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Cpe1786/IscR of Clostridium perfringens represses expression of genes involved in Fe-S cluster biogenesis.

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Gaëlle André, Elise Haudecoeur, Emmanuelle Courtois, Marc Monot, Bruno Dupuy, et al.. Cpe1786/IscR of Clostridium perfringens represses expression of genes involved in Fe-S cluster biogenesis.. Research in Microbiology, 2016, 10.1016/j.resmic.2016.03.002 . pasteur-01370878

HAL Id: pasteur-01370878

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Submitted on 23 Sep 2016

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Manuscript Number:

Title: Cpe1786/IscR of *Clostridium perfringens*, represses expression of genes involved in Fe-S cluster biogenesis

Article Type: Special Issue Paper

Keywords: Rrf2 repressor; cysteine-dependent regulation; FeS clusters; clostridia; fermentation

Corresponding Author: Prof. Isabelle Martin-Verstraete, PhD

Corresponding Author's Institution: Institut Pasteur

First Author: Isabelle Martin-Verstraete, PhD

Order of Authors: Isabelle Martin-Verstraete, PhD; Gaelle André, PhD; Elise Haudecoeur, PhD; Emmanuelle Courtois, bachelor; Marc Monot, PhD; Bruno Dupuy, PhD; Dmitry Rodionov, PhD

Abstract: Cpe1786 of *Clostridium perfringens* is an Rrf2-type regulator containing the three-cysteine residues coordinating a Fe-S in IscR, the repressor controlling Fe-S homeostasis in enterobacteria. The cpe1786 gene formed an operon with iscSU involved in Fe-S biogenesis and tmrU. This operon was transcribed from a σ A-dependent promoter. We showed that in the heterologous host *B. subtilis*, Cpe1786 renamed IscRCp negatively controlled its own transcription. We constructed an iscR mutant in *C. perfringens*. We then compared the expression profile of the strain 13 and of the iscR mutant. IscRCp controlled the expression of genes involved in Fe-S biogenesis, in amino-acid or sugar metabolisms, in fermentation pathways and in host compounds utilization. We then demonstrated using a ChIP-PCR experiment that IscRCp interacted with its promoter region in vivo in *C. perfringens* and with the promoter of cpe2093 encoding an amino-acid ABC transporter. We utilized a comparative genomic approach to infer a candidate IscR-binding motif in clostridia and reconstruct IscR regulons in clostridia. We showed that point mutations in the conserved motif of 29 bp identified upstream of iscR decreased the cysteine-dependent repression of iscR mediated by IscRCp.

Dear Editor,

Please find enclosed a copy of an article entitled Cpe1786/IscR of *Clostridium perfringens*, represses expression of genes involved in Fe-S cluster biogenesis. This paper described the characterization of IscR in the spore former pathogen, *Clostridium perfringens*. We combined studies in the heterologous host *Bacillus subtilis*, the inactivation of *iscR* in *C. perfringens*, a comparative transcriptome analysis of the wild-type strain and of the *iscR* mutant and a ChIP-PCR experiment to study the role of IscR in *C. perfringens*. This is to our knowledge the first physiological characterization of an IscR regulator in Gram-positive bacteria.

This paper is a contribution to the Special Issue of Research in Microbiology on "Beneficial and detrimental spore-formers" edited by Véronique Broussole, Christina Nielsen-Leroux, Vincent Sanchis, Frédéric Carlin and Didier Lereclus.

We hope that this paper will be of sufficient importance and quality to be considered suitable for publication in Research in Microbiology.

Yours sincerely

Isabelle Martin-Verstraete

1 **Cpe1786/IscR of *Clostridium perfringens*, represses expression of genes**
2 **involved in Fe-S cluster biogenesis**

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5 Gaëlle André^{1,2}, Elise Haudecoeur^{1,2}, Emmanuelle Courtois^{1,2}, Marc Monot¹, Bruno Dupuy¹,
6 Dmitry A Rodionov³ and Isabelle Martin-Verstraete^{1,2*}

7
8 1. Institut Pasteur, Laboratoire de Pathogénèse des Bactéries Anaérobies, 28 rue du Docteur
9 Roux, 75015 Paris, France.

10 2. Univ Paris 7-Denis Diderot, Sorbonne Paris Cité, 75205 Paris, France.

11 3. Institute for Information Transmission Problems, Russian Academy of Sciences, Moscow
12 127994, Russia.

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14
15
16 Key words: Rrf2 repressor, cysteine-dependent regulation, Fe-S clusters, clostridia, fermentation

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19 * corresponding author. mail: isabelle.martin-verstraete@pasteur.fr. Tel: 33 (1) 40 61 35 61
20

21 **Abstract**

22 Cpe1786 of *Clostridium perfringens* is an Rrf2-type regulator containing the three-cysteine
23 residues coordinating a Fe-S in IscR, the repressor controlling Fe-S homeostasis in
24 enterobacteria. The *cpe1786* gene formed an operon with *iscSU* involved in Fe-S biogenesis and
25 *tmrU*. This operon was transcribed from a σ^A -dependent promoter. We showed that in the
26 heterologous host *B. subtilis*, Cpe1786 renamed IscR_{Cp} negatively controlled its own
27 transcription. We constructed an *iscR* mutant in *C. perfringens*. We then compared the
28 expression profile of the strain 13 and of the *iscR* mutant. IscR_{Cp} controlled the expression of
29 genes involved in Fe-S biogenesis, in amino-acid or sugar metabolisms, in fermentation
30 pathways and in host compound utilization. We then demonstrated using a ChIP-PCR
31 experiment that IscR_{Cp} interacted with its promoter region *in vivo* in *C. perfringens* and with the
32 promoter of *cpe2093* encoding an amino-acid ABC transporter. We utilized a comparative
33 genomic approach to infer a candidate IscR-binding motif and reconstruct IscR regulons in
34 clostridia. We showed that point mutations in the conserved motif of 29 bp identified upstream
35 of *iscR* decreased the cysteine-dependent repression of *iscR* mediated by IscR_{Cp}.

36

37 **Introduction**

38

39 *Clostridium perfringens* is a Gram-positive, spore forming anaerobic bacterium. Toxinotype A
40 strains are the causative agent of food poisoning and gas gangrene. Numerous toxins and
41 degradative enzymes secreted by this bacterium contribute to the pathogenicity of fatal infection
42 like gas gangrene [30, 34]. The synergistic actions of these toxins and enzymes on the host tissue
43 are needed for the infection and a coordinated regulation of such virulence factors is observed. In
44 *C. perfringens* strain 13, the VirS/VirR two-component system is involved in the global
45 regulation of production of several toxins (α -, θ -, κ -toxin) and of other virulence factors
46 (capsular polysaccharide, sialidase, hyaluronidases or other enzymes able to degrade host
47 compounds) [24, 35]. Genes involved in carbon, energy, base and amino acid metabolisms are
48 also controlled by VirS/VirR [24, 35]. The response regulator VirR directly regulates the
49 expression of *pfoA* encoding the θ -toxin and of three non-coding RNAs, the VR-RNA, VirU and
50 VirT, which in turns control the expression of *plc* and *colA* encoding the α - and κ -toxin,
51 respectively [26, 36].

52 Less is known about the physiology of *C. perfringens*. The *ubiG* operon involved in methionine
53 to cysteine conversion and in AI-2 production is controlled by VirS/VirR [25]. Several genes
54 involved in sulfur metabolism are regulated in response to cysteine availability in *C. perfringens*

55 strain 13 [1]. These genes are controlled by premature termination of transcription [10] through a
56 cysteine specific T-box or a S-box dependent riboswitch. The *ubiG* operon is submitted to a
57 complex regulation [1]: i) an induction during cysteine starvation via a cysteine specific T-box
58 present upstream of *ubiG* that senses the level of charge of tRNA_{cys}; ii) a control by VirR through
59 the VR-RNA and iii) a regulation by VirX, a regulatory RNA, which controls toxin production
60 independently from VirR.

61 The expression of genes involved in Fe-S cluster biogenesis, in the maintenance of the cell redox
62 and in the fermentation pathways is also induced during cysteine limitation via mechanisms
63 different from the T-box and S-box systems [1]. Among the genes induced during cysteine
64 limitation, there is *cpe1786* encoding a regulator, which is a good candidate to participate in
65 cysteine-dependent regulation in *C. perfringens*. Cpe1786 belongs to the widespread Rrf2 family
66 of transcription factors. Notably, this family of regulators comprises: i) the Fe-S cluster
67 biogenesis regulator, IscR [32]; ii) the NsrR repressor controlling the expression of NO
68 detoxification systems [40]; iii) the global iron-responsive regulator RirA in *Rhizobiaceae* [15];
69 and iv) the global repressor of cysteine metabolism, CymR [7, 37]. Several mechanisms for the
70 modulation of Rrf2-type regulator activity in response to environmental signals are involved.
71 IscR, NsrR and RirA coordinate a Fe-S cluster via three conserved cysteines [8, 32, 40]. The Fe-
72 S cluster in IscR and NsrR contributes in sensing the pool of Fe-S or the presence of NO. These
73 metal centers are sensitive to oxidation by NO or peroxide but also to iron availability [32].
74 Interestingly, the cysteine residues conserved other Rrf2-type regulators are absent in CymR,
75 which displays a different mechanism of control of its activity with the formation of a complex
76 with CysK, an *O*-acetyl-serine-thiol-lyase [39]. CysK via its substrate *O*-acetyl-serine, is the
77 sensor of the cysteine pool and transmits this information to CymR.

78 In this work, we analyzed the regulatory role of the unique Rrf2-type regulator of *C. perfringens*,
79 Cpe1786.

Materials and Methods

80

81 **Bacterial strains and culture conditions:**

82 *Escherichia coli* strains were grown in LB medium and *B. subtilis* strains in SP or a sulfur-free
83 minimal medium [7] containing either 1 mM methionine or 1 mM cystine. For the experiments
84 involving expression of genes under the control of the *xylA* promoter, threonine (50 mg L⁻¹) and
85 0.1% xylose were added. When needed, ampicillin (100 µg/ml) or chloramphenicol (15 µg/ml)
86 was added to *E. coli* cultures while chloramphenicol (5 µg/ml) or spectinomycin (60 µg/ml) was
87 added for *B. subtilis* cultures. *C. perfringens* strains were grown under anaerobic conditions
88 (10% H₂, 10% CO₂, 90% N₂) in TY (30 g L⁻¹ bacto tryptone, 2g L⁻¹ yeast extract, 1 g L⁻¹
89 thioglycolate, pH7.4), BHI (37 g L⁻¹) or a sulfur-free minimal medium [1] containing either 1
90 mM cystine or 1 mM homocysteine. When necessary, thiamphenicol (5 µg/ml) or erythromycin
91 (10 µg/ml) was added to *C. perfringens* cultures.

92 **Strain and plasmid constructions**

93 The *cpe1786* promoter regions (-131,+ 61 or -41,+61 from the transcriptional start site) were
94 amplified by PCR with the creation of EcoRI and BamHI sites. These DNA fragments were
95 inserted between the EcoRI and BamHI sites of pAC6 [38] resulting in plasmids pDIA5820 and
96 pDIA5837, respectively. These plasmids linearized by ScaI were used to transform the *B. subtilis*
97 BSIP1798 or BSIP 2018 strains (Table 1).

98 Plasmid pDIA5820 was used to perform site directed mutagenesis with the Quikchange site-
99 directed mutagenesis Kit (Stratagene). Synthetic oligonucleotides complementary to opposite
100 strands and containing different mutations (a T→G or G→A at position -22 or -19, respectively)
101 were used to amplify pDIA5820 (Table S1). The presence of the mutation was verified by
102 sequencing. A plasmid containing the double mutation (T-22G and G-19A) was obtained by site
103 directed mutagenesis using pDIA5840 (P(T-22G)*cpe1786-lacZ*) as template.

104 The complete coding sequence of *cpe1786* (+3 to +503 from the transcriptional start site) was
105 amplified by PCR. This fragment was inserted into pXT [21]. The resulting plasmid, pDIA5744,
106 was introduced in strain BSIP1978 ($\Delta cymR$ P(-131 +61)*cpe1786_{CP}-lacZ*) (Table 1).

