.

445 Furthermore, despite the fact that the difference in the median fluorescence between an
446 efficient (NprE) and a less efficient (YwmC) signal peptide was rather small in the FACS
447 analysis (i.e. 12.1%, see Fig. 5A and B), we could show that, when setting an appropriate
448 sorting gate, the difference in specific fluorescence within the respective single cells is
449 sufficient to allow a significant enrichment of the superior signal peptide out of a 1:1 or even
450 a 1:100 mixture by only one FACS sorting and enrichment step. However, for the separation
451 of signal peptides with even smaller differences in their secretion efficiencies than the two
452 signal peptides examined in this study or for the screening of large signal peptide libraries,
453 two or even more rounds of FACS sorting and enrichment might be required to identify the
454 optimal signal peptide for the given target protein of choice.
455

456 **Conclusions**

457 In this study, we have constructed a *C. glutamicum* biosensor strain that allows for the
458 monitoring of Sec-dependent secretion of heterologous proteins in a dose-dependent manner,
459 independent of a direct assay for the respective target protein. The availability of such a
460 biosensor now opens up the way for various applications, such as (1) the screening of large
461 signal peptide libraries for any desired heterologous target protein, (2) the optimization of a
462 given signal peptide by random or saturation mutagenesis approaches, (3) the optimization of
463 the *C. glutamicum* host chassis by directed laboratory evolution, (4) the analysis of population
464 heterogeneities in microfluidic cultivation devices, and (5) the online monitoring of secretory
465 protein production processes.
466

467 **Methods**

468 **Bacterial strains, media, and growth conditions**

469 The bacterial strains used in this study are listed in Table 3. *E. coli* DH5α was used as
470 cloning host and grown in LB (lysogeny broth) medium [39] at 37°C. *C. glutamicum* strains
471 were growth at 30°C in brain heart infusion medium (BHI, Difco Laboratories), BHIS
472 medium (BHI medium containing 0.5 M sorbitol), CGIII medium [40] or CGXII medium [41]
473 containing 1 to 4% (w/v) glucose as indicated. If required, isopropyl-ß-D-
474 thiogalactopyranoside (IPTG) was added to final concentrations between 5 µM and 1 mM as
475 indicated. Antibiotic supplements were at the following concentrations: kanamycin 25 µg ml^{-1}
476 (*C. glutamicum*) and 50 µg ml^{-1} (*E. coli*).
477

478 **Microtiter plate cultivation**

479 Online monitoring of growth and biosensor fluorescence of *C. glutamicum* strains was done
480 in 48-well FlowerPlates (m2p-labs, Aachen/D) using the BioLector micro reactor cultivation
481 device (m2p-labs, Aachen/D). 750 µl CGXII medium containing 2% glucose per well were
482 inoculated with *C. glutamicum* cells from a preculture and grown at 30°C, a shaking
483 frequency of 1200 rpm, and a shaking diameter of 3 mm. After 4 h of cultivation, the gene
484 expression from pEKEx2-derived plasmids was induced by addition of various concentrations
485 of IPTG as indicated. During the entire cultivation process, the production of biomass was
486 measured as the backscattered light intensity of sent light with a wavelength of 620 nm (signal
487 gain factor of 20) and the eYFP fluorescence of the cultures was measured at an excitation of
488 508 nm and an emission of 532 nm (signal gain factor of 90). The specific fluorescence for
489 the cells is defined as eYFP fluorescence per scattered light intensity (given in a.u.) [42]. For
490 each experiment, three independent biological replicates were used.
491

492 **Plasmid constructions**

493 The plasmids used in this study are listed in Table 3. Oligonucleotides and primers used are
494 listed in Additional file 1: Table S1. All DNA manipulation followed standard procedures

495 [43]. All newly constructed plasmids were verified by DNA sequencing (Eurofins, Ebersberg,
496 Germany). Plasmids pEKEx2-NprE-cutinase, pEKEx2-YpjP-cutinase, and pEKEx2-YwmC-
497 cutinase have been described previously [10]. For the expression of the *B. subtilis* amylase
498 AmyE in *C. glutamicum*, the *amyE* gene was amplified by PCR using chromosomal DNA of
499 *B. subtilis* DB104 as template and primers AmyE-His-fw and AmyE-His-rv. The resulting
500 PCR fragment was purified, digested with *Bam*HI and *Sac*I and ligated into *Bam*H/*Sac*I-
501 digested pEKEx2, resulting in pEKEx2-AmyE. For the expression of a signal peptide less
502 variant of the *B. subtilis* amylase AmyE, the DNA region encoding the mature AmyE protein
503 was amplified by PCR using chromosomal DNA of *B. subtilis* DB104 as template and
504 primers ΔSP-AmyE-His-fw and AmyE-His-rv. The resulting PCR fragment was purified,
505 digested with *Bam*HI and *Sac*I and ligated into *Bam*HI/*Sac*I-digested pEKEx2, resulting in
506 pEKEx2-ΔSP-AmyE.
507
508 **Strain constructions**
509 For the construction of *C. glutamicum* K9 containing a replacement of the chromosomal
510 *htrA* gene by a DNA fragment encompassing the first 51 nucleotides of the *htrA* structural
511 gene followed by a TAG stop codon, a 16 bp spacer sequence containing a ribosome binding
512 site, and the *eyfp* gene (Fig.1B), three different DNA fragments were generated by PCR. The
513 first fragment containing approximately 580 bp of the *htrA* upstream region followed by 51
514 bp of the *htrA* structural gene and a TAG stop codon (FK9-1) was obtained by using
515 chromosomal DNA of *C. glutamicum* ATCC13032 as template and primers up-fw and up-
516 0998-rv. The second fragment containing the *eyfp* gene preceded by a ribosome binding site
517 (FK9-2) was obtained by using pSenLys [44] as template and primers RBS-eyfp-fw and eyfp-
518 rv. The third DNA fragment (FK9-3) containing aproximately 580 bp of the *htrA* downstream
519 region was generated by using chromosomal DNA of *C. glutamicum* ATCC13032 as template
520 and primers dw-fw and dw-rv. The corresponding PCR fragments were purified and fused

521 together in cross-over PCR reactions. First, fragments FK9-1 and FK9-2 were fused using
522 both fragments as template and primers up-fw and eyfp-rv. The resulting fused fragment
523 (FK9-4) was then fused with fragment FK9-3 using FK9-4 and FK9-3 as template and
524 primers up-fw and dw-rv. The resulting DNA fragment FK9-5 was then ligated into *Sma*I-
525 digested pK19mobsacB [45], yielding pK19mobsacB-K9. pK19mobsacB-K9 was introduced
526 into *C. glutamicum* ATCC13032 by electroporation [46] and cells that had integrated the
527 plasmid into the chromosome via homologous recombination were selected on plates
528 containing kanamycin. A second homologous recombination event leading to the loss of the
529 *sacB* gene (via excision of the integrated plasmid) was positively selected on BHIS agar
530 plates containing 10% (w/v) sucrose. Colonies were subsequently analyzed for the successful
531 gene replacement on the chromosome by colony PCR using primers proof-fw and proof-rv.
532 One of the isolates that contained the desired replacement was designated *C. glutamicum* K9.
533

534 **Flow cytometry**

535 *C. glutamicum* strains were first cultivated in flowerplates in 800 µl CGIII medium
536 containing 2% (w/v) glucose at 900 rpm for 8 h at 30°C (preculture 1). Subsequently, 100 µl
537 of the respective cultures were added to 700 µl CGXII medium containing 1% (w/v) glucose
538 and grown overnight at 900 rpm and 30°C (preculture 2). The resulting cultures were then
539 used to inoculate the main cultures (800 µl) in CGXII medium containing 2% (w/v) glucose
540 to an OD_{600} of 0.5. The cultures were then grown in a BioLector at 1200 rpm and 30°C, and,
541 after 4 h of growth, ITPG was added (250 µM final concentration). After further 6 h of
542 growth, cells from the respective cultures were subjected to FACS analysis or cell sorting.
543 For the separation of two differently efficient signal peptides out of a 1:1 or a 1:100
544 mixture of the respective cells by FACS, an enrichment step was performed. For this purpose,
545 $5x10^4$ (in the case of the 1:1 mixture) or $2x10^4$ cells (in the case of the 1:100 mixture) were
546 sorted out of the preselected gate 1 into 800 µl CGXII medium containing 2% (w/v) glucose

