This is the Author's Pre-print version of the following article: ***Juan A. Rojas Contreras, Mario Pedraza-Reyes, Leandro G. Ordoñez, Norma Urtiz Estrada, Ana P. Barba de la Rosa, Antonio De León-Rodríguez, Replicative and integrative plasmids for production of human interferon gamma in Bacillus subtilis, Plasmid, Volume 64, Issue 3, 2010, Pages 170-176,*** **which has been published in final form at: https://doi.org/10.1016/j.plasmid.2010.07.003 This article may be used for non-commercial purposes in accordance with Terms and Conditions for Self-Archiving.**

Replicative and integrative plasmids for production of human interferon gamma in *Bacillus subtilis*

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Running Title: Secretion of heterologous proteins in *Bacillus subtilis*

Submitted to: *Plasmid*

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1 **Abstract**

2 Integrative and replicative plasmids for the expression driven by the P_{43} promoter and
3 secretion of recombinant proteins in *Bacillus subtilis* were constructed. The plasmids
4 named pInt and pRep respectively were tested for the production of recombinant human
5 interferon gamma (rhIFN-γ). A synthetic *hIFN-γ* gene employing the optimized *B. subtilis*
6 codon-usage was fused with the *Bacillus licheniformis* α-amylase signal peptide (*sp-amyL*)
7 encoding sequence. The integrative construct produced 2.5±0.2 mg l^{-1} and the replicative
8 system produced 20.3±0.8 mg l^{-1} of total recombinant rhIFN-γ . The results showed that
9 secretion of hIFN-γ was the bottleneck for the overexpression of mature rhIFN-γ by *B.*
10 *subtilis*.

11

12

13 **Keywords:** heterologous, integrative, probiotic, therapeutic, recombinant, replicative
14 plasmid and secretion.

15

16 **1. Introduction**

17 A large collection of cloning and expression plasmids is available for the Gram-negative
18 bacterium *Escherichia coli*. Therefore, most protein expression strategies in biotechnology
19 research focus on this microorganism. However, aside from the advantages of *E. coli*
20 systems, several problems can occur during the process of heterologous gene expression
21 and purification, such as formation of inclusion bodies, protein misfolding, and toxicity
22 problems.

23

24 *Bacillus subtilis* is a generally recognized as safe (GRAS) organism and considered as an
25 alternative expression host for production of heterologous proteins. This Gram-positive
26 bacterium does not produce endotoxins and directly secrete proteins to the culture medium
27 simplifying the purification of recombinant proteins (Simonen and Palva, 1993). Several
28 vectors have been assessed for the production of microbial proteins in *B. subtilis* (see as
29 recent publications: Özdamar et al. 2009; Lin et al., 2009; Liu et al., 2010). They are based
30 on replicative plasmids, that have the risk of the segregational instability, which is one of
31 the main problems using *B. subtilis* as expression system (Fleming and Patching, 1994). To
32 our knowledge the choice of available integrative expression plasmids is still limited
33 (Wang et al., 2004). In addition, few studies on the production of human cytokines in *B.*
34 *subtilis* have been reported and the overexpression is still a challenge (Lam et al., 1998;
35 Min-Young et al., 2002; Westers et al. 2006; Westers et al., 2004).

36

37 Human interferon gamma (hIFN-γ) is an immunomodulatory cytokine involved in a broad
38 range of biological activities. The hIFN-γ is a glycoprotein of 143 amino acid residues with

39 a molecular mass of 20~25 kDa produced by $CD4^+$ and $CD8^+$ T lymphocytes as well as
40 activated NK cells. Recombinant hIFN-γ (rhIFN-γ) produced in *E. coli* is a non-
41 glycosylated 16.7 kDa protein, however it is physiologically active and it has been
42 approved for treatment of severe malignant osteopetrosis and chronic granulomatous
43 disease (Madyastha et al., 2000; Khalilzadeh et al., 2003; Marciano et al., 2004, Schroder et
44 al., 2004). The potential therapeutic applications of the rhIFN-γ have increased the interest
45 for developing new efficient systems for its production and purification with the less
46 downstream steps (Medina-Rivero et al., 2007; Balderas Hernández et al., 2008b).
47
48 The goal of this wok was to evaluate the expression and secretion of rhIFN-γ by *B. subtilis*
49 using replicative multicopy plasmids driven by the P_{spac} or P_{43} strong promoters and an
50 integrative plasmid containing the P_{43} promoter. To our knowledge, this is the first report
51 showing the production of human cytokines using both optimized synthetic genes and
52 integrative plasmids in *B. subtilis*.
53
54 **2. Materials and methods**
55
56 *2.1 Bacterial strains and media*
57
58 *Escherichia coli* DH5α (Invitrogen) and *Bacillus subtilis* WB600 (kindly provided by Prof.
59 Wong, University of Calgary) were routinely grown at 37°C in Luria Bertani broth
60 (Invitrogen); the genotypic characteristics of these strains are shown in table 1. The rhIFN-γ
61 production and secretion assays in *B. subtilis* were done in 2xL-Mal medium described by