107 A DNA fragment corresponding to the *cpe1786* gene (-131 to +497 from the transcriptional start
108 site) fused to a sequence encoding the X-flag motif was amplified by PCR. This PCR product
109 was inserted by TA cloning into the pGEM-Teasy vector (Promega, Madison, USA) to give
110 pDIA5925. A 2.4 kb fragment corresponding to the pCB102 replicon and the *catP* marker of
111 pMTL83151 (PmeI-AscI fragment treated by Klenow) [13] was cloned into the NaeI site of
112 pDIA5925 giving pDIA5928.

113 The ClosTron gene knockout system [12] was used to inactivate *cpe1786*. Primers IBS, EBS1d,
114 EBS2 to retarget the group II intron on pMTL007 to this gene (Table S1) were designed by the
115 Targetron design software (<http://www.sigmaaldrich.com>). We generated by overlap extension
116 PCR a 353 bp product that would facilitate intron retargeting to *cpe1786*. The PCR product was
117 cloned into pMTL007. The plasmid pMTL007-*cpe1786*-28a obtained was then introduced in *C.*
118 *perfringens* strain 13 by electroporation. Transformants were selected on BHI agar containing
119 thiamphenicol and then plated on BHI agar containing erythromycin. Chromosomal DNA of
120 clones resistant to erythromycin and sensitive to thiamphenicol was extracted using the kit
121 QIAamp DNA Mini Kit (Qiagen). PCR using the ErmRAM primers confirmed that ErmR
122 phenotype was due to the splicing of the group I intron from the group II intron following
123 integration. PCRs with primers IMV484 and IMV485 flanking the *cpe1786* gene or with
124 IMV484 located in the *cpe1786* gene (Table S1, Fig. S1) and EBSu were then performed to
125 verify the integration of the LI.LtrB intron in *cpe1786*.

126 **Enzyme assays and volatile fatty acid analysis**

127 β -Galactosidase specific activity was measured as previously described [7]. Concentration of
128 proteins was determined by the method of Bradford. One unit of β -galactosidase is defined as the
129 amount of enzyme that produces 1 nmol min⁻¹ of *O*-nitrophenol (ONP) at 28°C. The mean value
130 of at least three independent experiments was presented.

131 The end products of fermentation were detected in supernatants of strain 13 and the *cpe1786*
132 mutant after growth in minimal medium with 1 mM cystine for 48h at 37°C by gas-liquid
133 chromatography as previously described [2]. The amount of fatty acids was calculated by
134 comparison with an internal standard [6].

135 **RNA isolation, quantitative RT-PCR and 5'RACE analysis**

136 Total RNA from strain 13 and its derivative *cpe1786* mutant grown in minimal medium with
137 cystine or homocysteine was extracted as previously described [1]. cDNAs were synthesized
138 with Superscript II Reverse Transcriptase (Invitrogen) using 1 μ g of RNA and 1pmol of hexamer
139 oligonucleotide primers (pDN6, Roche). Real-time quantitative PCR was performed with gene-
140 specific primers (Table S1) as previously described [1]. In each sample, the quantity of cDNAs
141 of a gene was normalized to the quantity of cDNAs of the *gyrA* gene. The relative change in
142 gene expression was recorded as the ratio of normalized target concentrations ($\Delta\Delta$ Ct) [16].

143 5'RACE assays were performed on total RNA extracted from strain 13 grown in minimal
144 medium in the presence of homocysteine using a 5'RACE System kit (Invitrogen). After reverse
145 transcription, the cDNAs were treated with Terminal deoxynucleotidyl transferase to add a

146 polyC tail. PCR was then performed using a primer hybridizing with the polyC tail and gene-
147 specific primers (Table S1). PCR amplification products were sequenced.

148 **DNA-array hybridization and microarray analysis.**

149 The microarray analysis was performed using previously described Agilent microarrays (GEO
150 Database, GPL 9765) [1]. RNA was labeled with either Cy3 or Cy5 fluorescent dye (GE
151 healthcare) using the SuperScript Indirect cDNA labelling system kit (Invitrogen) according to
152 the manufacturer's recommendations. The cDNA synthesis, the hybridization, the washing and
153 the scanning were performed as previously described [1]. All the slides were analyzed using R
154 and limma software (Linear Model for Microarray Data) from Bioconductor project
155 (www.bioconductor.org) and the normalization was performed as previously described [1]. The
156 complete experience dataset was deposited in the GEO database with accession numbers
157 GSE19359.

158 **Chromatine immunoprecipitation of Cpe1786-XFlag bound to DNA**

159 Strains CPIP01 (*cpe1786*) and CPIP11 (*cpe1786* pDIA5927-*cpe1786*-XFlag) grown in minimal
160 medium containing 1 mM cystine were incubated with 1 % formaldehyde 10 minutes at 37°C.
161 Cross-linking was quenched by addition of glycine (125 mM). Cells were then collected by
162 centrifugation, washed twice with 20 mM Tris-HCl pH7.5, 150 mM NaCl and stored at -80°C.
163 Pellets were resuspended in 10 ml of buffer A (50 mM Tris-HCl pH7.5, 150 mM NaCl, 1 mM
164 EDTA, 1% Triton X-100, 0.1% SDS) and sonicated in a water bath sonicator (Bioruptor,
165 Diagenode) to shear DNA to an average size of 300-500 bp. After removal of cells debris, the
166 supernatant was incubated in the presence of magnetic beads (Sigma) on a rotating wheel. The
167 magnetic beads were washed twice with buffer A, once with buffer A plus 500 mM NaCl, once
168 with buffer B (10 mM Tris-HCl pH 7.5, 1 mM EDTA, 0.1 % Nonidet-P40) and once with 10
169 mM Tris-HCl pH 7.5, 1 mM EDTA. Proteins were then eluted for 24h at 37°C with 100 µl of
170 elution buffer (50 mM Tris-HCl pH 7.5, 10 mM EDTA, 0.5% SDS, 150 mM NaCl, Triton X-100
171 0.5%). Samples were treated 1h at 56°C in 0.5X elution buffer containing 50 µg of Proteinase K.
172 DNA was purified using the QIAquick PCR purification Kit (QIAGEN). Chromatine
173 immunoprecipitation (ChIP) was analyzed by q-PCRs using primer pairs flanking the *cpe1786*,
174 *cpe0664*, *cpe1031*, *cpe1371*, *cpe2093* and *ldh* promoter regions (Table S1). The reaction mixture
175 contained 1µl of DNA obtained by ChIP from strains CPIP11 or CPIP01, 400 nM primers and
176 10 µl of SYBR Green Mix (Roche) in a total volume of 20 µl. We used *gyrA* a gene not
177 regulated by Cpe1786 in transcriptome as a control. The ratio of enrichment by ChIP for a target
178 was calculated as follows: $2^{(Ct_{\text{target}}-Ct_{\text{gyrA}})_{\text{CPIP11}} / (Ct_{\text{target}}-Ct_{\text{gyrA}})_{\text{CPIP01}}}$. The means of two

179 independent experiments was obtained.

180

181

Results

182 The Cpe1786 regulator of *C. perfringens*

183 Cpe1786 of *C. perfringens*, a Rrf2-type regulator, shares 50 % identity with the cysteine
 184 metabolism repressor CymR from *B. subtilis* [7] and 37 % and 49 % identity with the Fe-S
 185 cluster biogenesis regulator IscR from *E. coli* [33] and *Thermincola potens* [31], respectively. An
 186 alignment of CymR-type regulators, IscR-type regulators and Cpe1786 (Fig. S2) indicates that
 187 the three-cysteine residues coordinating the Fe-S cluster in IscR are conserved in Cpe1786 [8].
 188 Three genes are located downstream of *cpe1786*. The *cpe1785/iscS* and *cpe1784/iscU* genes
 189 encode a cysteine desulfurase and a scaffold protein for Fe-S assembly, respectively [3] while
 190 *trmU* (*cpe1783*) encodes an enzyme involved in thio-uridylation of tRNAs (Fig. S3A). The
 191 expression of these genes increased during cysteine starvation [1] suggesting that they can form
 192 an operon. RT-PCR experiments using primers hybridizing with *cpe1786* and *iscS*, *iscS* and *iscU*
 193 or *iscU* and *trmU* were performed and confirmed that these genes were cotranscribed (Fig. S1B).
 194 So, *cpe1786* of *C. perfringens* forms an operon with an *iscS*-type gene and with *trmU* as found in
 195 *B. subtilis* [7] and with *iscS* and *iscU* as observed in *E. coli* and in *T. potens* [31, 32] (Fig. S3A).
 196 We then characterized the *cpe1786* promoter by determining its transcriptional start site by
 197 5'RACE analysis. Transcription is initiated 39 bp upstream of the translational start site. -10
 198 (TATAAT) and -35 (TTGACA) boxes that perfectly match with the consensus for σ^A -dependent
 199 promoters are found upstream of this transcriptional start site (Fig. 1).

200

201 Cpe1786 controls *cpe1786* transcription in the heterologous host *B. subtilis*

202 To determine whether Cpe1786 controls its own transcription, we constructed a transcriptional
 203 fusion between the *cpe1786* promoter region (-131,+61) and *lacZ* and tested the effect of the
 204 addition of Cpe1786 on the expression of this fusion in the heterologous host *B. subtilis*. The
 205 fusion was integrated at the *amyE* locus of a *B. subtilis* $\Delta cymR$ mutant or a $\Delta cymR$ mutant
 206 containing at the *thrC* locus a copy of P_{xyIA} -*cpe1786*. The resulting strains were grown in
 207 minimal medium in the presence of cystine or methionine corresponding to conditions of
 208 cysteine limitation [39]. The P(-131,+61)*cpe1786-lacZ* fusion was constitutively expressed in a
 209 *B. subtilis* *cymR* mutant. The introduction of Cpe1786 in this mutant led to a cysteine-dependent
 210 repression of this fusion (Table 2) as observed for *cpe1786* expression in *C. perfringens* [1]. This
 211 result indicates that Cpe1786 represses its own transcription as observed for IscR in *E. coli* [33]

212 and we renamed this regulator IscR_{Cp}. The expression of a second P(-41, +61)*cpe1786-lacZ*
213 fusion was also repressed in the presence of cysteine indicating that the DNA binding motif of
214 IscR_{Cp} is located between position -41 and +61 from the transcriptional start site as classically
215 observed for repressors.

216

217 **Global regulation by the Cpe1786/IscR regulator in *C. perfringens***

218 To study the role of IscR_{Cp} in *C. perfringens*, we constructed a *cpe1786/iscR* mutant using the
219 Clostron system [12]. The group II intron was inserted into the *iscR* gene in antisense orientation
220 immediately after the 28th nucleotide in its coding sequence. To verify the insertion of the group
221 II intron into *iscR*, PCRs were carried out using a primer internal of the target gene and the
222 intron-specific primer EBSu or primers flanking the insertion site of the intron (Fig. S1C). To
223 identify genes regulated by IscR_{Cp} in *C. perfringens*, we further compared the expression profiles
224 of strain 13 and the *iscR* mutant after growth in a minimal medium in the presence of cystine.
225 250 genes were differentially expressed in these two strains. We confirmed these transcriptome
226 data by qRT-PCR analysis for several genes (Table 3). Some of the controlled genes including
227 those with expression ratio above three-fold between these two strains, those associated with
228 iron-sulfur clusters biogenesis, carbon or sulfur metabolism and the degradation of host
229 compounds are presented in Table 3. Most of these genes (62 %) were derepressed in an *iscR*
230 mutant as expected for the inactivation of a repressor. A large set of genes regulated by IscR_{Cp}
231 (50 %) was also regulated in response to cysteine availability [1].

232

233 **Regulation of genes involved in iron-sulfur clusters biogenesis**

234 The expression of genes involved in Fe-S cluster biogenesis was up-regulated in the *iscR* mutant
235 (Table 3). IscR_{Cp} regulates its own transcription in response to cysteine availability in *B. subtilis*
236 (Table 2). Our transcriptome data also showed a 2-fold increase of *iscS*, *iscU* and *trmU*
237 expression in the *iscR* mutant. This up-expression of genes located downstream from
238 *cpe1786/iscR* might be due to the presence of a promoter in the group II intron or in the *erm*
239 cassette and indicated the absence of a major polar effect of the *iscR* gene disruption on the
240 expression of the downstream genes. The expression of *cpe0664*, encoding a 114 amino-acid
241 protein belonging to the HesB family that probably corresponds to an A-type carrier required for
242 Fe-S biogenesis [41], increased in the *iscR* mutant (12.5-fold in transcriptome/27-fold in qRT-
243 PCR). This gene is also induced during cysteine starvation [1]. IscR_{Cp} controls *iscS*, *iscU* and
244 *cpe0664* involved in Fe-S clusters biogenesis likely in response to cysteine availability.