547 and subsequently grown for 24 h in a Biolector at 1200 rpm and 30°C. The respective culture
548 was then used to inoculate 800 µl CGXII medium containing 2% (w/v) glucose to an OD_{600} of
549 0.5 and treated further as described above for subsequent FACS analysis. Finally, after the
550 enrichment step, 46 cells were sorted out of the preselected gate onto BHI agar plates
551 containing 25 µg ml^{-1} kanamycin. A graphical workflow of the FACS analysis and sorting
552 experiments is shown in Additional file 1: Figure S3.
553 Flow cytometric measurements and sorting were performed on a FACS ARIA III high
554 speed cell sorter (BD Biosciences, Franklin Lakes, NJ, USA). A 488 nm blue solid state laser
555 was used for the detection of eYFP fluoresence with a 502-nm long-pass and a 530/30-nm
556 band-pass filter set. Forward scatter (FSC) and side-scatter (SSC) were detected as a voltage
557 pulse of height (H) and width (W). Sorting was performed with a custom four-way purity
558 single-cell precision mode at a threshold rate up to 10^4 events/s and a sample pressure of 70
559 psi. For cell sorting, a preselection of cells was performed to exclude cell doublets and debris
560 by electronic gating using FSC-W against FSC-H (gate 0). All FACS data were analyzed
561 using BD DIVA 8.0.1 and FlowJo 10.6.0 software (Tree Star, Inc., Ashland, OR, USA).
562
563 **Miscellaneous procedures**
564 Transcriptome analyses by DNA microarray experiments were performed as described by
565 Kleine et al. [20]. Specifically, precultures of the respective *C. glutamicum* strains in CGXII
566 medium containing 4% glucose were grown to an OD_{600} of 5-6 and used to inoculate the main
567 cultures in the same medium to an OD_{600} of 0.5, which subsequently were grown to the mid-
568 exponential growth phase. The main cultures were then splitted into two cultures and IPTG
569 was added to a final concentration of 1 mM to one of the cultures. The cells were harvested 30
570 min after IPTG addition by pouring the cultures into ice-containing tubes precooled to -20°C
571 followed by centifugation (3 min, 4200 x g, 4°C). The cell pellets were quickly frozen in
572 liquid nitrogen and stored at -80°C until use for RNA isolation and synthesis of fluorescently

573 labeled cDNA as described by Möker et al. [47]. All DNA microarray analyses were
574 performed with custom-made DNA microarrays based on 70mer oligonucleotides obtained
575 from Operon Biotechnologies. The comparisons were performed in three independent
576 biological replicates. The experimental details for handling these microarrays are described
577 elsewhere [48]. Processed and normalized data as well as experimental details were stored in
578 an inhouse microarray database for further analysis [49]. The full microarray data sets of this
579 study have also been deposited in the NCBI Gene Expression Omnibus and can be found
580 under the GEO accession number GSE140735. Preparation of whole cell extracts and
581 supernatant fractions of *C. glutamicum* strains for subsequent analysis by SDS-PAGE were
582 performed as described by Meissner *et al.* [50]. Cutinase activity in the culture supernatant
583 fractions was determined as previously described [51]. Alkaline phosphatase (PhoA) activity
584 in the culture supernatant fractions was determined as previously described by Darmon *et al.*
585 [52].
586
587
588
589
590 **Supplementary information**
591 File name: Additional file 1
592 File format: .pdf
593 Title of data: Supplementary information.
594 Description of data: Additional file 1: Table S1. Oligonucleotides and primers used in this
595 study. Figure S1. Expression and localization of heterologous proteins in *C. glutamicum* K9.
596 Figure S2. *C. glutamicum* wild-type does not possess any significant intrinsic fluorescence.
597 Figure S3. Graphic workflow for the FACS analysis and sorting experiments. Figure S4.

598 FACS analysis of *C. glutamicum* K9 strains. Figure S5. Secretory production of cutinase and
599 PhoA by *C. glutamicum* wild-type and *C. glutamicum* K9 strains.
600
601
602 **Declarations**
603 **Ethics approval and consent to participate**
604 Not applicable
605
606 **Consent for publication**
607 Not applicable
608
609 **Availability of data and materials**
610 All data generated or analysed during this study are included in this published article and its
611 supplementary information files
612
613 **Competing interests**
614 Janko Potzkei, Stephan Binder and Georg Schaumann are working for SenseUp GmbH, a
615 German company focused on commercial development of microbial production strains by
616 natural evolution and biosensor-based screening, including application of the biosensor
617 presented here. All other authors declare that they have no competing interests.
618
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Authors´ contributions

RF, GS, SB: conception and design of the study; SJ, AB, DD-O, BK, JP, PJB, RF: acquisition, analysis and interpretation of the data; RF: writing of the manuscript. All authors read and approved the final manuscript.

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Legends to Figures

Figure 1. The *htrA* locus of *C. glutamicum*. (A) Genomic organization of the chromosomal *cgtR2-cgtS2-htrA-moa*-cg1000 locus in *C. glutamicum* ATCC13032 wild-type. The promoters (P) upstream and within the operon and the transcripts derived from the respective promoters [25] are indicated. (B) In *C. glutamicum* K9, the *htrA* gene was replaced by a DNA fragment encompassing the first 51 bp of *htrA* (*htrA´*) followed by a TAG stop codon (underlined), a 16 bp spacer containing a ribosome binding site (RBS, bold letters in the nucleotide sequence) and the *eyfp* gene.

864 **Figure 2.** Cutinase secretion by *C. glutamicum* K9. Cells of *C. glutamicum* K9 possessing the
865 pEKEx2 empty vector (EV) or pEKEx2-NprE-cutinase were inoculated to an OD_{600} of 0.5 in
866 750 µl CGXII medium in a 48-well FlowerPlate and subsequently cultivated in a BioLector
867 for 24 h at 30°C, 1200 rpm and constant 85% relative humidity. After 4 h of cultivation, IPTG
868 was added at the indicated final concentrations. (A) Growth of the respective cultures was
869 monitored as backscattered light in 15 min intervals starting at the beginning of the
870 cultivation. The growth curves show one representative experiment of three independent
871 biological replicates. Standard deviations are given for selected time points. (B) Specific
872 fluorescence of the respective cultures during the BioLector cultivation. Also here, one
873 representative experiment of three independent biological replicates is shown and the standard
874 deviations are given for selected time points. (C) Cutinase activity in the supernatant (black
875 symbols) and specific fluorescence values (green symbols) after 24 h of cultivation of *C.*
876 *glutamicum* K9 (pEKEx2-NprE-cutinase) induced by different IPTG concentrations. (D)
877 After 24 h of growth, samples of the culture supernatant corresponding to an equal number of
878 the respective *C. glutamicum* K9 cells that had been induced by the IPTG concentrations
879 indicated below the lanes were analyzed by SDS-PAGE and proteins were visualized by
880 Coomassie Brilliant Blue staining. EV, *C. glutamicum* K9 (pEKEx2 empty vector) as negative
881 control; NprE-cutinase, *C. glutamicum* K9 (pEKEx2-NprE-cutinase); M, molecular weight
882 protein markers in kDa. The position of the secreted cutinase protein is indicated by an
883 asterisk. AU: arbitrary units.
884
885 **Figure 3.** PhoA secretion by *C. glutamicum* K9. Cells of *C. glutamicum* K9 possessing the
886 pEKEx2 empty vector (EV) or pEKEx2-Pre^{Lip}-PhoA were inoculated to an OD_{600} of 0.5 in
887 750 µl CGXII medium in a 48-well FlowerPlate and subsequently cultivated in a BioLector
888 for 24 h at 30°C, 1200 rpm and constant 85% relative humidity. After 4 h of cultivation, IPTG
889 was added at the indicated final concentrations. (A) Growth of the respective cultures was