62 Morimoto et al. (2008). Antibiotics were used at the following final concentrations:

63 ampicillin (Ap) 100 μg ml^{-1}; erithromycin (Em) 5 μg ml^{-1}; kanamycin (Kan) 30 μg ml^{-1}.

64

65 2.2 *DNA manipulation and transformation procedures*

66

67 Procedures for DNA purification, restriction, ligation, agarose gel electrophoresis and

68 transformation of competent *E. coli* cells were done using standard techniques (Sambrook

69 et al., 1989). *B. subtilis* was transformed as described by Anagnostopoulos and Spizizen

70 (1961).

71

72 *2.3 Construction of plasmids*

73

74 A synthetic optimized *hIFN-γ* gene was fused with the signal peptide sequence of the *B.*

75 *licheniformis* α-amylase gene (sp-*amyL*) as follows: the *hIFN-γ* gene was amplified by

76 PCR from pBAL (Balderas-Hernández et al., 2008a) using the sense 5'-GCTGCCTCATT

77 CCGCGGCCGCGCAGGACCCATACGTG-3' and antisense 5'-TCTAGATTACTGACT

78 TGCACGACGACC-3' primers. The *B. licheniformis sp-amyL* sequence was amplified

79 from pAX01-B.L.*amyL* using the sense 5'-AAGCTTAAAGGAGGTGATCCAG-3' and

80 antisense 5'- CACGTATGGGTCCTGCGCGGCCGCGGAATGAGGCAGC-3' primers.

81 The *Hind*III and *Bam*HI restriction sites are underlined in the primers. The two PCR

82 fragments were joined by the overlapping extension technique (Ho et al., 1989), subcloned

83 in pCR2.1 (Invitrogen) and digested with *Hind*III and *Bam*HI enzymes. The *Hind*III-

84 *spamyL-hIFNγ-Bam*HI restriction product was cloned into pDG148 (Stragier et al., 1988),

85 which contains the P_{spaC} promoter, and the resulting plasmid was named pSpac-gam (Fig.
86 1). For construction of the replicative plasmid driven by the P_{43} promoter, the downstream
87 sequence of the *cdd* gene of *B. subtilis* 168 (containing the P_{43} promoter) was amplified
88 using the sense 5'-GATATCCCGCCTGCGCTGTTCTCA-3' and anti-sense 5'-AAGCTTC
89 CTCTCTTACCTATAATGG-3' primers containing the *Eco*RV and *Hind*III sites
90 respectively (underlined). The *Eco*RV-P_{43}-*Hin*dIII fragment was cloned in pGEM T-Easy
91 vector (Promega), digested and subcloned in the pSpac-gam previously digested with
92 *Eco*RV and *Hind*III enzymes. The resulting plasmid was named pRep-gam (Fig. 1). The
93 integrative plasmid to express constitutively the *hIFN-γ* gene was based on the integrative
94 pAX01 vector (Hartl et al., 2001) and was made as follow: the Em^r cassette from pAX01
95 was replaced by the *Not*I-Kan^r-*Not*I fragment from pBEST501 (Itaya et al., 1989), to
96 generate pAX02. The expression cassette *Sac*I-P_{43}-*spamy*L-*hIFNγ-Bam*HI from pRep-gam
97 was cloned in the corresponding sites of pAX02. This procedure eliminated the *xylR* gene
98 and the resulting plasmid was named pInt-gam (Fig. 2). All constructions were verified by
99 restriction analysis and sequencing in Molecular Cloning Laboratories (MCLAB, San
100 Francisco, CA). The main characteristics of the plasmids are shown in Table 1.
101
102 *2.4 Expression of rhIFN-γ*
103
104 *B. subtilis* WB600 strains transformed with pSpac-gam, pRep-gam or pInt-gam were
105 propagated in 50 ml of 2xL-Mal medium supplemented with Kan (30 μg ml^{-1}) and
106 incubated at 37°C for 42 h. Culture samples were collected at different times and
107 centrifuged at 8000 x *g* for 10 min to separate the cells and the culture medium. The