245 The expression of *cpe2092-cpe2093* and *cpe1371* increased in the mutant in our transcriptome
246 (Table 3). Cpe2092 and Cpe2093, which correspond to the ATP binding cassette and a solute
247 binding protein fused to a permease of an ABC transporter, respectively, share 61 %, 47 % and
248 44% identity with YxeO, YxeN and YxeM from *B. subtilis*. The *yxe* operon, which is induced
249 under conditions of cysteine limitation and belongs to the CymR regulon, is probably involved in
250 the uptake and degradation of sulfur compounds including S-methyl-cysteine [5]. Cpe1371
251 shares similarity with symporters. The expression *cpe1371* is also 5-fold derepressed during
252 cysteine starvation [1] suggesting that Cpe1371 could also play a role in the uptake of sulfur
253 compounds. So, IscR_{Cp} might regulate the uptake systems for sulfur-containing metabolites that
254 might be required for the maintenance of pools of cysteine, the sulfur donor for Fe-S cluster
255 biogenesis.

256

257 **Regulation of fermentation pathways**

258 The expression of *ldh* encoding the lactate dehydrogenase increased 38-fold in transcriptome and
259 130-fold in qRT-PCR in the *iscR* mutant compared to the strain 13 (Table 3 and Fig. 2A). By
260 contrast, the expression of the *cpe2297-cpe2301* operon, encoding the enzymes responsible for
261 acetyl-CoA to butyryl-CoA conversion decreased in the mutant compared to strain 13 (0.1 to
262 0.3-fold). These genes are also regulated in response to cysteine availability [1]. To confirm the
263 role of IscR_{Cp} in the control of fermentation pathways, we analyzed the end products of
264 fermentation of strain 13 and the *iscR* mutant grown 48h in minimal medium containing cystine.
265 The butyrate production decreased 2.5-fold in the *iscR* mutant (Fig. 2B). By contrast, lactate
266 production drastically increased in the *iscR* mutant in agreement with the huge derepression of
267 *ldh* transcription in this mutant. So, the modulation of expression of key genes of fermentation
268 pathways correlates with changes in the amount of lactate and butyrate produced.

269

270 **Regulation of genes involved in host compounds utilization**

271 NagL is a hyaluronidase (μ -toxins), which is probably involved in hyaluronic acid degradation, a
272 host component. The *nagL* expression was induced in the *iscR* mutant (3.75 in transcriptome/ 8.8
273 in qRT-PCR) and during cysteine limitation [1] suggesting the involvement of IscR_{Cp} in its
274 regulation in response to cysteine availability. The *cpe0818* and *cpe0866* genes were also more
275 expressed in the *iscR* mutant (9.9- and 5.8-fold in transcriptome/16- and 15-fold in qRT-PCR)
276 (Table 3). Cpe0866, which shares similarity with α -N-acetyl-glucosaminidase, could also
277 degrade host compounds. Finally, Cpe0818 encodes a protein similar to endo- β -N-acetyl-

278 glucosaminidases. These enzymes could be involved either in peptidoglycan hydrolysis such as
279 *B. subtilis* LytD protein or in the hydrolysis between two N-acetyl-glucosamine residues of
280 glycoproteins. Two endo- β -N-acetyl-glucosaminidases that are active on (Man)₆(GlcNAc)₂Asn
281 and/or (Man)₅(GlcNAc)₂Asn substrates exist in *C. perfringens* [14]. Cpe0818 probably degrades
282 glycoproteins to provide mannose and N-Acetyl-glucosamine to *C. perfringens*. Interestingly, an
283 operon encoding a PTS system (Cpe1463 to Cpe1466) belonging to the Mannose/Fructose
284 family of PTS and a gene encoding a phosphomannomutase (Cpe1873) were also up-regulated in
285 the *iscR* mutant. It is tempting to speculate that Cpe0818, the PTS system and the
286 phosphomannomutase are involved in host glycoprotein degradation and in the uptake and
287 utilization of the released sugars and that IscR_{Cp} coordinately regulates the corresponding genes.

288

289 **Identification of IscR_{Cp} direct targets *in vivo* by chromatine immunoprecipitation**

290 Among the large set of genes negatively controlled by IscR_{Cp} in transcriptome, we would like to
291 identify some direct targets. For this purpose, we tested by ChIP the binding *in vivo* of IscR_{Cp} to
292 a selection of controlled promoters identified in transcriptome. The IscR_{Cp} protein was modified
293 by addition of a C-terminal 3XFlag-tag. The modified gene was expressed under the control of
294 its own promoter. Strain CPIP11 (*iscR* pDIA5928-*iscR*-XFlag) and CPIP01 (*iscR*) used as a
295 control were grown in minimal medium in the presence of cystine that corresponds to conditions
296 of repression by IscR_{Cp}. After *in vivo* cross-linking, DNA fragments bound to IscR_{Cp}-XFlag were
297 enriched by immunoprecipitation using monoclonal antibodies raised against the XFlag.
298 Immunoprecipitated DNAs were used as templates to amplify the promoter regions of 6 genes
299 derepressed in the *iscR* mutant in transcriptome. We then performed q-PCR experiments and
300 compared the relative quantity of DNA retained in strain CPIP11 compared to strain CPIP01
301 (*iscR*). The data were standardized using *gyrA*, a gene not regulated by IscR_{Cp}, as a control. After
302 ChIP, we detected a 25 +/-1 and 4.5 +/-1 fold enrichment for the *iscR* and the *cpe2093* promoter
303 regions, respectively with strain CPIP11 compared to strain CPIP01. This clearly indicated that
304 IscR_{Cp} controls its own transcription by binding to its promoter region and is a direct regulator of
305 *cpe2093-cpe2092* expression. By contrast, we observed an enrichment factor between 1 and 1.5
306 in ChIP experiments for the promoter regions of *ldh*, *cpe0664*, *cpe1031* and *cpe1371*. For these
307 genes, it is therefore difficult to discriminate between an absence of binding or a weak
308 interaction of IscR_{Cp} to some of these promoters in our conditions.

309

310 **Identification of an IscR_{Cp} binding motif**

311 To identify a putative binding motif for IscR_{Cp}, we first compared the promoter regions of *iscR*
312 and *cpe2093*. We identified a conserved sequence upstream of these two promoters (Fig. S3B).
313 In the *iscR* promoter region, the location of this motif (Fig. 1) is in agreement with the
314 requirement for the presence of a DNA sequence between position -41 and +61 to observe a
315 negative control by IscR_{Cp} of its own transcription in *B. subtilis* (Table 2). Interestingly, this
316 motif is very similar to the IscR binding sites of the *iscR* promoter regions of *E. coli* and *T.*
317 *potens* [9, 31] and is conserved in the *iscR* promoter regions of several clostridia (see
318 discussion). The alignment of the *iscR* promoters of *E. coli*, *T. potens* and *C. perfringens* and the
319 *cpe2093* promoter allowed proposing a conserved motif, AWWGTTGACMAWWW-
320 TRMTSGGNWWT (Fig. 3SB). In all cases, this motif overlaps the -35 boxes of the promoters.
321 To confirm the involvement of this motif in *iscR*_{Cp} regulation, two point mutations were
322 introduced in conserved nucleotides in this sequence. The *PiscR-lacZ* fusions containing
323 mutations were introduced at the *amyE* locus of a *B. subtilis* Δ *cymR thrC::P_{xyIA}-iscR_{Cp}* strain.
324 The level of β -galactosidase activity was determined after growth in the presence of methionine
325 or cystine (Table 2). The replacement of the T at position -22 by a G or the G at position -19 by a
326 A (Fig. 1) led to a partial derepression of *iscR* expression in the presence of cystine while in a
327 double mutant (T-22G/G-19A), the expression of *iscR* was only two-fold repressed in the
328 presence of cystine instead of 9-fold for the fusion containing the wild-type promoter region.
329 These results are in agreement with a role of the conserved motif for the cystine-dependent
330 repression of *iscR*_{Cp}, a repression mediated by IscR_{Cp}.

331 The RegPredict web-server [23] was subsequently used to search for similar DNA motifs and
332 reconstruct candidate IscR regulons in the genomes of 16 *Clostridium* spp. (Table S2). A
333 constructed positional-weight-matrix for the identified IscR-binding motif was applied to
334 upstream gene regions in the genomic sequences of clostridia to identify additional candidate
335 sites (Fig. 3). One or two copies of IscR motif were found upstream of the *iscRSU* gene cluster in
336 each analyzed genome. We also identified a second potential IscR binding site located
337 downstream of the transcriptional start site of *iscR*_{Cp} (Fig. 1 and 3). Additional candidate sites
338 were found upstream of the *suf* operons in *C. acetobutylicum* and *C. kluyveri*, and of the *cysK*
339 gene in *C. cellulolyticum* and *C. beijerinckii*. Finally, we found additional IscR sites upstream of
340 genes controlled by IscR_{Cp} in the obtained *C. perfringens* transcriptome (Table 3). These include
341 potential IscR binding sites in the promoter regions of *cpe0664* encoding an A type carrier for
342 Fe-S clusters biogenesis and the lactate dehydrogenase gene *ldh* (Fig. 3 and Table S2). We
343 mapped the promoters of *cpe0664* and *ldh* by RACE (Fig. S4). In both cases, the potential IscR

344 binding site is located downstream from the transcriptional start site for *ldh* or overlaps the -35
345 box for *cpe0664*. This is in agreement with the repression of their expression by IscR_{Cp}.

346

347 **Discussion**

348 In *E. coli* and several other bacteria, genes involved in Fe-S cluster biogenesis are regulated in
349 response to Fe-S availability through the Fe-S regulatory protein IscR, and are induced during
350 iron starvation and oxidative stress [32]. By contrast, only few data are available concerning the
351 control of Fe-S cluster synthesis in Gram-positive bacteria, in anaerobic bacteria [31] or in
352 response to sulfur availability [11]. In *C. perfringens*, we have previously shown a coordinated
353 regulation in response to cysteine availability of genes involved in Fe-S production (*iscS*, *iscU*,
354 *cpe0664*) [1]. We demonstrate in this work that the IscR_{Cp} repressor mediates this control. Other
355 genes encoding transporters (Cpe2093-Cpe2092 and Cpe1371) that might be involved in
356 supplying for sulfur required for Fe-S biogenesis are also under the coordinated control of
357 IscR_{Cp}. These regulations may allow *C. perfringens* maintaining its pools of Fe-S clusters, which
358 play a crucial role in the physiology of clostridia lacking the heme synthesis machinery [17].
359 Interestingly, IscR_{Cp} also controls the expression of genes involved in fermentation pathways
360 (Fig. 2) and we observe accordingly a decrease of butyrate production and a drastic increase of
361 lactate production in the *iscR* mutant. The pyruvate to acetyl-CoA conversion is catalyzed by the
362 pyruvate ferredoxin-oxido-reductase (PFOR). This Fe-S enzyme forms CO₂ and acetyl-CoA by
363 oxidizing pyruvate and reducing a 2Fe-4S ferredoxin. This step implies the utilization of several
364 Fe-S clusters while lactate production by the lactate dehydrogenase does not. So, we propose that
365 during cysteine limitation [1], IscR_{Cp} could reroute the fermentation metabolism increasing *ldh*
366 expression to reoxidize the NADH produced during glycolysis and limiting the utilization of Fe-
367 S clusters. It is intriguing to note that in *C. acetobutylicum*, lactate production increases under
368 conditions of iron limitation [4] while in *C. perfringens* *ldh* expression increases under
369 conditions of sulfur limitation [1]. In both cases, IscR that is probably able to sense iron and
370 sulfur availability might be involved in this control.