890 monitored as backscattered light in 15 min intervals starting at the beginning of the
891 cultivation. The growth curves show one representative experiment of three independent
892 biological replicates. Standard deviations are given for selected time points. (B) Specific
893 fluorescence of the respective cultures during the BioLector cultivation. Also here, one
894 representative experiment of three independent biological replicates is shown and the standard
895 deviations are given for selected time points. (C) PhoA activity in the supernatant (black
896 symbols) and specific fluorescence values (green symbols) after 24 h of cultivation of *C.*
897 *glutamicum* K9 (pEKEx2-Pre^{Lip}-PhoA) induced by different IPTG concentrations. (D) After
898 24 h of growth, samples of the culture supernatant corresponding to an equal number of the
899 respective *C. glutamicum* K9 cells that had been induced by the IPTG concentrations
900 indicated below the lanes were analyzed by SDS-PAGE and proteins were visualized by
901 Coomassie Brilliant Blue staining. EV, *C. glutamicum* K9 (pEKEx2 empty vector) as negative
902 control; Pre^{Lip}-PhoA, *C. glutamicum* K9 (pEKEx2-Pre^{Lip}-PhoA); M, molecular weight protein
903 markers in kDa. The position of the secreted PhoA protein is indicated by an asterisk. AU:
904 arbitrary units.
905
906 **Figure 4.** Cutinase secretion by *C. glutamicum* K9 using three different Sec signal peptides.
907 Cells of *C. glutamicum* K9 possessing the pEKEx2 empty vector (EV), pEKEx2-NprE-
908 cutinase (NprE), pEKEx2-YpjP-cutinase (YpjP), or pEKEx2-YwmC-cutinase (YwmC) were
909 inoculated to an OD_{600} of 0.5 in 750 µl CGXII medium in 48-well FlowerPlates and
910 subsequently cultivated in a BioLector system for 24 h at 30°C, 1200 rpm and constant 85%
911 relative humidity. After 4 h of cultivation, IPTG was added (250 µM final concentration). (A)
912 Growth of the respective cultures was monitored as backscattered light in 15 min intervals
913 starting at the beginning of the cultivation. The growth curves show one representative
914 experiment of three independent biological replicates. Standard deviations are given for
915 selected time points. (B) Specific fluorescence of the respective cultures during the BioLector

916 cultivation. Also here, one representative experiment of three independent biological
917 replicates is shown and the standard deviations are given for selected time points. (C)
918 Cutinase activity in the supernatant (gray bars) and specific fluorescence values (green dots)
919 after 24 h of cultivation. (D) After 24 h of growth, samples of the culture supernatant
920 corresponding to an equal number of the respective *C. glutamicum* K9 cells were analyzed by
921 SDS-PAGE and the proteins were visualized by Coomassie Brilliant Blue staining. M,
922 molecular weight protein markers in kDa. The position of the secreted cutinase protein is
923 indicated. AU: arbitrary units.
924
925 **Figure 5.** FACS analysis of *C. glutamicum* K9 strains and sorting strategy for the enrichment
926 of the best performing signal peptide. 10^5 cells from cultures of the respective strains were
927 analyzed, followed by a preselection of cells to exclude cell doublets and debris by electronic
928 gating using FSC-W against FSC-H (Table 2, gate 0). An overlay of *C. glutamicum* K9
929 carrying pEKEx2-NprE-cutinase (red) or pEKEx2-YwmC-cutinase (blue) is shown as dot plot
930 (A) and histogram (B). In the dot plot, the fluorescence intensity (eYFP) is plotted against cell
931 size (FSC-H), whereas the histogram shows the cell count against the fluorescence intensity
932 (eYFP). (C) Cells were inoculated to an OD_{600} of 0.5 in CGXII medium containing 2% (w/v)
933 glucose and cultivated at 30°C. After 4 h of growth, IPTG (250 µM final concentration) was
934 added to the cultures and, after 10 h of growth, cells were sampled from the respective
935 cultures and subjected to FACS analysis. For the enrichment of the better performing signal
936 peptide (NprE) out of a 1:1 or a 1:100 mixture with a less efficient signal peptide (YwmC),
937 we selected a gate (gate 1) in the dot plot of the respective preselected cells such that it
938 contains as many of the better perfoming cells (i.e. those containing pEKEx2-NprE-cutinase)
939 and excludes as many of the less productive cells (i.e. those containing pEKEx2-YwmC). For
940 the corresponding numbers of events falling in gate 1, see Table 2 and Additional file 1:
941 Figure S4.

942

943

944 **Table 1. *C. glutamicum* genes responding to Sec secretion stress in microarray**
945 **experiments.**

946

Gene locus	Gene	Annotation	Average mRNA ratio ΔSP-AmyE overproduction[a]	Average mRNA ratio AmyE overproduction[b]	Average mRNA ratio NrpE-cutinase overproduction[c]
cg0998	*htrA*	HtrA protease homologue	1.24	**4.22**	**4.40**
cg0706	*esrI*	EsrI, Inhibitor of the EsrSR two-component system	0.99*	**2.31**	0.80*
cg1325		Putative stress responsive transcriptional regulator	1.13*	**3.69**	0.96*
cg3264	*rsmP*	RsmP, conserved cytoskeletal protein	0.61	**2.67**	0.96*

947

948 Significant ($p \leq 0.05$) more than two-fold changes in the average mRNA ratios in the DNA
949 microarray experiments are indicated in bold numbers. Average mRNA ratios with $p > 0.05$
950 are marked by an asterisk. All microarray data were from three independent biological
951 samples.

952 [a]Average mRNA ratio of *C. glutamicum* (pEKEx2-ΔSP-AmyE) induced / uninduced by IPTG
953 for 30 min.

954 [b]Average mRNA ratio of *C. glutamicum* (pEKEx2-AmyE) induced / uninduced by IPTG for
955 30 min.

956 [c]Average mRNA ratio of *C. glutamicum* (pEKEx2-NprE-cutinase) induced / uninduced by
957 IPTG for 30 min.

958

959 **Table 2. FACS analysis of cutinase-secreting *C. glutamicum* K9 strains[a]**

Signal peptide	Events in gate 0	Events in gate 1	Ratio NprE : YwmC	Percentage NprE
A:				
Before enrichment				
NprE	68 173	309		
YwmC	69 699	7		
Mixture NprE : YwmC	68 915	87	1 : 1	50 %
After enrichment				
Enriched culture	68 199	148		
44 clones sequenced			1 : 0.023 (43 NprE : 1 YwmC)	97.7 %
B:				
Before enrichment				
NprE	71 804	458		
YwmC	71 923	46		
Mixture NprE : YwmC	72 151	50	1 :100	1 %
After enrichment				
Enriched culture	76 932	286		
43 clones sequenced			1 : 1.7 (16 NprE : 27 YwmC)	37 %

960

961 [a] The strains used in the experiments were *C. glutamicum* K9 (pEKEx2-NprE-cutinase), *C.*
962 *glutamicum* K9 (pEKEx2-YwmC-cutinase), or 1:1 (A), or 1:100 (B) mixtures of both strains.
963 100 000 cells of each strain or the mixture of strains were analyzed. For all sortings,
964 electronic gating was set in a dot plot of FSC-H against FSC-W to exclude cell doublets and
965 cell debris (gate 0). Selection of cells with respect to the better performing signal peptide was
966 performed by setting a gate in the dot blot (gate 1) that contains as many of the better
967 perfoming cells (*i.e.* those with the NprE signal peptide) and that excludes as many of the less
968 productive cells (*i.e.* those with the YwmC signal peptide) as possible. The number of cells
969 falling into the respective gates are shown.