108 proteins secreted into the culture medium were precipitated with five volumes of methanol;
109 the protein pellets were resuspended in 200 μl of PBS (140 mM NaCl, 7.2 mM Na_2HPO_4,
110 2.8 mM $NaH_2PO_4.H_2O$ at pH 7.5), 15 μl aliquots from this volume were analyzed by 4-
111 20% gradient SDS-polyacrylamide gel electrophoresis. The cell pellet was washed,
112 resuspended in PBS buffer and sonicated in an Ultrasonic processor GE 505 (Sonics,
113 Newton, CT) using 10 pulses of 10 s at 25% amplitude and 10 s resting among the pulses.
114 The cell homogenate was centrifuged at 1500 x *g* for 10 min and the recovered supernatant
115 was defined as the intracellular fraction; 15 μl from this fraction were analyzed by 4-20%
116 gradient SDS-PAGE. Independent experiments were done by triplicate and the average
117 values were given in the manuscript.
118
119 *2.5 Western blot and ELISA analysis*
120
121 Protein fractions were separated by 4-20% gradient SDS-PAGE and electroblotted onto
122 nitrocellulose membrane (Amersham Biosciencies, Piscataway, NJ). Recombinant hIFN-γ
123 was detected by immunoblotting and commercial rhIFN-γ (PreproTech, Rocky Hill, NJ)
124 was used as standard. The polyclonal rabbit anti-hIFN-γ (PreproTech) was used as the
125 primary antibody to final concentration of 0.15 μg ml^{-1}. The goat anti-rabbit IgG coupled to
126 alkaline phosphatase (Invitrogen) diluted to 1:3000 was used as secondary antibody. The
127 blot was developed with *p*-nitro blue tetrazolium and sodium-5-bromo-4-chloro-3-indolyl-
128 phosphate (NBT/BCIP, Amersham Biosciences; Piscataway, NJ). Analysis of immunoblots
129 was carried out using a photo-documenter Gel-Doc 2000 (BioRad Laboratories, Segrate,
130 Milan) and the Quantity One™ v4.5 software (BioRad). Quantification of soluble rhIFN-γ

131 was assayed by ELISA kit (R&D Systems, Minneapolis, MN) following the instructions of
132 the manufacturer.
133
134 **3. Results and discussion**
135
136 *3.1 Construction of the expression vectors*
137
138 To assess the production of the rhIFN-γ by replicative and integrative expression systems in
139 *B. subtilis*, we made the respective constructions using an optimized synthetic *hIFN-γ* gene
140 and the P_{spaC} or P_{43} promoters. Codon bias is one of the most important factors that affects
141 the expression of eukaryotic genes in prokaryotic systems (Gustafsson et al., 2004). To
142 avoid problems in translational efficiency we decided to use a synthetic *hIFN-γ* gene since
143 the wild-type *hIFN-γ* gene has 16 codons reported as "rares". We modified the sequence
144 based in accordance with the codon preference in *B. subtilis* (Table 2). The resultant
145 optimized gene had 75.69% of identity with respect to the wild-type gene (Fig. 1). The
146 synthetic *hIFN-γ* gene fused to the secretion signal peptide *sp-amyL* was cloned in the
147 *Hin*dIII-*Bam*HI sites of pDG148. This procedure eliminated the *lacI* repressor gene in the
148 vector, generating a plasmid to drive the constitutive expression of the rhIFN-γ by the P_{spaC}
149 promoter. The P_{spaC} promoter is a modified version of the *E. coli* P_{lac} promoter that
150 functions in *B. subtilis* (Yansura and Henner, 1984; Nguyen et al., 2005). Additionally, the
151 *Not*I, *Sac*II and *Xma*III restriction sites were added as a multicloning site downstream of *sp-*
152 *amyL*. The P_{43} is a highly efficient promoter that comprises two overlapping promoters
153 recognized by the *B. subtilis* σ^A and σ^B containing RNA polymerase holoenzymes. There

154 are few reports on expression systems in *B. subtilis* using integrative plasmids (Jan et al.,
155 2001; Wang et al., 2004), and to our knowledge no reports exist on the use of integrative
156 systems for the production of eukaryotic proteins. We built an integrative vector to test the
157 production of the rhIFN-γ based on the P_{43} strong promoter. The pInt-gam plasmid also
158 contains the kanamycin antibiotic resistance marker, the target gene open reading frame,
159 and a multicloning site between the two ends of the *B. subtilis lacA* gene. Following
160 transformation of *B. subtilis*, the integration of this construct occurs by double homologous
161 recombination event in the *lacA* locus. A schematic representation of the integrative and
162 replicative plasmids is shown in Fig. 2.