371 We then establish using ChIP experiments that IscR_{Cp} directly interacts with the promoter region
372 of *iscR_{Cp}* and of *cpe2093* *in vivo* in *C. perfringens*. We further identify a conserved motif
373 overlapping the -35 boxes of these two promoters that share similarities with the IscR binding
374 motifs of *E. coli*, *Erwinia chrysanthemi*, *Pseudomonas aeruginosa* and *T. potens* [9, 28, 29, 31].
375 We also propose that a second site is present in the *iscR* promoter region of *C. perfringens* as
376 observed in *E. coli* and *T. potens*. We further show that this motif is conserved upstream of *iscR*

377 in clostridia (Fig. 3 and Table S2). For *cpe0664* encoding an A-type scaffold protein involved in
378 Fe-S biogenesis and *ldh*, we identify DNA sequences located at position allowing repression
379 (Fig. 3 and S4) that share similarities to the IscR_{Cp} binding motif. However, we fail to detect a
380 clear binding of IscR_{Cp} to the *cpe0664* or the *ldh* promoter region *in vivo* in our conditions. It is
381 known that the binding of several proteins to a promoter region may lead to false negatives in
382 ChIP experiments [20]. So, it is interesting to note that a Rex binding site is identified in the *ldh*
383 promoter region [27] and we cannot exclude that interference might exist between IscR and Rex.
384 In conclusion, *in vitro* experiments will be required to determine if IscR_{Cp} binds to the candidate
385 IscR binding motifs in the *cpe0664* and *ldh* promoter regions. This will deserve further
386 investigations.

387 Other genes involved in stress response, in metabolism or encoding proteins of unknown
388 functions are regulated both by IscR_{Cp} and in response to cysteine availability [1]. A second class
389 of genes including *cpe1031* encoding a regulator, genes involved in host compound utilization or
390 encoding proteins of unknown function seems to be regulated only by IscR_{Cp}. In *E. coli* and
391 probably also in *T. potens*, two classes of IscR-controlled genes with distinct binding motifs
392 exist. Type I sites are only bound by IscR associated to a Fe-S cluster while type II sites interact
393 both with Apo-IscR and IscR containing a Fe-S cluster [9, 22, 31, 32]. The possible existence of
394 type II promoters in *C. perfringens* and the determination of direct or indirect regulation by
395 IscR_{Cp} for most of the IscR-controlled genes remain to be established and will deserve further
396 investigations.

397 The IscR regulator is absent in aerobic firmicutes but is present in several clostridia. With a few
398 exceptions, the *iscR* gene is in operon with *iscS* and *iscU* (Fig. 3). The presence of an IscR
399 binding motif upstream of these *iscR* operons strongly suggests a conserved mechanism of
400 control of Fe-S cluster homeostasis in these clostridia. Interestingly, a putative *iscR* binding site
401 is also present upstream of the *suf* operons in *C. acetobutylicum* and *C. kluyveri*. In *C.*
402 *acetobutylicum*, the Isc system is probably absent while in *C. kluyveri* like in *E. coli* and *T.*
403 *potens*, both the Isc and Suf machineries are present. In *E. coli*, the *suf* genes are positively
404 controlled by IscR in aerobic conditions and IscR is able to bind to the *suf* promoter region in *E.*
405 *coli* and *T. potens* through a type II binding site [9, 31]. Moreover, we also identify a potential
406 IscR binding motif upstream of *cysK* of *C. cellulolyticum* and *C. beijerinckii* (Fig. 3 and Table
407 S2). While the *cysK* gene encoding the O-acetylserine-thiol-lyase in *C. perfringens* is induced
408 under conditions of cysteine starvation through a cysteine specific T-box [1], the *cysK* gene in *C.*
409 *cellulolyticum* and *C. beijerinckii* might be under the control of IscR. Cysteine is the sulfur donor

410 for Fe-S biogenesis and the possible existence of a coordinated regulation of the *isc* genes and
 411 the *cysK* gene might allow supplying for sulfur required for Fe-S production.
 412 Finally, the control by IscR_{Cp} of genes encoding proteins involved in host compound degradation
 413 suggests that IscR_{Cp} might play a role during the host colonization or the infection by *C.*
 414 *perfringens*. This is in agreement with recent works showing that in several pathogenic Gram-
 415 negative bacteria, IscR controls expression of factors involved in virulence or allowing
 416 adaptation to hostile conditions encounters in the host [18, 28].

417
 418 **Acknowledgments.** We are thankful to Olga Soutourina for helpful discussion. This research
 419 was supported by a grant from the Institut Pasteur (PTR N°256). G. André was the recipient of
 420 a grant from the Ministère de l'enseignement supérieur et de la recherche and from the Pasteur-
 421 Weizmann foundation. D.Rodionov was supported by the Russian Foundation for Basic
 422 Research (14-04-91154).

423
 424

425 **Figure legends**

426 **Figure 1. *C. perfringens* *cpe1786/iscR_{Cp}* promoter region.**

427 The initiation of transcription start site « +1 » identified by 5'RACE is indicated by an arrow.
 428 The -10 and -35 boxes of the σ^A -dependent promoter are underlined. The ribosome-binding site
 429 is indicated in bold while the translational start site of *cpe1786* is indicated in bold and italic. In
 430 the genome of *C. perfringens* 13, the translational start codon of *cpe1786* is a GTG
 431 corresponding to a valine leading to a truncation of 25 amino acids and the absence of the $\alpha 1$
 432 helix of the WHTH motif (Fig. S2). We rather proposed the ATG preceded by a ribosome
 433 binding site (GAGG). This start codon corresponds to the start of CPF_2040, the homologs of
 434 Cpe1786 in the genome of *C. perfringens* ATCC 13124 [19]. Broken arrows indicate the end
 435 points of the *iscR-lacZ* fusions. The conserved motif present upstream of the *iscR* genes in
 436 clostridia is boxed. Point mutations obtained in this motif are indicated. A second potential IscR
 437 binding motif (see Table S2) is over-lined.

438 **Figure 2. Effect of *iscR_{Cp}* gene inactivation on fermentation pathways.** (A) Genes involved in
 439 fermentation pathways differentially expressed in an *iscR* mutant compared to strain 13. Arrows
 440 ↗ and ↘ indicated genes whose expression increased or decreased in an *iscR* mutant compared
 441 to strain 13. (B) Changes in metabolic end products in the *C. perfringens* strain 13 and the *iscR*
 442 mutant. Chromatography analysis from these two strains after 48 h of growth in minimal
 443 medium containing cystine was performed. The mean and standard error of two experiments are
 444 shown.

445 **Figure 3. Candidate IscR regulons reconstructed by comparative genomics approach in**
 446 **Clostridia spp.** (A) Chromosomal clusters of candidate IscR-regulon genes in Clostridia and
 447 *Thermincola potens*. Homologous genes are marked by the same color. Potential IscR-binding
 448 sites are shown by red arrows. (B) Consensus sequence logo of the predicted IscR-binding sites
 449 in Clostridia. The logo was generated from the sequence alignments with WebLogo
 450 (<http://weblogo.berkeley.edu>).

451

452 **Table 1 Strains used in this study**

Strain	Genotype	Origin
<i>E. coli</i>		
DH5	F- ϕ 80 <i>lacZ</i> Δ M15 Δ (<i>lacZYA-argF</i>)U169 <i>deoR recA1 endA1 hsdR17</i> (r_k^- , m_k^-) <i>phoA supE44 thi-1 gyrA96 relA1 λ</i>	Invitrogen
Top10	F ⁻ <i>mcrA</i> D(<i>mrr-hsdRMS-mcrBC</i>) f80 <i>lacZ</i> Δ M15 D <i>lacX74 deoR, recA1 araD139 D(ara-leu)7697 galK rpsL</i> (StrR) <i>endA1 nupG</i>	Invitrogen
<i>B. subtilis</i>		
168	<i>trpC2</i>	Laboratory stock
BSIP1798	<i>trpC2 ΔcymR_{Bs} amyE::aphA3 lacZ</i>	[7]
BSIP1978	<i>trpC2 ΔcymR_{Bs} amyE::P(-131 +61) iscR_{Cp}-lacZ cat</i>	pDIA5820→BSIP1798
BSIP1981	<i>trpC2 ΔcymR_{Bs} amyE::P(-131 +61)iscR_{Cp}-lacZ cat thrC::PxylA-iscR_{Cp} spc</i>	pDIA5744→BSIP1978
BSIP2018	<i>trpC2 ΔcymR_{Bs} amyE::aphA3 lacZ thrC::PxylA-iscR_{Cp} spc</i>	PAC7→BSIP1981
BSIP2020	<i>trpC2 ΔcymR_{Bs} amyE ::P(-41,+61)iscR_{Cp}-lacZ thrC::PxylA-iscR_{Cp} spc</i>	PDIA5837→BSIP2018
BSIP2022	<i>trpC2 ΔcymR_{Bs} amyE ::PiscR_{Cp}T-22G-lacZ cat thrC::PxylA-iscR_{Cp} spc</i>	pDIA5840 →BSIP2018
BSIP2024	<i>trpC2 ΔcymR_{Bs} amyE ::PiscR_{Cp}G-19A-lacZ cat thrC ::PxylA-iscR_{Cp} spc</i>	pDIA5842 →BSIP2018
BSIP2029	<i>trpC2 ΔcymR_{Bs} amyE ::PiscR_{Cp}T-22G/G-19A-lacZ cat thrC ::PxylA-iscR_{Cp} spc</i>	pDIA5846 →BSIP2018
<i>C. perfringens</i>		
13	Wild-type	[34]
CPIP01	<i>cpe1786/iscR::erm</i>	pMTL007-Cpe1786-28a → strain 13
CPIP11	<i>cpe1786/iscR::erm</i> pDIA5928- <i>iscR</i> -Xflag	pDIA5928→ CPIP01
Plasmids		
pMTL007	group II intron, ErmBtdRAM2 and ltrA ORF from pMTL20lacZTTErmBtdRAM2, Cm ^R /Tm ^R	[12]
pMTL83151	PCB102 replicon, Cm ^R	[13]
pAC6		[38]
pXT	Ap ^R , Sp ^R	[21]
pMTL007- <i>cpe1786-28a</i>	Cm ^R /Tm ^R	This work
pDIA5744	pXT- <i>iscR_{Cp}</i>	This work
pDIA5820	pAC6 P(-131, +61) <i>iscR_{Cp}-lacZ</i>	This work
pDIA5837	pAC6 P(-41, +61) <i>iscR_{Cp}-lacZ</i>	This work
pDIA5840	pAC6 P(-131 +61) T-22G <i>iscR_{Cp}-lacZ</i>	This work
pDIA5842	pAC6 P(-131 +61) G-19A <i>iscR_{Cp}-lacZ</i>	This work
pDIA5846	pAC6 P(-131 +61) T-22G / G-19A <i>iscR_{Cp}-lacZ</i>	This work

pDIA5852	pAC6 P(-131 +61) T-15C <i>iscR_{Cp}-lacZ</i>	This work
pDIA5925	pGEMTeasy- <i>iscR</i> -XFlag	This work
pDIA5928	pGEMTeasy/pCB3 replicon/cat- <i>iscR</i> -XFlag	This work

- 453 (1) Ec, Bs and Cp correspond to *E. coli*, *B. subtilis* and *C. perfringens*, respectively
 454 (2) *cat*, *erm* and *spc* encode proteins leading to chloramphenicol (Cm), erythromycin (Em) or spectinomycin (Sp)
 455 resistance. Tm corresponds to thiamphenicol.
 456 (3) the arrow indicates a construction by transformation
 457

458 **Table 2. Regulation of *iscR_{Cp}* expression by *IscR_{Cp}* in *B. subtilis*.**