970

971 **Table 3. Bacterial strains and plasmids used in this study.**

972

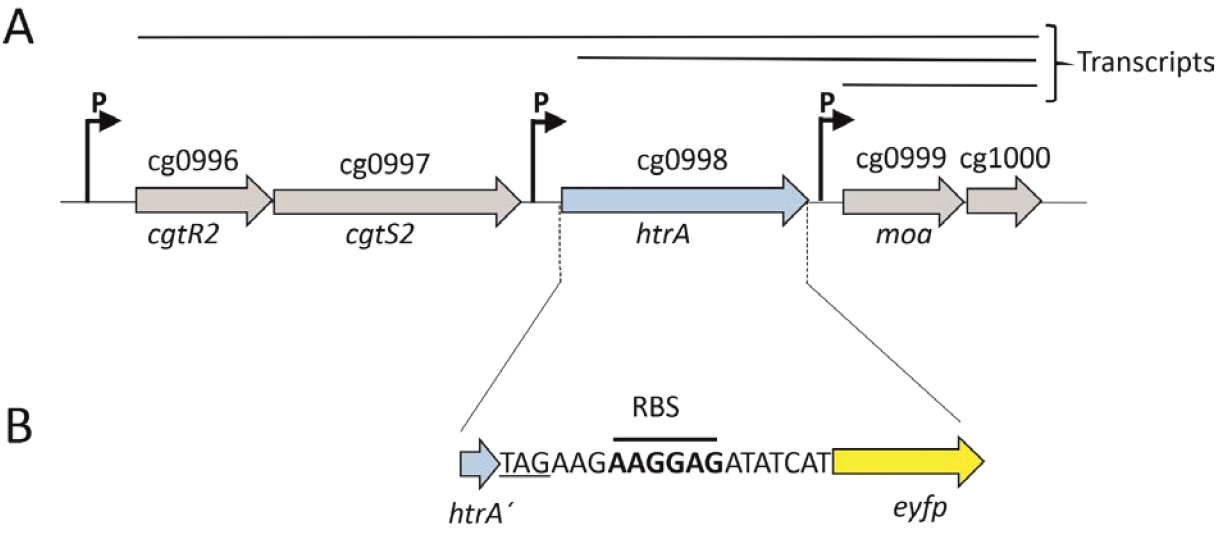
Strains or plasmids	Relevant properties[a]	Source of reference
***E. coli* strains**		
DH5α	*supE44, ΔlacU169*(*ϕ80 lacZΔM15*) *hsdR17 recA1 endA1 hsdR gyrA relA thi*	[53]
***C. glutamicum* strains**		
ATCC13032	wild-type	[54]
ATCC13032 K9	htrA::htrA´-eypf (replacement of htrA by htrA´-eyfp)	This study
***C. glutamicum* plasmids**		
pSenLys	lysine sensor plasmid encoding LysG and its target promoter P*lysE* fused to *eyfp*	[44]
pK19mobsacB	Vector for allelic exchange in *C. glutamicum*, pK18 *oriV*$_{E.coli}$, *oriT*, *mob*, *sacB*, *lacZ*α, Km^R	[45]
pK19mobsacB-K9	pK19mobsacB containing the *eyfp* gene flanked by the upstream region and the first 51 bp of the *htrA* structural gene and the *htrA* downstream region	This study
pEKEx2	*C. glutamicum*/*E. coli* shuttle vector for regulated gene expression, pBL1 oriV$_{C.glutamicum}$, pUC18 oriV$_{E. coli}$, P$_{tac}$, *lac*I^q, Km^R	[55]
pEKEx2-AmyE	pEKEx2 containing a gene encoding *B. subtilis* AmyE with its authentic signal peptide and a carboxl-terminal His-tag	This study
pEKEx2-ΔSP-AmyE	pEKEx2 containing a gene encoding a signal peptide-less variant of *B. subtilis* AmyE with a carboxyl-terminal His-tag	This study
pEKEx2-NprE-cutinase	pEKEx2 containing a gene encoding the mature part of the cutinase from *F. solani pisi* fused to the NprE signal peptide	[10]
pEKEx2-YpjP-cutinase	pEKEx2 containing a gene encoding the mature part of the cutinase from *F. solani pisi* fused to the YpjP signal peptide	[10]
pEKEx2-YwmC-cutinase	pEKEx2 containing a gene encoding the mature part of the cutinase from *F. solani pisi* fused to the YwmC signal peptide	[10]

973

974 [a] Km^R, kanamycin resistance

975

Figure 1.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Transcripts
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B
RBS
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htrA´
eyfp

Figure 2.