163

164 *3.2 Production of hIFN-γ using the replicative and integrative plasmids in B. subtilis*

165

166 Typical batch cultures of the *B. subtilis* WB600 transformed with pSpac-gam, pRep-gam or
167 pInt-gam are shown in Fig. 3. A similar specific growth rate of 0.18 h^{-1} was observed in the
168 different strains used in this study. For the culture with WB600/pInt-gam strain, the
169 biomass increased up to a maximum of 2.1±0.14 g_{DCW} l^{-1} at 24 h of culture, and thereafter it
170 remained constant (Fig. 3A). The WB600/pSpac-gam and WB600/pRep-gam strains
171 reached 2.29±0.15 and 2.53±0.21 g_{DCW} l^{-1} as maximum, respectively. The total and secreted
172 rhIFN-γ concentrations are shown in the Fig. 3B and 3C, respectively. The maximum
173 rhIFN-γ production was attained with the WB600/pRep-gam strain, which generated
174 20.3±0.8 mg l^{-1}, whereas the WB600/pSpac-gam and WB600/pInt-gam strains only
175 produced 2.1±0.3 and 2.5±0.2 mg l^{-1} respectively; these maximum values were observed in
176 the 30 h of cultures, and thereafter they remained constant. Since the replicative plasmid

177 driven by the P_{spaC} promoter showed low yield of hIFN-γ, we decided do not make an
178 integrative construction with this promoter. The Western blot analysis of cell pellet showed
179 a main band of 19 kDa, which corresponded to the non-processed form of the rhIFN-γ
180 suggesting that the secretion machinery was rapidly saturated (Fig. 4). A band
181 corresponding to the expected molecular mass of the processed form was observed in all
182 cases. WB600/pRep-gam and WB600/pInt-gam strains produced a second degradation
183 product resulting of subsequent proteolytic degradation (Fig. 4).
184
185 The maximum secreted rhIFN-γ concentration attained by the WB600/pSpac-gam,
186 WB600/pRep-gam and WB600/pInt-gam strains was 76±24, 200±39 and 90±15 μg l^{-1},
187 respectively (Fig. 3C). For the WB600/pRep-gam strain the maximum secreted rhIFN-γ
188 concentration was reached at 30 h of the culture; afterwards the concentration of the rhIFN-
189 γ diminished. A similar behavior was observed in the other cultures, although in these
190 cases, the maximum concentrations of secreted rhIFN-γ were detected during 24 h of
191 culture. These results suggest that after 24-36 h, the rhIFN-γ is subjected to proteolysis, and
192 it is supported by the Western blotting analysis shown in Fig. 5. We observed a unique 16.7
193 kDa band the 30 h of culture corresponding to the expected weight of the processed protein.
194 The presence of this band, strongly suggest that the signal sequence *amyL*-SAAA of *B.*
195 *licheniformis* attached to rhIFN-γ was recognized and processed by the signal peptidases of
196 *B. subtilis*. As shown in Figs. 3 and 5 the degradation of secreted rhIFN-γ coincided with
197 the beginning of the stationary growth phase. It is known that at the end of the exponential
198 growth phase, when nutrients become limiting, *B. subtilis* produces proteases, lipases and
199 others degradative enzymes (Hamoen et al., 2003). Although *B. subtilis* WB600 is a