Strain	Relevant genotype	β-galactosidase activity (nmol ONP/min/mg of protein)	
		Methionine	Cystine
BSIP1978	$\Delta cymR_{Bs}$ <i>amyE</i> ::P(-131,+61) <i>iscR_{Cp}-lacZ</i>	1070+/-90	1025+/-60
BSIP1981	$\Delta cymR_{Bs}$ <i>amyE</i> ::P(-131,+61) <i>iscR_{Cp}-lacZ thrC</i> ::P _{Xyl} - <i>iscR_{Cp}</i>	1085+/-2	115+/-20
BSIP2020	$\Delta cymR_{Bs}$ <i>amyE</i> ::P(-41,+61) <i>iscR_{Cp}-lacZ thrC</i> ::P _{Xyl} - <i>iscR_{Cp}</i>	730+/-17	58.5+/-1.5
BSIP2022	$\Delta cymR_{Bs}$ <i>amyE</i> ::P(-131,+61) <i>iscR_{Cp}-T-22G-lacZ thrC</i> ::P _{Xyl} - <i>iscR_{Cp}</i>	1025+/-30	370+/-20
BSIP2024	$\Delta cymR_{Bs}$ <i>amyE</i> ::P(-131,+61) <i>iscR_{Cp}-G-19A-lacZ thrC</i> ::P _{Xyl} - <i>iscR_{Cp}</i>	1070+/-30	380+/-5
BSIP2029	$\Delta cymR_{Bs}$ <i>amyE</i> ::P(-131,+61) <i>iscR_{Cp}-T-22G/G-19A-lacZ thrC</i> ::P _{Xyl} - <i>iscR_{Cp}</i>	1050+/-24	515+/-10

459 β-galactosidase activity was measured on crude extracts from strains grown in minimal medium in the presence of 1
 460 mM methionine or 1 mM cystine. *iscR_{Cp}*= *cpe1786*. For strains BSIP1981, BSIP2020, BSIP2022, BSIP2024 and
 461 BSIP2029 0.1% xylose and 50 mg/L of threonine were added.
 462

463 **Table 3. Genes differentially expressed between strain 13 of *C. perfringens* and a *iscR_{Cp}*
 464 mutant.**
 465

gene (Synonym)	Functions/similarities	Transcriptome	quantitative RT-PCR
		<i>iscR_{Cp}</i> /13	<i>iscR_{Cp}</i> /13
Fe-S clusters and redox functions			
<i>cpe0664</i>	HesB-like protein (114 aa)	12.5	27
<i>cpe1785 (iscS)</i>	Cysteine desulfurase, Fe-S clusters biosynthesis	2.1	
<i>cpe1784 (iscU)</i>	Fe-S clusters assembly	2	
<i>cpe1783 (trmU)</i>	Methylaminomethyl-2-Thiouridylate-Methyltransferase	2	
<i>cpe2511 (fer)</i>	Ferredoxin [3Fe-4S]	0.29	0.2
<i>cpe0855 (rubY)</i>	rubrerythrin	0.17	
Transporters, membrane or exported proteins			
<i>cpe2092</i>	Amino acid ABC transporter, ATP binding cassette	10.2	26
<i>cpe2093</i>	Amino acid ABC transporter, permease	5.5	
<i>cpe2295 (lepW)</i>	Type I Signal peptidase	6.6	
<i>cpe1371</i>	Na ⁺ -dependent symporter	3.6	7.5
<i>cpe1621</i>	putative cation efflux protein	3.4	
<i>cpe1343</i>	probable galactoside ABC transporter	0.1	
<i>cpe1341</i>	probable galactoside ABC transporter	0.21	
Regulators			
<i>cpe1031</i>	ArsR-SmtB family regulator	7.8	20

<i>cpe2304</i>	ArsR-SmtB family regulator	1.99	
Carbon metabolism			
<i>cpe0103 (ldh)</i>	L-lactate dehydrogenase	38.6	130
<i>cpe2531(adhE)</i>	Aldehyde alcohol dehydrogenase	3.03	
<i>cpe2195 (atoB)</i>	Acetyl-CoA acetyltransferase	0.13	
<i>cpe2297</i>	3-hydroxybutyryl-CoA dehydrogenase	0.163	
<i>cpe2298 (fixB)</i>	α subunit electron transfer flavoprotein	0.22	
<i>cpe2299 (etfB)</i>	β subunit electron transfer flavoprotein	0.29	
<i>cpe2300 (bcd)</i>	Acyl-CoA dehydrogenase	0.21	
<i>cpe2301 (crt)</i>	3-hydroxybutyryl-CoA dehydratase	0.33	0.5
<i>cpe0892</i>	butanol dehydrogenase NADPH dependent	0.618	
<i>cpe2347 (buk)</i>	butyrate kinase	0.63	
<i>cpe2348 (ptb)</i>	phosphotransbutyrylase	0.625	
<i>cpe1185 (pfk)</i>	6-phosphofructokinase	0.5	
<i>cpe1299 (eno)</i>	enolase	0.54	
<i>cpe2149 (pykA)</i>	Pyruvate kinase	0.59	
<i>cpe2267</i>	glucose-6-phosphate isomerase	1.65	
<i>cpe1463</i>	PTS system IID component (IIDMan)	2.2	
<i>cpe1464</i>	PTS system IIC component (IICMan)	1.45	
<i>cpe1465</i>	PTS system IIB component (IIDMan)	1.53	
<i>cpe1466</i>	PTS system IIA component (IIAMan)	2.65	
<i>cpe1873(manB)</i>	phosphomannomutase	1.587	
Other metabolisms			
<i>cpe1050</i>	5'-MTA/SAH nucleosidase	3.8	
<i>cpe0056</i>	5'-MTA/SAH nucleosidase	1.58	
<i>cpe1573</i>	Probable glutamate cysteine ligase	2.9	
<i>cpe0447 (lguL)</i>	Lactoylglutathione lyase	2	
Host compounds degradation			
<i>cpe0818</i>	endo- β -N-acetyl-glucosaminidase	9.9	16
<i>cpe0866</i>	α -N-acetyl-glucosaminidase	5.8	15
<i>cpe1523 (nagL)</i>	hyaluronidase	3.8	8.8
Unknown function			
<i>cpe0554</i>	Unknown	6.7	
<i>cpe2261</i>	Unknown	5.3	
<i>cpe2262</i>	Unknown	4.35	
<i>cpe2063</i>	Unknown	5.2	
<i>cpe1079</i>	Unknown	5.2	
<i>cpe1257</i>	Unknown	4.6	
<i>cpe1875</i>	Unknown	4.3	
<i>cpe0106</i>	Unknown	3.5	
<i>cpe0105</i>	Unknown	3.15	
<i>cpe1169</i>	Unknown	3.42	
<i>cpe1735</i>	Unknown	3.66	
<i>cpe1173</i>	Unknown	0.15	
<i>cpe2111</i>	Unknown	0.26	
<i>cpe1078</i>	Unknown	0.27	
<i>cpe0098</i>	Unknown	0.29	
<i>cpe1472</i>	Unknown	0.29	

466 Gene names and functions correspond to those indicated in the GenoList data-base
467 (<http://genodb.pasteur.fr/cgi-bin/WebObjects/GenoList>). A gene was considered as differentially
468 expressed when the p-value is < 0.05 using the statistical analysis described in Materials and

469 Methods. Controlled genes with expression ratio above three-fold between strains 13 and *iscR_{Cp}* and
 470 those associated with iron-sulfur clusters biogenesis, redox functions, carbon and sulfur metabolisms
 471 and the degradation of host compounds are presented in this Table. We confirmed the transcriptome
 472 data by qRT-PCR analysis for several genes.
 473

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 579

580 Supplementary data

581 **Figure S1.** Genetic organization of the *cpe1786/iscR* locus and inactivation of the *cpe1786* gene.

582 The ClosTron delivery system (A) encoded on plasmid pMTL007 consists of a group II intron
 583 (black arrow) with an internal Retrotransposition-Activated Marker (RAM) conferring
 584 erythromycin resistance (white arrow) that is itself interrupted by the *td* group I intron (hatched
 585 box) [12]. The group II intron is retargeted to the *cpe1786* gene (grey arrow) by altering the
 586 sequence of the IBS/EBS region using overlap PCR. Splicing of the group II intron into *cpe1786*
 587 disrupts it and the splicing out of the *td* group I intron from the *erm* RAM restores a functional
 588 *ermB* gene allowing positive selection of *cpe1786* mutants. B) Analysis of genetic organization
 589 of the *cpe1786* locus by RT-PCR experiments. cDNA were synthesized using RNA extracted
 590 from cells grown in the presence of homocysteine. Couples of primers used for PCRs are
 591 indicated in panel A. Agarose gel corresponds to the migration of the different PCR fragments
 592 obtained from RNA without (lane 1,3,5) or with (lane 2,4,6) a reverse transcriptase treatment.
 593 Lane 1 and 2: IMV489-IMV490, lane 3 and 4: GA54-IMV491, lane 5 and 6: IMV492-493.

594 C) Confirmation of *cpe1786* knockout by PCR was performed using chromosomal DNA of *C.*
 595 *perfringens* CP13 (lane 1, 3 and 5) or an erythromycin resistant clones containing an intron in the
 596 *cpe1786* gene (lane 2, 4 and 6) and the primer pairs RAM-F/RAM-R (lane 1 and 2),
 597 IMV485/EBSu (lane 3 and 4) and IMV485-IMV484. PCR products were visualized on 1% w/v
 598 agarose gel.

599 **Figure S2. Alignment of CymR and IscR-type regulators.** The alignments were performed
 600 using the CLUSTALW algorithm. SA: *Staphylococcus aureus*; BS: *B. subtilis*; CA: *C.*
 601 *acetobutylicum*; CP: *C. perfringens*; CD: *C. difficile*; TP: *Thermincola potens*; EC: *E. coli*;
 602 ERWCT: *Erwinia carotovora*. The winged helix-turn-helix (HTH) motif is indicated by a blue

603 arrow and the amino acids shown to interact with DNA of type 2 sites in *E. coli* are underlined.
604 The Glutamate at position 43 proposed to be involved in discrimination between type 2 and
605 type 1 site is indicated in blue. The region containing the Fe-S binding motif corresponds to the
606 red arrow. The 3 conserved cysteine residues coordinating the Fe-S cluster are indicated in red
607 while the histidine residue involved in Fe-S coordination in *E. coli* is indicated in green [8].

608 **Figure S3. The *iscR* and *cymR* operons.** **A.** Comparison of the genetic organization of the
609 *cpe1786* operon of *C. perfringens* with that of the *cymR* operon of *B. subtilis* and the *iscR*
610 operons of *E. coli* and *T. potens*. Genes encoding regulators are indicated by blue arrows. The %
611 of identity between each regulator and Cpe1786 is indicated. **B.** IscR binding motif. Alignment
612 of IscR sites experimentally validated in *E. coli* [9], *T. potens* [31] and *C. perfringens* (this
613 work). The -35 boxes are underlined. The promoters have been mapped for *iscR* and *yadR* of *E.*
614 *coli* [9] and *iscR* of *C. perfringens* (Fig 1).

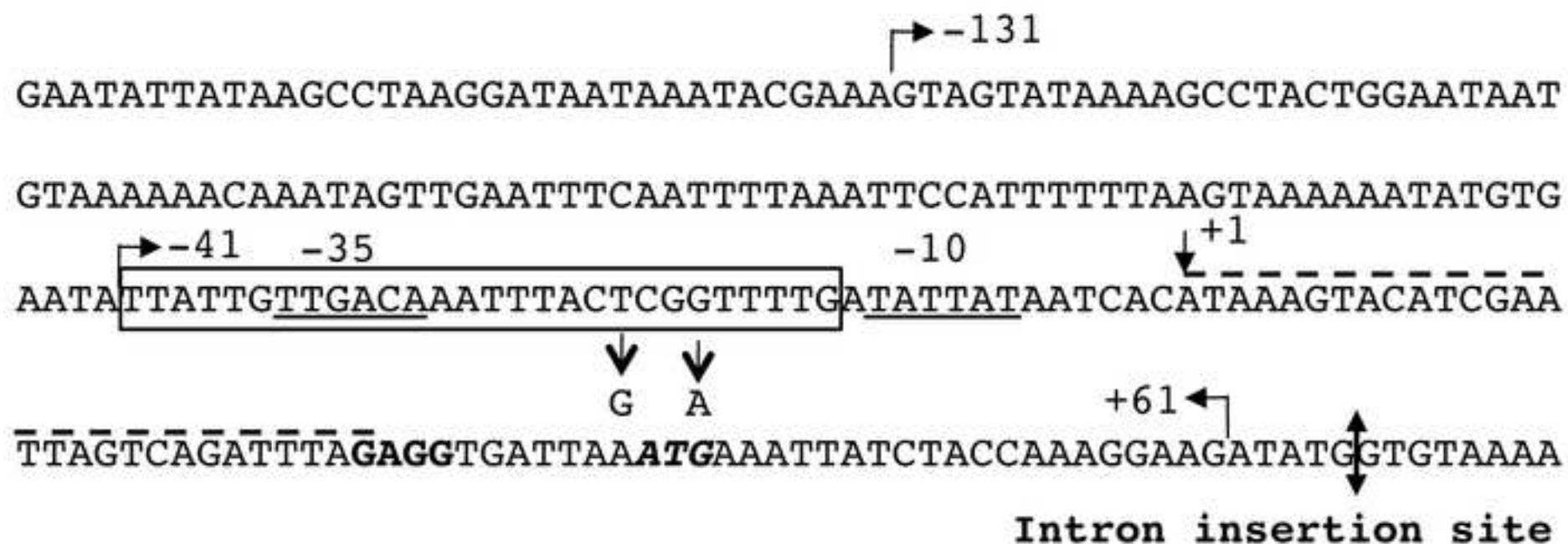
615 **Figure S4. Mapping of *ldh* and *cpe0664* promoters by 5'RACE.** The promoters region of *ldh*
616 (*cpe0103*) and *cpe0664* encoding an A-type scaffold protein for Fe-S assembly are presented.
617 The ATG of *ldh* and *cpe0664* are indicated in green. The ribosome bonding sites (RBS) are
618 underlined and the -10 and -35 corresponding to SigA consensus are boxed. The IscR binding
619 motifs identified using Reg-Predict [23] are indicated by blue arrows. In the case of *cpe0664*, a
620 second motif with a score <5 is also indicated.