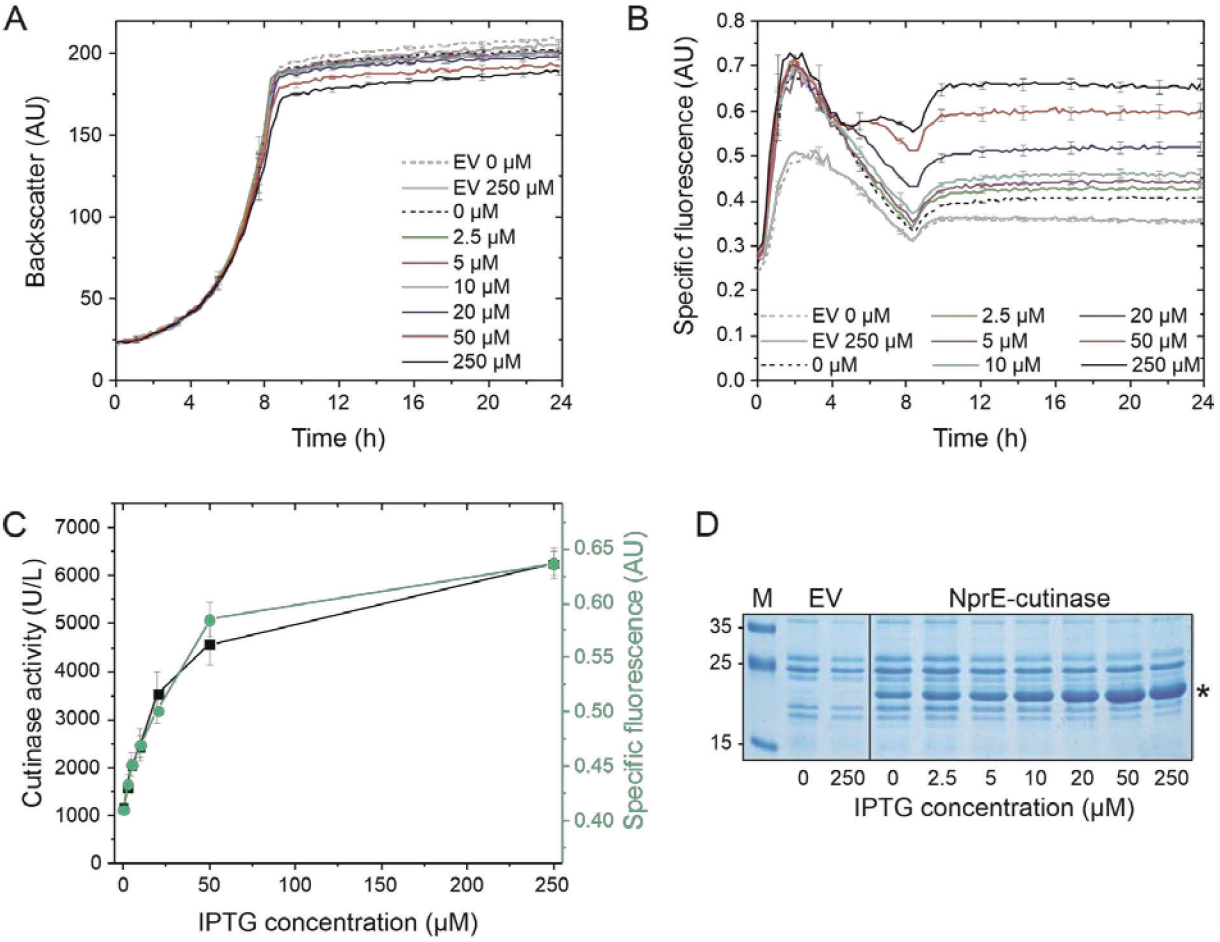
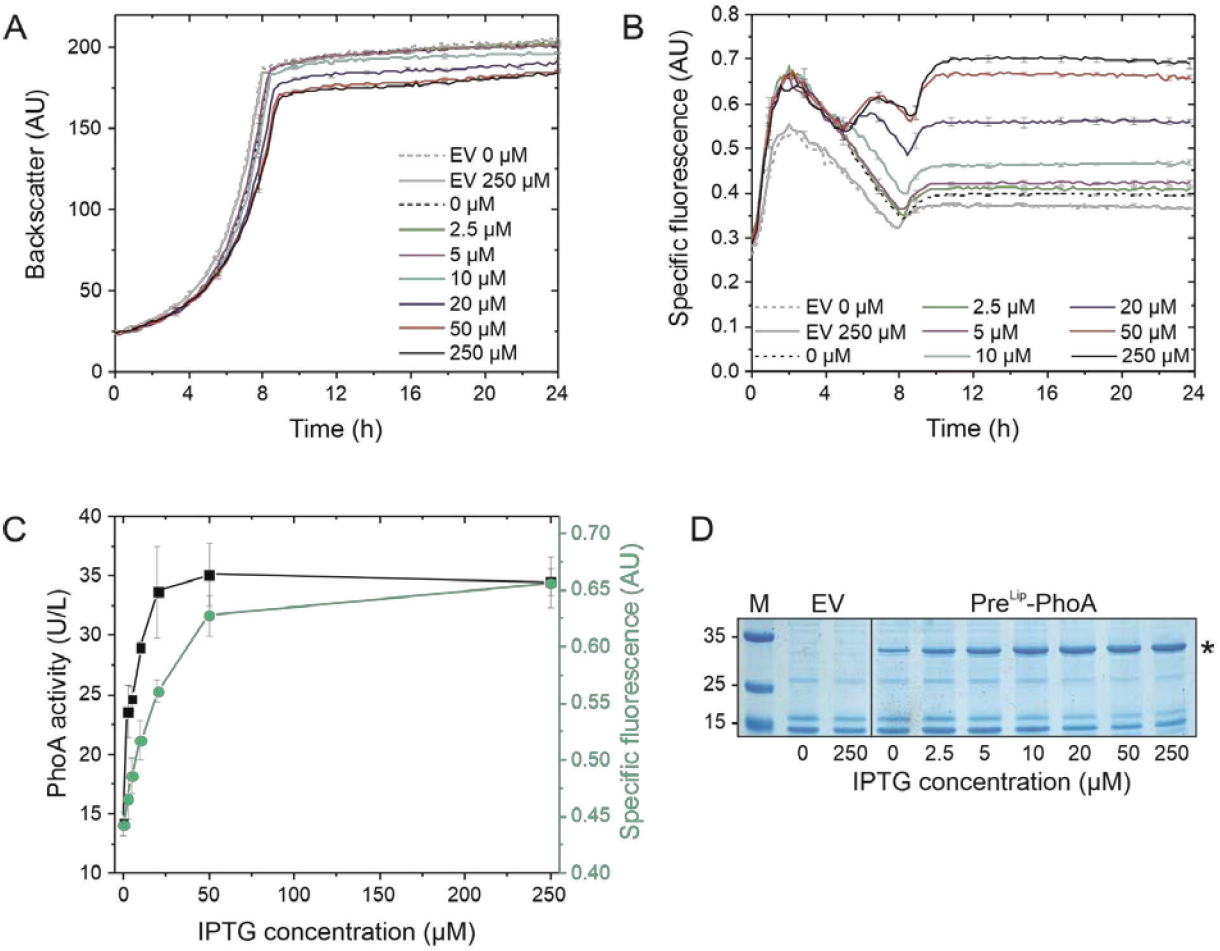

A
Backscatter (AU)
Time (h)
EV 0 μM
EV 250 μM
0 μM
2.5 μM
5 μM
10 μM
20 μM
50 μM
250 μM
B
Specific fluorescence (AU)
Time (h)
C
Cutinase activity (U/L)
Specific fluorescence (AU)
IPTG concentration (μM)
D
M
EV
NprE-cutinase
35
25
15
*
0 250 0 2.5 5 10 20 50 250
IPTG concentration (μM)

Figure 3.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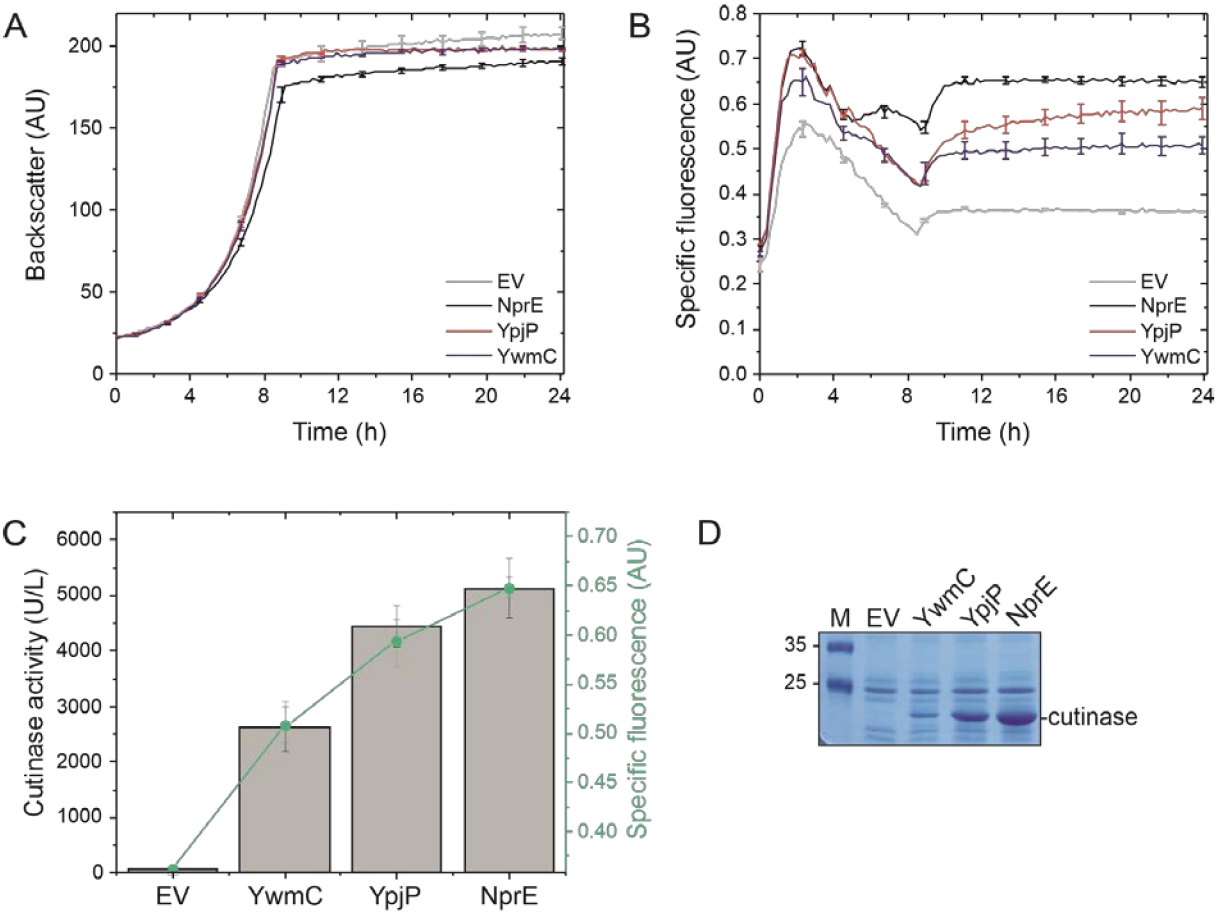
Time (h)
EV 0 μM
EV 250 μM
0 μM
2.5 μM
5 μM
10 μM
20 μM
50 μM
250 μM
B
Specific fluorescence (AU)
Time (h)
C
PhoA activity (U/L)
Specific fluorescence (AU)
IPTG concentration (μM)
D
M
EV
Pre^Lip-PhoA
*
IPTG concentration (μM)