200 deficient strain in six extracellular proteases, it retains 0.5% of extracellular protease
201 activity. This strain has been efficiently used for the production of β-lactamase,
202 streptokinase, and the antidigoxin single-chain antibody fragment (Westers et al., 2004),
203 however our results suggest that the rhIFN-γ is sensitive to intra and extracellular proteases.
204 The use of a *B. subtilis* WB800 strain, which in addition to the six extracellular proteases
205 absent in the strain WB600, also lacks the Vpr and the WprA proteases resulted in a
206 significant increase of the hIL-3 secretion, compared to concentration obtained with the
207 WB600 strain (Westers et al., 2006). Therefore, the use of WB800 strain as expression host
208 could increase the production of rhIFN-γ using the expression vectors described here.
209
210 The total amount of the rhIFN-γ produced here is comparable to the values of other
211 cytokines produced in *B. subtilis* (Table 3), and it could be increased through the
212 optimization of the operational conditions in bioreactors (Balderas Hernández et al.,
213 2008a). The hIFN-γ is distinguished from other types of interferons on its instability by
214 changes in the pH and proteases (Hsu and Arakawa 1985). Until now, the maximum
215 amount of recombinant cytokines produced by *B. subtilis* is 100 mg l^{-1} of hIL-3 using an
216 expression system with the HpaII and P_{43} promoters in tandem and an eight deficient
217 proteases strain. It has been reported that the processing efficiency and secretion depend on
218 aminoacid sequence and intrinsic characteristics of each protein (Koshland and Botstein,
219 1980). To our knowledge, this is the first report showing the production of the rhIFN-γ
220 using a synthetic gene and integrative or replicative plasmids in *B. subtilis*. Although the
221 production of the rhIFN-γ in *B. subtilis* using the integrative plasmid was 10 times less than
222 that obtained with the use of the replicative plasmid, it should be noted that the integrative

223 construction has solely one copy of the *hIFN-γ* gene in the *lacA* locus from *B. subtilis*
224 chromosome, whereas the copy number of the replicative plasmid can vary from about 50
225 to 100 copies per cell (Wu and Wong, 1999). Thereby, the use of distinct *B. subtilis loci* to
226 increase the copy number of the target gene could improve expression systems based on
227 integrative plasmids. The use of integrative plasmids could also avoid the segregational
228 instability of the replicative plasmids, which is one of the main problems using *B. subtilis*
229 as expression system (Fleming and Patching, 1994). *B. subtilis* secreting rhIFN-γ
230 constructed here could be used as an expression system for the production and purification
231 of the rhIFN-γ in bioreactors. But the strains also could be used directly in humans as a
232 functionalized probiotic as occurred for a *B. subtilis* strain secreting rhIFN-α2, which is
233 already commercially available under the name of Subalin (Beliavskaia et al., 2003).
234 However, further work and clinical trials showing the effectivity and safety must be
235 performed.
236
237 **Acknowledgments**
238
239 This work was partially supported by CONACyT Grant 82010. Juan A. Rojas Contreras
240 acknowledges the PhD fellowship 204213 from CONACyT. We thank Sydney Robertson
241 for English correction.
242
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345 operator to control gene expression in *Bacillus subtilis*. Proc. Natl. Acad. Sci. USA
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348 Table 1. Plasmids and strains used in this work.

Strains and plasmids	Genotype/relevant properties	Source/reference
Strains		
E. coli DH5α	F^{-} ϕ80d*lac*Z*Δ*M15 *end*A1 *rec*A1 *gyr*A96 *thi*-1 *hsd*R17(rK^{-} mK^{-}) *sup*E44 *rel*A1 *deo*R Δ(*lac*ZYA-argF) U169	Invitrogen Life Technologies (USA)
B. subtilis		
WB600	*trpC2 nprE aprE epr bpr mpr nprB*; Em^{r}	(Wu et al. 1991)
Plasmids		
pCR2.1	pUC derivative, replicating in *E. coli*, f1 ori, P_{lac}, Ap^{r}, Kan^{r}	Invitrogen
pBAL	pET12a derivative, containing the synthetic hIFN-γ gene cloned in the *Nde*I-*Bam*HI restriction sites; Ap^{r}	(Balderas Hernández et al. 2008a)
pAX01	Expression vector capable of integrating into the *B. subtilis* chromosome at the *lacA* locus; Em^{r}	(Hartl et al. 2001)
pAX01-B.L.*amy*L	pAX01 derivative, containing the *Bacillus licheniformis* α-amylase gene cloned in the *Bam*HI restriction site; Em^{r}	Provided by Prof. C. Harwood, Newcastle University, UK
pAX02	pAX01 derivative, the fragment *Not*I-*Not*I containing the Em^{r} gene was replaced by the Kan^{r} gene of pBEST501; Kan^{r}	This study
pBEST501	pGEM4 containing the Kan^{r} gene from pUB110	(Itaya et al. 1989)
pDG148	Shuttle vector, replicating in *E. coli* and *B. subtilis*, ColE1, *repB*, Ap^{r}, Kan^{r}, P_{spaC} promoter regulated by *lac*I repressor, IPTG inducible; Kan^{r}	(Stragier et al. 1988)
pSpac-gam	pDG148 derivative, containing the *spamyL-hIFN*γ cassette downstream of the P_{spaC} promoter, the fragment *Hind*III-*Bam*HI containing the *lacI* repressor was deleted; Kan^{r}	This study
pRep-gam	pSpac derivative, containing the P_{43} promoter upstream of the *spamyL-hIFN*γ cassette; Kan^{r}	This study
pInt-gam	pAX02 derivative, carrying a fragment *Sac*I-*Bam*HI of pDG43G containing the P_{43}-*spamyL-hIFN*γ cassette; Kan^{r}	This study

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350 Note: Kan^r, kanamycin resistance; Em^r, erythromycin resistance; Ap^r, ampicillin resistance.