621 **Table S1. List of oligonucleotides**

622 **Table S2. Genomic identification of candidate IscR-binding sites in Clostridia spp.**

Figure

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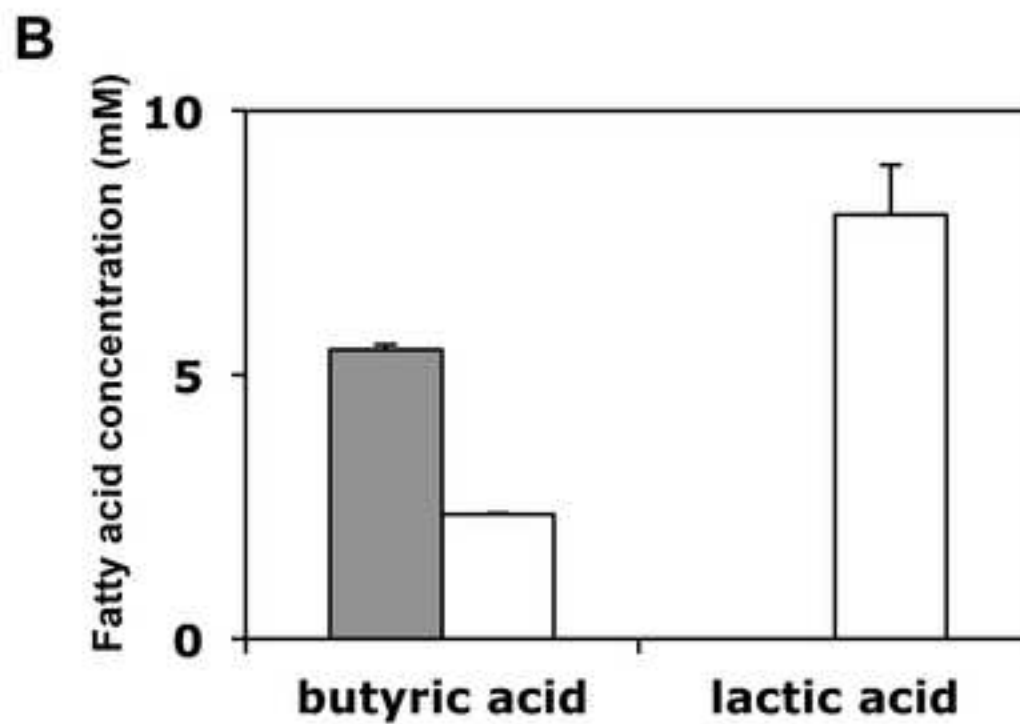
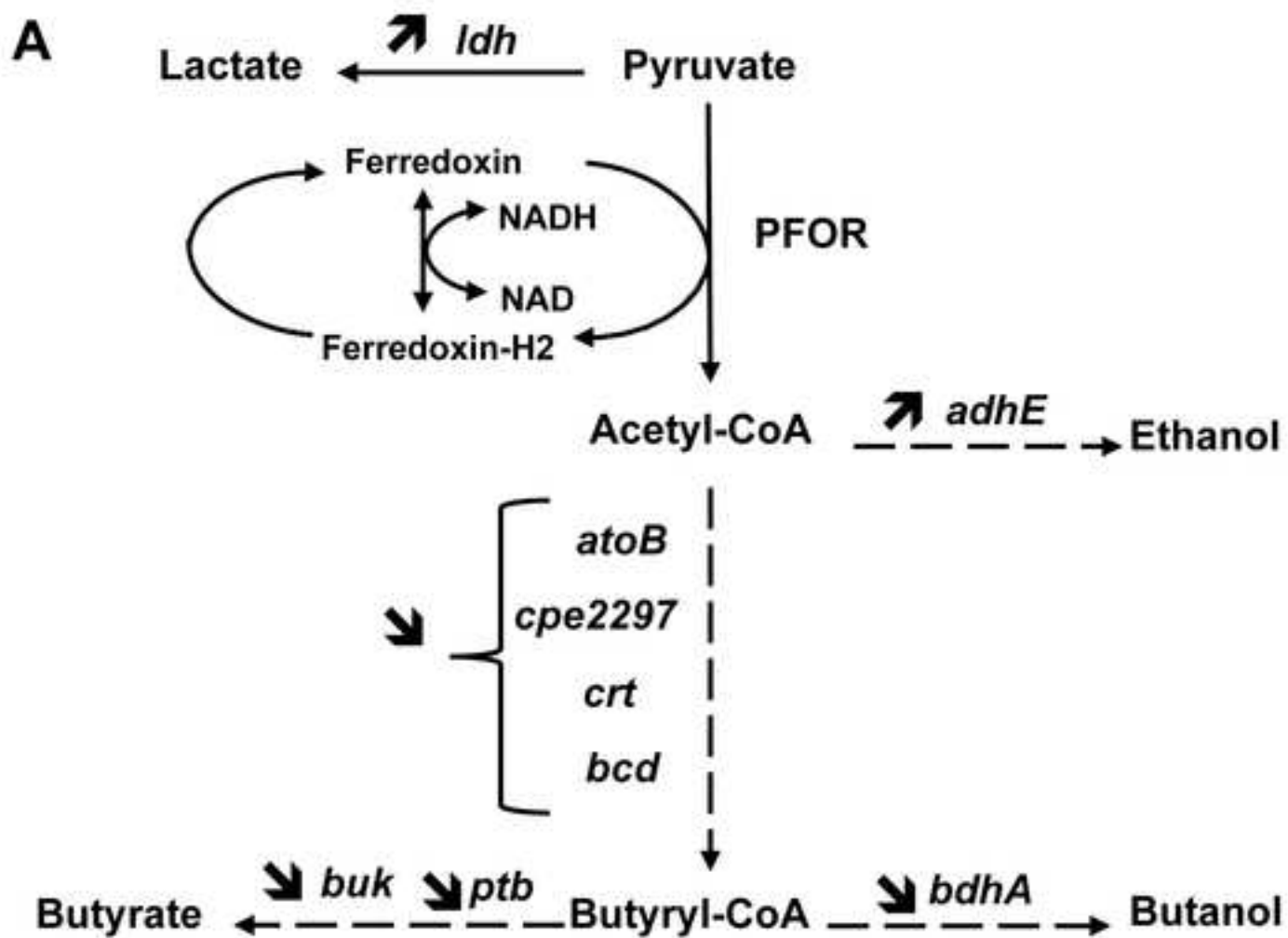