Figure 4.


A
Backscatter (AU)
Time (h)
EV
NprE
YpjP
YwmC
B
Specific fluorescence (AU)
Time (h)
EV
NprE
YpjP
YwmC
C
Cutinase activity (U/L)
Specific fluorescence (AU)
EV
YwmC
YpjP
NprE
D
M
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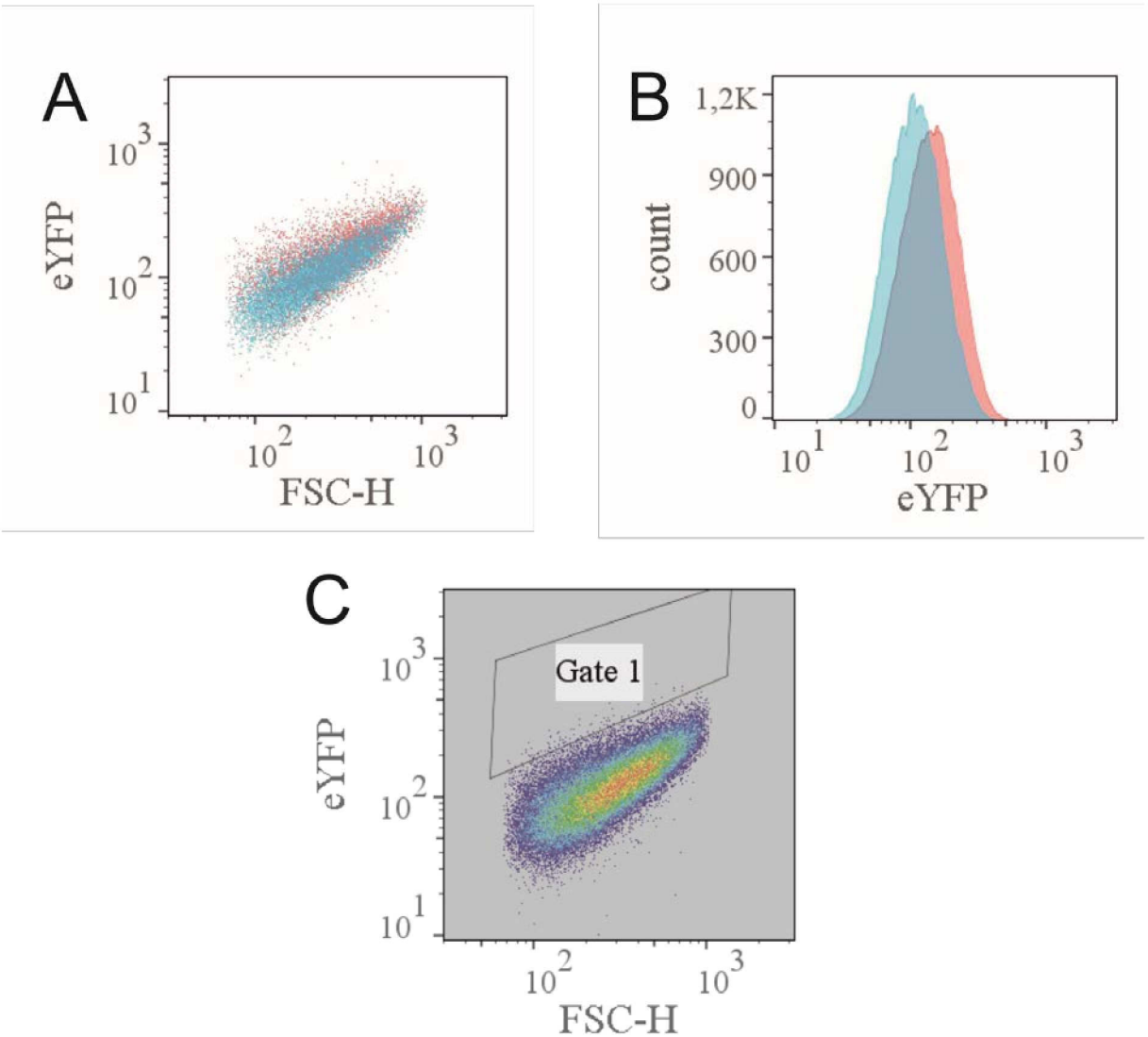
YwmC
YpjP
NprE
35
25
cutinase

Figure 5.



Additional file

A secretion biosensor for monitoring Sec-dependent protein export in *Corynebacterium glutamicum*

by S. Jurischka, A. Bida, D. Dohmen-Olma, B. Kleine, J. Potzkei, S. Binder, G. Schaumann, P. J. Bakkes, and R. Freudl

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Table S1. Oligonucleotides and primers used in this study

Name	Sequence (5' → 3')
up-fw	GAAGAAACCGCCGAAACGTCAAGC
eyfp-rv	GCAGATTAACTCTTTTAAACTTATTACTTGTACAGCTCGTCCATGCCG
dw-fw	GTTTAAAAGAGTTAATCTGCATCTAATCAAGTAGCC
dw-rv	GCCATCACGAATTGCCGAACGAG
up-0998-rv	CGATGCACGGTCCGGGTTCTC
RBS-eyfp-fw	GAGAACCCGGACCGTGCATCGTAGAAGAAGGAGATATCATATGG
proof-fw	GTTGATGGCAACCAACAGTG
proof-rv	CTCGTCTACGTTGTCTACTC
AmyE-His-fw	GAGGGGATCCCGAAGGAGATATAGATATGTTTGCAAAACGATTCA
ΔSP-AmyE-His-fw	CTAGAGGATCCCGAAGGAGATATAGATATGGAAACGGCGAACAAATC
AmyE-His-rv	GATCTAGAGCTCCTAGTGATGGTGATGGTGATGATGGGGAAGAGAACCGCTTAA