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Table 2. Summary of codon preference in *Bacillus subtilis* and codon usage in the wild-type and the optimized synthetic *hIFN-γ* gene.

Aminoacid	**Wild-type *hIFN-γ* gene**	**Synthetic *hIFN-γ* gene**	**Codon Usage in *B. subtilis* (%)**
Arg	CGC, CGA, AGA, AGG	CGT	19
Leu	TTA, TTG, CTT, CTC, CTG	TTA, TTG, CTT, CTG	23, 15, 24, 23
Gly	GGT, GGC, GGA, GGG	GGT	21
Pro	CCA	CCA	21
Thr	ACT, ACC, ACA	ACT, ACC, ACA	18, 14, 45
Phe	TTT, TTC	TTT, TTC	67, 33
Ile	ATT, ATC, ATA	ATT, ATC	50, 34
Val	GTC, GTA, GTG,	GTT, GTC, GTA, GTG	30, 23, 23, 24
Ser	TCC, TCA, TCG, AGT, AGC	TCT, TCC, TCA	24, 13, 20
Ala	GCT, GCA, GCG	GCT, GCC, GCA	26, 18, 32
Tyr	TAT, TAC	TAT, TAC	67, 33
His	CAT	CAT, CAC	68, 32
Gln	CAA, CAG	CAA, CAG	55, 45
Asn	AAT, AAC	AAT, AAC	53, 47
Lys	AAA, AAG	AAA	73
Asp	GAT, GAC	GAT, GAC	64, 36
Glu	GAA, GAG	GAA, GAG	70, 30
Stop	TAA	TAA	66

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368 Table 3. Some heterologous proteins produced in *B. subtilis.*

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Protein	Production (mg l^{-1})	Strain/plasmid	Reference
CII	6.4	WB600/pUBEX-CII	Min-Yong et al. 2002
mIFN-β	2.0 x10^{-5*}	207-25/pTUB502	Shiroza et al. 1985
hEGF	7.0	DB104/pM2Veg	Lam et al. 1998
hIFN-α 2	0.5-1	IH6140/pKTH93	Palva et al. 1983
hIL-3	100	WB800/pP43LatIL3	Westers et al. 2006
hIL-3	0.1	DB104/pLatIL3	Westers et al. 2006
hIFN-γ	20.3	WB600/pRep-gam	This work
hIFN-γ	2.5	WB600/pInt-gam	This work

370 *Equivalent to 4000 U of activity.

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397 **Figure caption**

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399 **Fig. 1.** Alignment of nucleotide sequence of the wild-type and the optimized synthetic

400 *hINF-γ* gene used in this work.

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402 **Fig. 2** Schematic representation of the pSpac-gam, pRep-gam and pInt-gam plasmids, the

403 relevant molecular elements are shown.

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405 **Fig. 3** Growth kinetics and rhIFN-γ production by the distinct *B. subtilis* WB600 strains

406 cultured in 2xL-Mal. A) Biomass concentration, WB600/pSpac-gam (□), WB600/pRep-

407 gam (△) and WB600/pInt-gam (○); B) Total rhIFN-γ concentration; C) Secreted rhIFN-γ

408 concentration. Vertical bars indicate the standard deviation of three independent

409 experiments.

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411 **Fig. 4** Immunoblot detection of the rhIFN-γ in cell-pellet homogenate of the distinct strains

412 of *B. subtilis* WB600 at the time of maximum production. Lanes 1-3: WB600/pSpac-gam,

413 WB600/pRep-gam and WB600/pInt-gam. Lane 4: hIFN-γ standard (PeproTech).

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415 **Fig. 5** Immunoblot detection of the rhIFN-γ in the culture medium of the *B. subtilis*

416 WB600/pRep-gam. Lane 1: 20 ng of the rhIFN-γ standard, Lanes 2-9 show samples taken

417 at different culture times.