Figure 3
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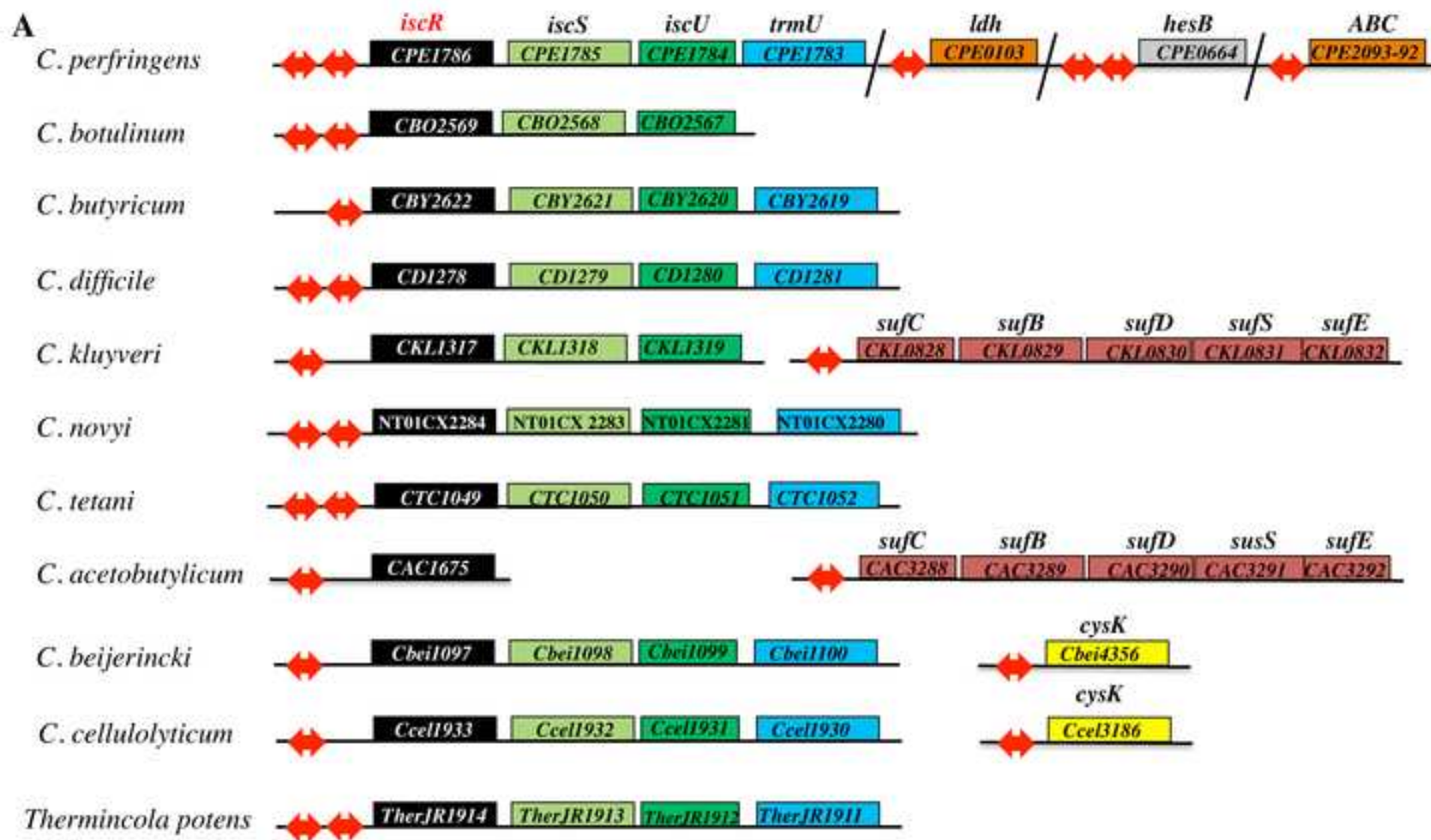
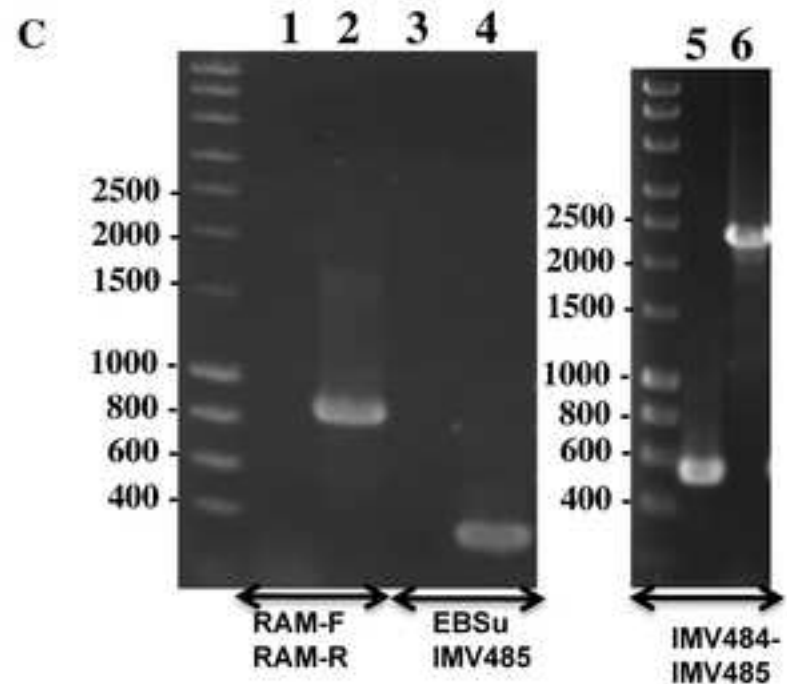
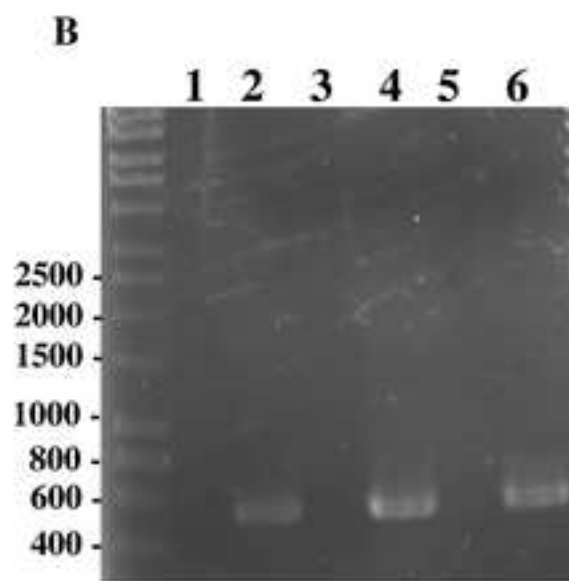
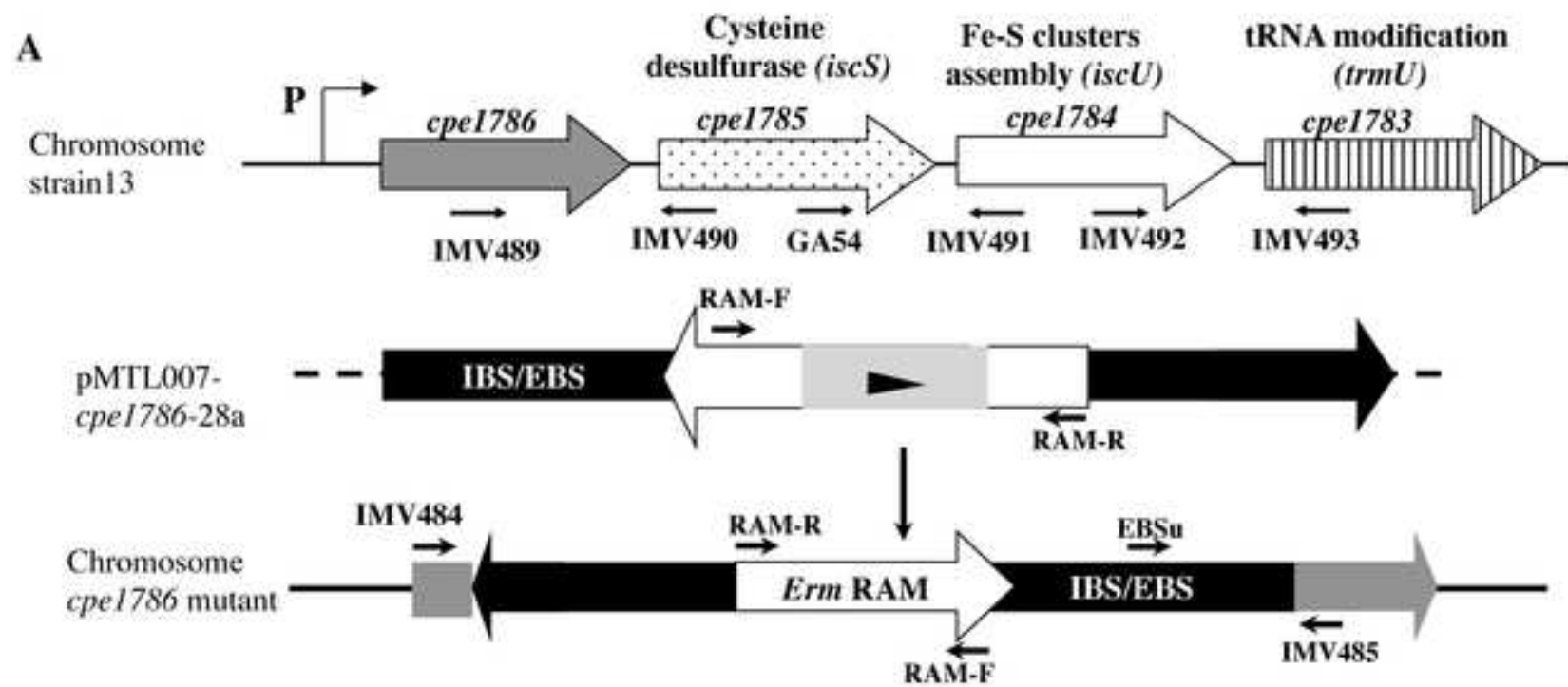
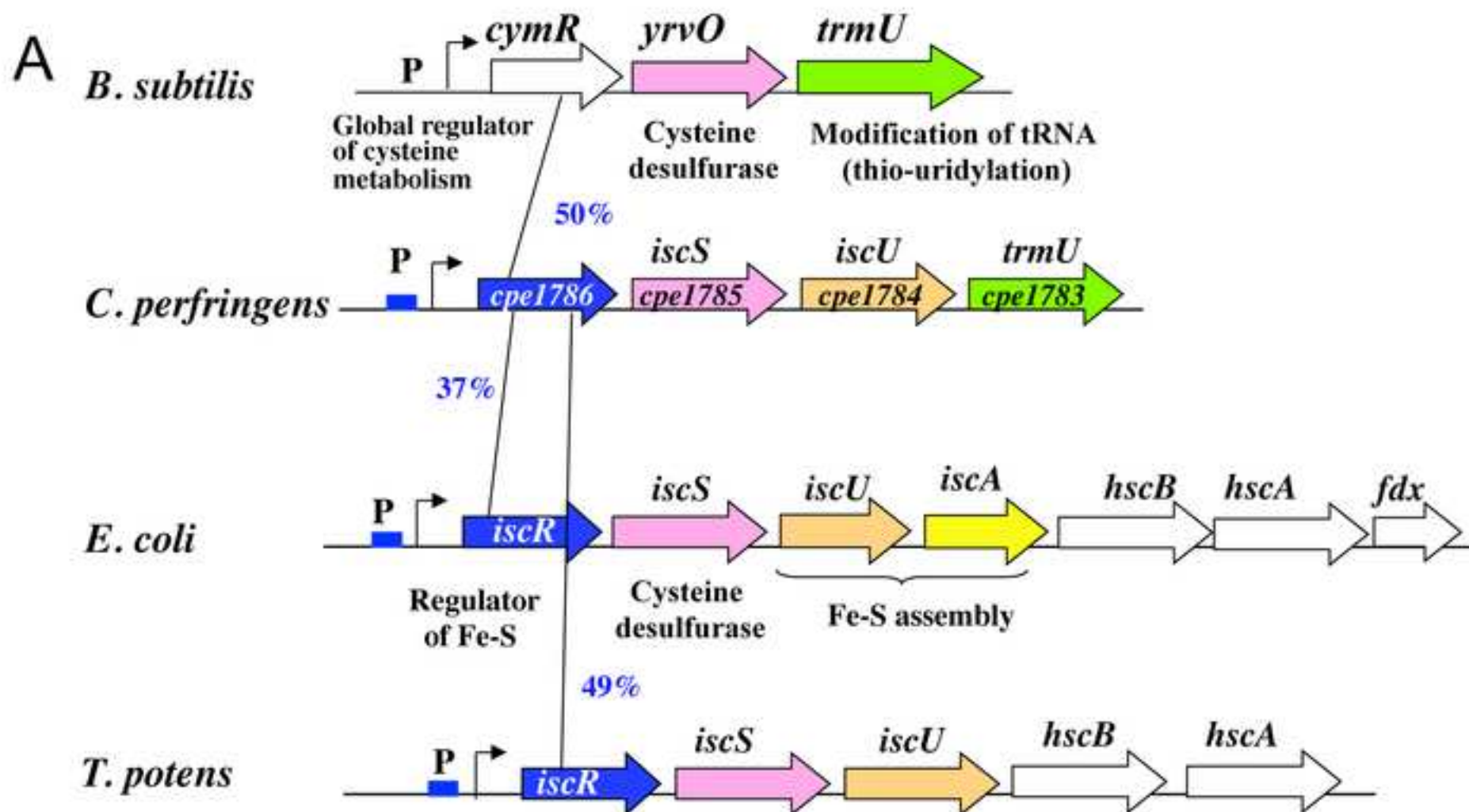


Figure S1
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**B**

```

iscR E.C ATAGTTGACCAATTTACTCGGGAAT
iscR T.P ATAGTTGACCAATTTACTCGGGAAT
iscR C.P ATTGTTGACAAATTTACTCGGTTTT
cpe2093 AAAGTTGACAATTTTGATGGGAATT
        AWWGTTGACMAWWWTRMTSGGNWWT

```

Figure S4
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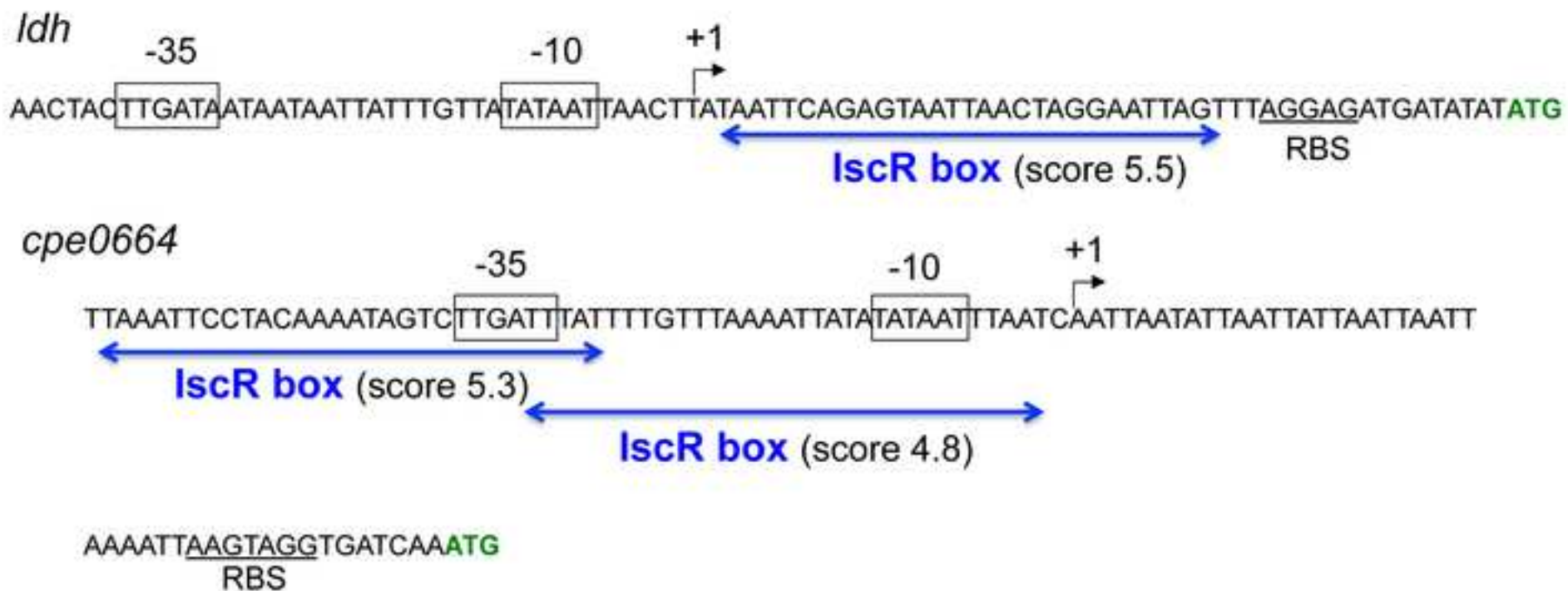


Table S1. Oligonucleotides used in this study.