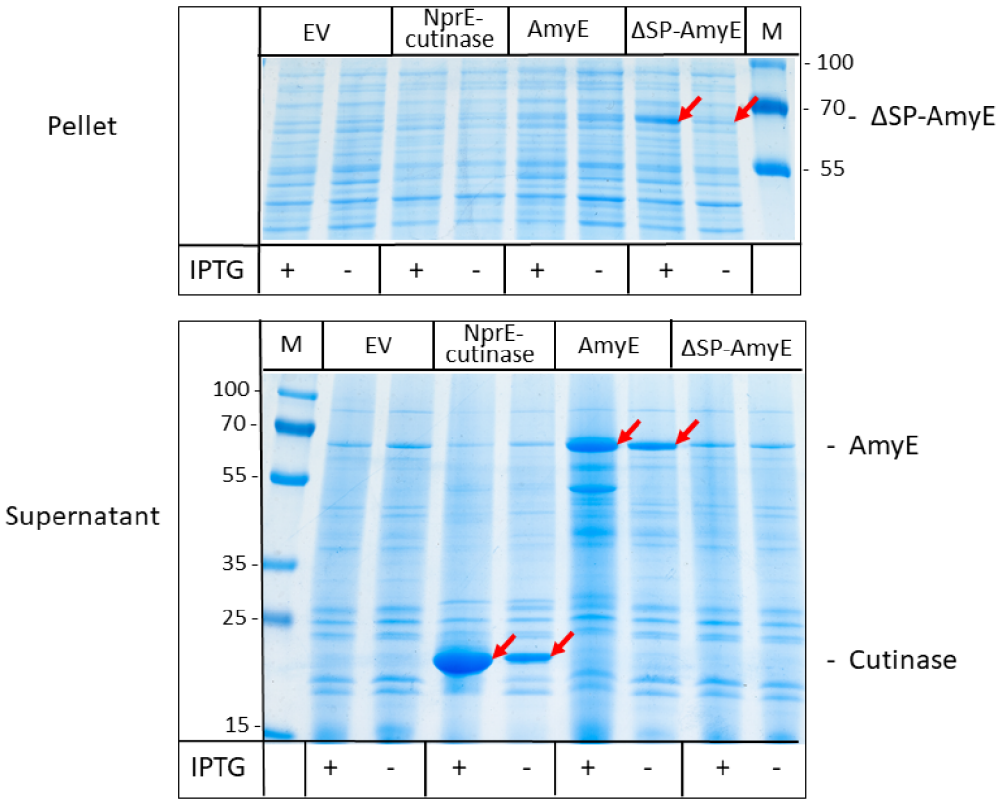

Figure S1. Expression and localization of heterologous proteins in *C. glutamicum* K9. Cultures of *C. glutamicum* K9 strains harboring the pEKEx2 empty vector (EV), pEKEx2-AmyE (AmyE), pEKEx2-ΔSP-Amy (ΔSP-Amy), or pEKEx2-NprE-cutinase (NprE-Cutinase) that were grown in the presence (+) or absence (-) of 250 µM IPTG were fractionated into cells (pellet) and culture supernatant. Samples of both fractions corresponding to an equal number of cells were analyzed by SDS-PAGE and proteins were visualized by Commassie Brillant Blue staining. The positions of the different heterologous proteins within the respective fractions are marked by red arrows and are indicated on the right margin. M, molecular weight protein markers in kDa.

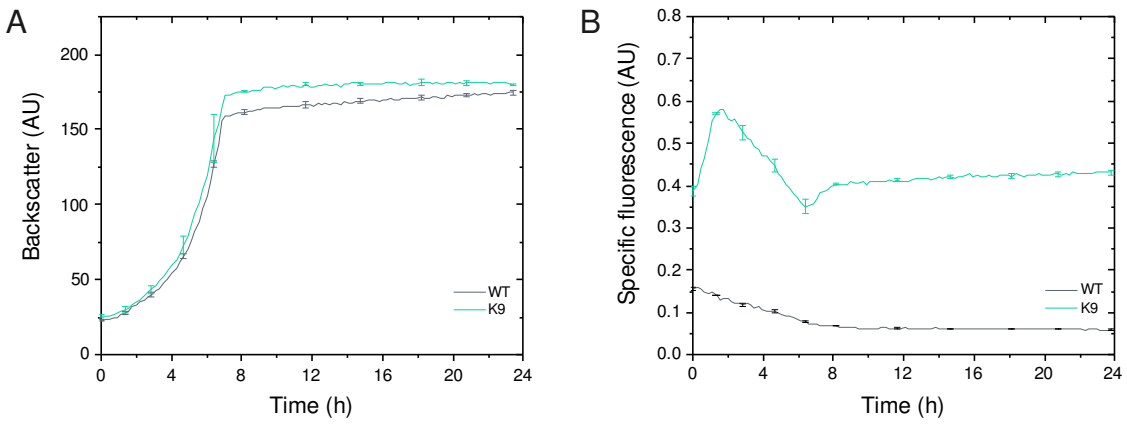

Figure S2. *C. glutamicum* wild-type does not possess a significant intrinsic fluorescence. Cells of *C. glutamicum* wild-type (WT) or *C. glutamicum* K9 (K9) were inoculated to an OD_{600} of 0.5 in 750 µl CGXII medium in a 48-well FlowerPlate and subsequently cultivated in a BioLector system for 24 h at 30°C, 1200 rpm and constant 85% relative humidity. (A) Growth of the respective cultures was monitored as backscattered light in 15 min intervals starting at the beginning of the cultivation. The growth curves show one representative experiment of three independent biological replicates. Standard deviations are given for selected time points. (B) Specific fluorescence of the respective cultures during the BioLector cultivation. Also here, one representative experiment of three independent biological replicates is shown and the standard deviations are given for selected time points.

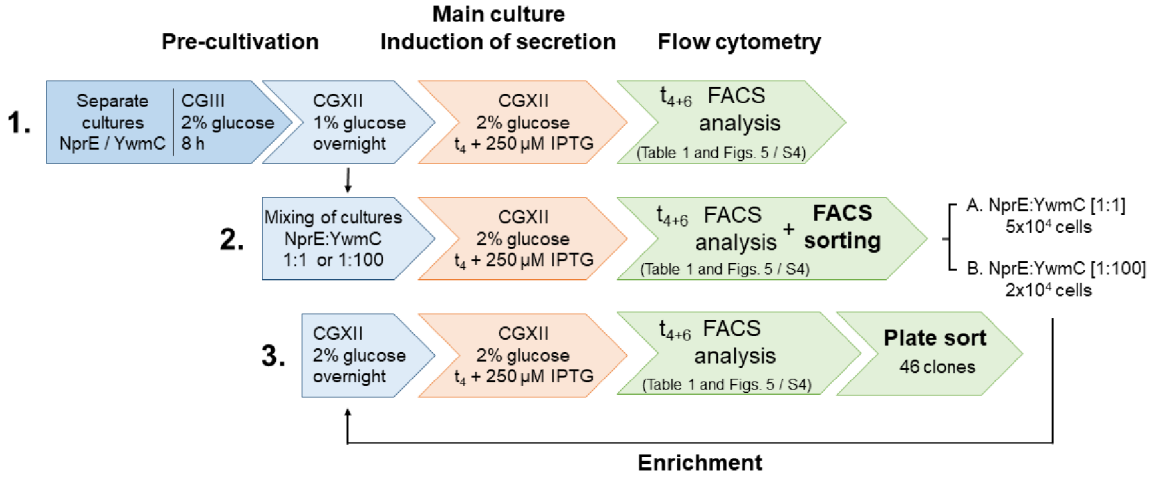

Figure S3. Graphical workflow for the FACS analysis and sorting experiments.