Name	sequence	function
Quantitative RT-PCR		
GA99	TGCAGGATTTGTTGGTTCAA	5' <i>ldh</i>
GA100	GCACCTTGTGCTAGGTCCAT	3' <i>ldh</i>
GA103	GGTAGCAATGAGCGATGATG	5' <i>cpe0664</i>
GA104	TCCGTTCCCACCAAAGTTTA	3' <i>cpe0664</i>
GA105	CCTGGCTCTTTTGAAGGTGT	5' <i>cpe1371</i>
GA106	ACAACCATACCGCATCCATT	3' <i>cpe1371</i>
GA108	TCCCAATGTTACCCTTTTGA	5' <i>cpe2092</i>
GA109	TTGCAAAGCTCATTTCATGG	3' <i>cpe2092</i>
GA110	CTCCTTACAAGGCACCAGGA	5' <i>cpe0818</i>
GA111	CCCTTTGGCTACTGGAACAC	3' <i>cpe0818</i>
GA112	ATGCCTGAAACAATGCCTTC	5' <i>cpe0866</i>
GA113	TTGATCCCAATCCCAGAAAG	3' <i>cpe0866</i>
GA114	TGGTACTGCTCCAAC TTTCG	5' <i>cpe1031</i>
GA115	TTCCACCAACAAC TACAGCATC	3' <i>cpe1031</i>
GA124	GAGGGAAACATTGGGGTTTT	5' <i>crt</i>
GA125	ATCAGCACCTGCAACAAATG	3' <i>crt</i>
GA126	TCAAAGCAAGCTGAGTGGA	5' <i>nagL</i>
GA127	CATTGTTGCATGTCCTGTCC	3' <i>nagL</i>
GA30	TGCCAGAATAGTTGGGGAAAG	5' <i>gyrA</i>
GA31	TACCATGTCCGTCAACAAGC	3' <i>gyrA</i>
5'RACE		
GA130	TTCATAGTCGCTCATTAATGGAT	5'RACE <i>cpe0664</i>
GA131	AGAAGAATCAATAACAAAAGTTAA	5'RACE <i>cpe0664</i> int
GA132	TACTAAAAGTATTGAGTTTGGGC	5'RACE <i>ldh</i>
GA133	GTTATTATAACTATGTCAGAG	5'RACE <i>ldh</i> int
GA51	TCCTCCTTGAGCACCTCTAA	5'RACE <i>cpe1786</i>
GA62	GTCTAGTCGCACAACAA	5'RACE <i>cpe1786</i> int
Plasmid constructions		
pMTL007-F	TTAAGGAGGTGTATTTTCATATGACCATGATTACG	intron variable region
pMTL007-R	AGGGTATCCCCAGTTAGTGTTAAGTCTTGG	intron variable region
RAM-F	ACGCGTTATATTGATAAAAATAATAATAGTGGG	Erm cassette
RAM-R	ACGCGTGCGACTCATAGAATTATTTCTCCCG	Erm cassette
EBSu	CGAAATTAGAACTTGCCTTCAGTAAAC	intron internal
TBD043	AAAAAAGCTTATAATTATCCTTAGGATACGATACTGTGCGC CCAGATAGGGTG	IBS <i>cpe1786</i>
TBD044	CAGATTGTACAAATGTGGTGATAACAGATAAGTCGATACT GGTAACTTACCTTTCTTTGT	EBS1d <i>cpe1786</i>
TBD045	TGAACGCAAGTTTCTAATTTTCGATTTATCCTCGATAGAGGA AAGTGTCT	EBS2 <i>cpe1786</i>
IMV484	GACAAATTTACTCGGTTTTGAT	5' <i>cpe1786</i>
IMV485	CTCTATTATTCATTTTCTTCACTC	3' <i>cpe1786</i>
IMV390	GGCGAATTCGTAGTATAAAAGCCTACTGG	<i>cpe1786</i> -P-131 EcoRI
IMV391	GGCGAATTCCTTATTGTTGACAAATTTACT	<i>cpe1786</i> P-41 EcoRI
IMV394	GGCGGATCCCTTCTTTGGTAGATAA	<i>cpe1786</i> P+61 BamHI
IMV439	TTGTTGACAAATTTACGCGGTTTTGATATTATA	T-22G P- <i>cpe1786</i>
IMV440	TATAATATCAAACCGCGTAAATTTGTCAACAA	T-22G P- <i>cpe1786</i>
IMV441	TTGACAAATTTACTCGATTTTGATATTATAATC	G-19A P- <i>cpe1786</i>
IMV442	GATTATAATATCAAATTCGAGTAAATTTGTCAA	G-19A P- <i>cpe1786</i>
IMV455	TTGTTGACAAATTTACGCGATTTTGATATTATAATC	T-22G/G-19A P- <i>cpe1786</i>
IMV456	GATTATAATATCAAATTCGCGTAAATTTGTCAACAA	T-22G G-19A P- <i>cpe1786</i>
EH18	AAATCTAGATCACTACTTG7CA7CGTCATCCTTGATGCGATGTCATGATCT TTATAATCACCGTCATGGTCTTTGTAGTCTTCATTTCTTCACTCTACT	Fusion of a XFlag at the end of <i>cpe1786</i>
ChIP-PCR		
IMV524	ACGAAATAAAAATCAAAGTTGACAAT	<i>cpe2093</i> ChIP-PCR

IMV525	TTGCTACAACCAACTAATACCATCA	<i>cpe2093</i> ChIP-PCR
IMV520	GAAATTTCCATTTTAAATTCCTACTAAAA	<i>cpe0664</i> ChIP-PCR
IMV521	AGCATCATCGCTCATTGCTA	<i>cpe0664</i> ChIP-PCR
EH40	TTGACAAATTTACTCGGTTTTGA	<i>cpe1786</i> ChIP-PCR
EH41	ATCAACCATGGCTTTTACAC	<i>cpe1786</i> ChIP-PCR
EH44	GAGGTGGCTTATGGAAGATAAAA	<i>cpe1031</i> ChIP-PCR
EH45	CTAGCTATCCCTTTGCACACA	<i>cpe1031</i> ChIP-PCR
EH50	GTGTTGGTGCCGCTTTAGG	<i>cpe1371</i> ChIP-PCR
EH51	GACCTGTAGTTCCAAGTATAAAAACAA	<i>cpe1371</i> ChIP-PCR

Table S2. Genomic identification of candidate IscR-binding sites in *Clostridia* spp.

Genome	First gene locus tag	regulated operon	Site		Candidate	IscR-binding site
			Position	Score		
<i>Clostridium perfringens</i> str. 13	CPE1786	<i>iscRSU-trmU</i>	-80	5.8	TTATTGTTGACAAATTTACTCGGTTTTGA	
			-39	5	ATAAAGTACATCGAATTAGTCAGATTTAG	
	CPE0103	<i>ldh</i>	-46	5.5	ATAATTCAGAGTAATTAAC TAGGAATTAG	
	CPE0664	<i>hesB</i>	-101	5.3	TAAATTCCTACTAAAATAGCTCTGATTTA	
			-76	4.8	TTATTTTGT TAAAAATATAAATTTAA	
	CPE2093	<i>yx eONM</i>	-69	5.3	TCAAAGTTGACAATTTTGATGGGAATTAA	
<i>Clostridium acetobutylicum</i> ATCC 824	CAC1675	<i>iscR</i>	-41	5.8	ACAAAGTAGAGTATTATGTCCGGAATTAA	
	CAC3288	<i>sufCBDSE</i>	-58	5.4	TGAATTCGACTTAAATGGTCAAGATTAA	
<i>Clostridium novyi</i> NT	NT01CX_228	<i>iscRSU-trmU</i>	-82	5.9	TTAATGTTGACAAAAACACTCGGATTTGA	
			-41	5.6	AGAATTCATAGTATAA CACTCGGATTTAA	
<i>Clostridium kluyveri</i> DSM 555	CKL_1317	<i>iscRSU</i>	-71	5.8	TTATTGTTGACAAGTTTACTCTGATTGA	
	CKL_0828	<i>sufCBDSE</i>	-41	5.1	TTAAATATGAGTCAA TACTCGGATTTAA	
<i>Clostridium butyricum</i> 5521	CBY_2622	<i>iscRSU-trmU</i>	-61	5.3	CAATAGCCGAGTATTTTGATAAGATATAA	
<i>Clostridium beijerincki</i> NCIMB 8052	Cbei_1097	<i>iscRSU-trmU</i>	-105	5.7	CAAAGCCGAGTATTTTGGTAAGATATAA	
	Cbei_4356	<i>cysK</i>	-55	5.2	TTATAACATAGTAAACTGATAAGAATTAT	
<i>Clostridium tetani</i> E88	CTC01049	<i>iscRSU-trmU</i>	-92	5.2	TTATAGTTGACAGGA TACTCGGATTTGT	
			-52	5.5	TGAAAGTTTAGTAAAGCAGTAGACTTTCA	
<i>Clostridium botulinum</i> A str. ATCC 35 CBO2569	<i>iscRSU</i>	-82	4.8	ATATTGTTGACAGGTTTAGTCTGATTTGC		
		-43	6	ATAAATCATAGTAAAGTGGTCAACATTAA		
<i>Clostridium bartlettii</i> DSM 16795	CLOBAR_021	<i>iscRSU-trmU</i>	-113	6.6	TTAATCTGACTAAAATAGTCAAATTTAA	
			-88	5.7	TTAATGTTGACAAAAACACTCAAGTATAA	
			-41	4.7	AGAAATCCTACTAAAAAGTAGGAATAAG	
<i>Clostridium hiranonis</i> DSM 13275	CLOHIR_0115	<i>iscRSU-trmU</i>	-103	5.9	ACAAATCATACTAAAATAGTAGGAAATAA	
			-77	5.7	TAATACTTGACAAAAATAGTCCGATATAA	
			-43	6	TTAAACCATAGTAAACAGTAGGAATTAA	
<i>Clostridium difficile</i> 630	CD1278	<i>iscRSU-trmU</i>	-98	6	TTAATCTTGACAAAATTAGTAGGGTATAA	
			-53	5.4	GTAATTCCTACTAAACAGTATGAATTAG	
<i>Clostridium</i> sp. OhILAs	Clos_1671	<i>iscRSU</i>	1	5.2	TGAATGTTGACAAAATAGTAGGGTATAC	
	Clos_0333	<i>cysK</i>	-43	5	ATAAAACCTATCGGTTTACTATGAATTAA	
<i>Clostridium cellulolyticum</i> H10	Ccel_1933	<i>iscRSU-trmU</i>	-93	6.1	TTAAATCATAGTAATTTAATTGGAATTAA	
	Ccel_3186	<i>cysK</i>	-106	5.2	TTAATTCATACTAATTAGATAGGAAATAT	
<i>Clostridium nexile</i> DSM 1787	CLONEX_030	<i>iscRSU-trmU</i>	-51	5.9	ATAAACCTAGTAATTC ACTAGGAATTAA	
<i>Clostridium scindens</i> ATCC 35704	CLOSCI_0139	<i>iscRSU-trmU</i>	18	5.7	AGAAATCCTAGCAAATGTCTAGGAATTAA	
<i>Clostridium</i> sp. SS2/1	CLOSS21_000	<i>iscRSU-trmU</i>	-38	5.4	TGAAATCCGAGTTATATAGTCCGGATTGA	
<i>Thermincola potens</i> JR	TherJR_1914	<i>iscRSU-trmU</i>	-120	5.2	AAAAATCTTAGTATTTTAGTTGGAATTTT	
			-50	4.8	CCATAACCGAGTGCAACTAGGAATTAA	