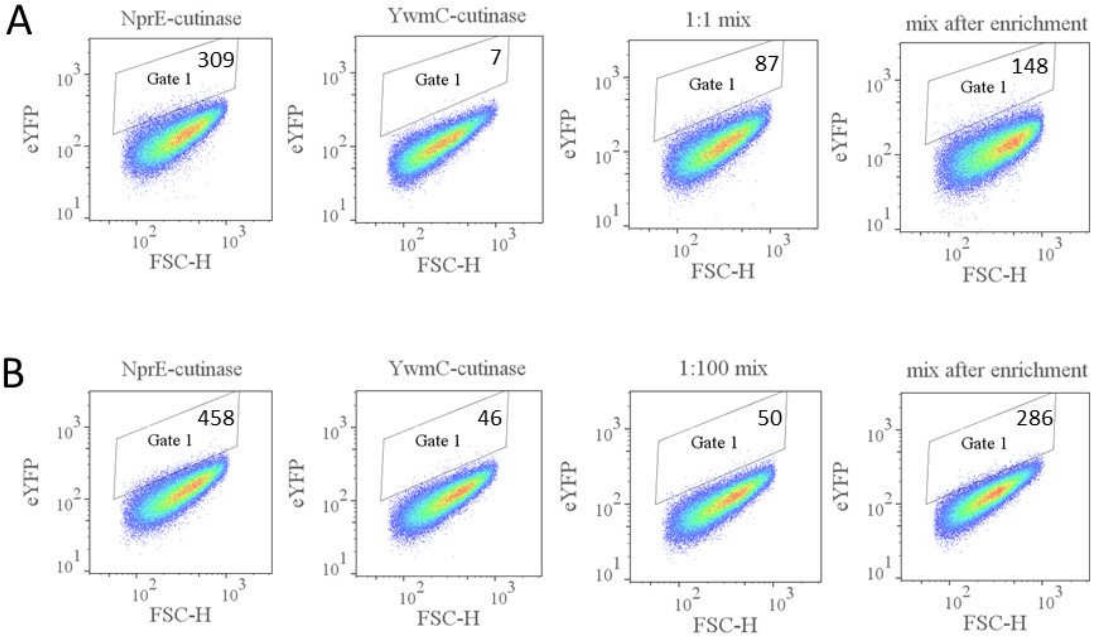

Figure S4. FACS analysis of *C. glutamicum* K9 strains. The strains used in the experiments were *C. glutamicum* K9 (pEKEx2-NprE-cutinase), *C. glutamicum* K9 (pEKEx2-YwmC-cutinase), or 1:1 (A) or 1:100 (B) mixtures of both strains. 100 000 cells of each strain or the mixture of strains were analyzed. To exclude doublets and debris, cells were preselected by electronic gating using FSC-W against FSC-H. Selection of cells with respect to the better performing signal peptide was performed by setting a gate in the dot blot (gate 1) that contains as many of the better performing cells (*i.e.* those with the NprE signal peptide) and that excludes as many of the less productive cells (*i.e.* those with the YwmC signal peptide) as possible. The number of cells falling into the respective gates 1 are indicated in the upper right corner (see also Table 2 in the main manuscript).

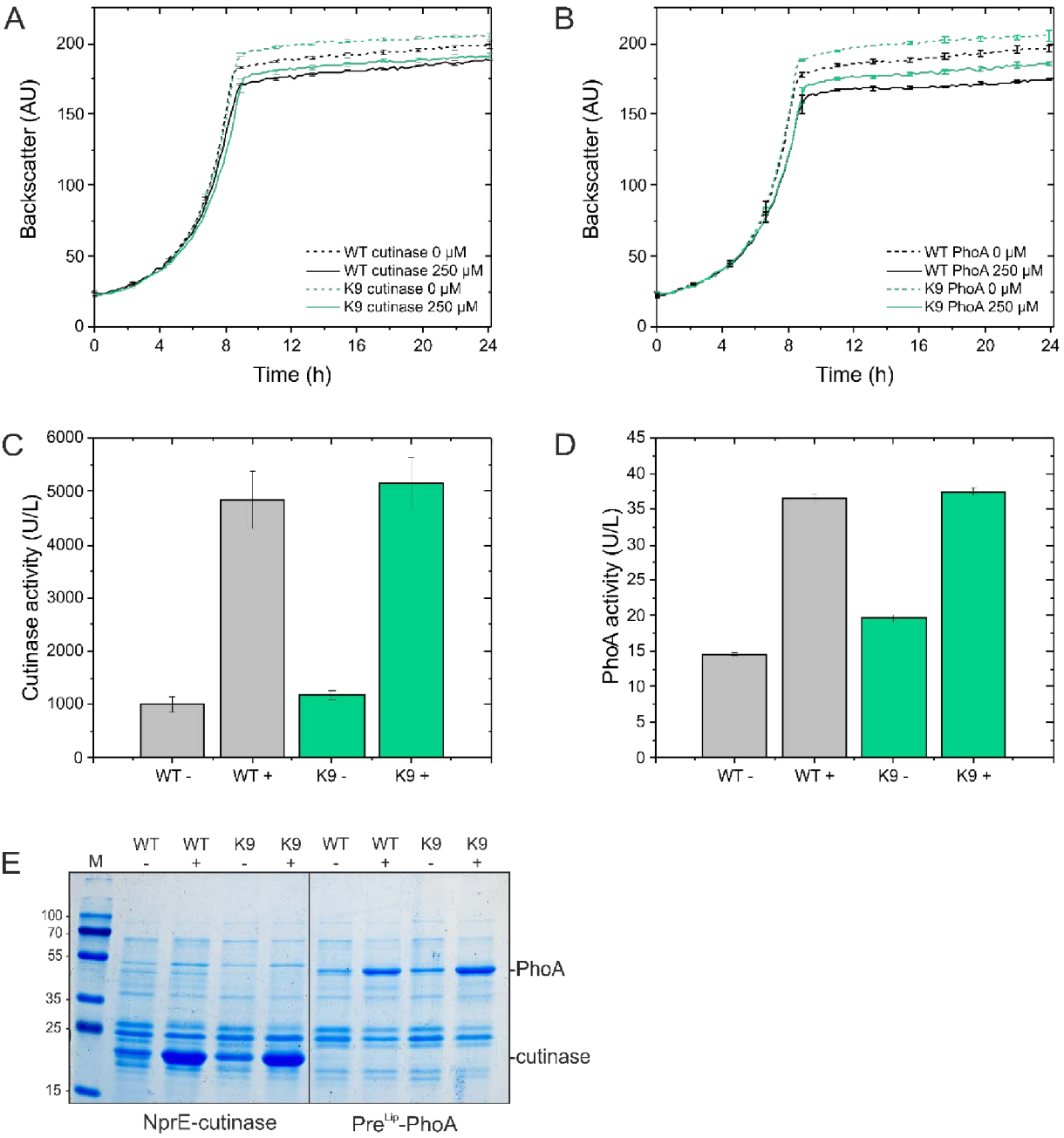

Figure S5. Secretory production of cutinase and PhoA by *C. glutamicum* wild-type and *C. glutamicum* K9 strains. Cells of *C. glutamicum* wild-type (WT) or *C. glutamicum* K9 containing (A) pEKEx2-NprE-cutinase (cutinase) or (B) pEKEx2-Pre^{Lip}-PhoA (PhoA) were inoculated to an OD_{600} of 0.5 in 750 µl CGXII medium in a 48-well FlowerPlate and subsequently cultivated in a BioLector system for 24 h at 30°C, 1200 rpm and constant 85% relative humidity. For the full induction of gene expression, IPTG was added to a 250 µM final concentration (+). Uninduced cells showing a basal level of gene expression received no IPTG (-). Growth of the respective cultures in (A) and (B) was monitored as backscattered

light in 15 min intervals. The growth curves show one representative experiment of three independent biological replicates. Standard deviations are given for selected time points. (C) Cutinase activity in the supernatant of *C. glutamicum* WT and K9 cells harboring pEKEx2-NrpE-cutinase after 24 h of cultivation with (+) or without (-) added IPTG. (D) PhoA activity in the supernantant of *C. glutamicum* WT and K9 cells harboring pEKEx2-Pre^{Lip}-PhoA after 24 h of cultivation with (+) or without (-) added IPTG. (E) Supernatant fractions of *C. glutamicum* WT or K9 cells harboring pEKEx2-NprE-cutinase or pEKEx2-Pre^{Lip}-PhoA after 24 h of cultivation with (+) or without (-) added IPTG were analyzed by SDS-PAGE and proteins were visualized by Coomassie Brillant Blue straining. The positions of the secreted cutinase and PhoA proteins are indicated. M, molecular weight protein markers in kDa.