

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

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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 σA-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

Manuscript

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

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

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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 $IscR_{Cp}$ 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. $IscR_{Cp}$ controlled the expression of genes involved in Fe-S biogenesis, in amino-acid or sugar metabolisms, in fermentation pathways and in host compound utilization. We then demonstrated using a ChIP-PCR experiment that $IscR_{Cp}$ 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 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 $IscR_{Cp}$.

Introduction

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

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

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

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

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

Materials and Methods

Bacterial strains and culture conditions:

Escherichia coli strains were grown in LB medium and *B. subtilis* strains in SP or a sulfur-free minimal medium [7] containing either 1 mM methionine or 1 mM cystine. For the experiments involving expression of genes under the control of the *xylA* promoter, threonine (50 mg L^{-1}) and 0.1% xylose were added. When needed, ampicillin (100 μg/ml) or chloramphenicol (15 μg/ml) was added to *E. coli* cultures while chloramphenicol (5 μg/ml) or spectinomycin (60 μg/ml) was added for *B. subtilis* cultures. *C. perfringens* strains were grown under anaerobic conditions (10% H_2, 10% CO_2, 90% N_2) in TY (30 g L^{-1} bacto tryptone, 2g L^{-1} yeast extract, 1 g L^{-1} thioglycolate, pH7.4), BHI (37 g L^{-1}) or a sulfur-free minimal medium [1] containing either 1 mM cystine or 1 mM homocysteine. When necessary, thiamphenicol (5 μg/ml) or erythromycin (10 μg/ml) was added to *C. perfringens* cultures.

Strain and plasmid constructions

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

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

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

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

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

Enzyme assays and volatile fatty acid analysis

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

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

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

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

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

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

DNA-array hybridization and microarray analysis.

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

Chromatine immunoprecipitation of Cpe1786-XFlag bound to DNA

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

independent experiments was obtained.

Results

The Cpe1786 regulator of *C. perfringens*

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

Cpe1786 controls *cpe178 6*transcription in the heterologous host *B. subtilis*

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

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

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

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

Regulation of genes involved in iron-sulfur clusters biogenesis

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

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

Regulation of fermentation pathways

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

Regulation of genes involved in host compounds utilization

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

glucosaminidases. These enzymes could be involved either in peptidoglycan hydrolysis such as *B. subtilis* LytD protein or in the hydrolysis between two N-acetyl-glucosamine residues of glycoproteins. Two endo-β-N-acetyl-glucosaminidases that are active on $(Man)_6(GlcNAc)_2Asn$ and/or $(Man)_5(GlcNAc)_2Asn$ substrates exist in *C. perfringens* [14]. Cpe0818 probably degrades glycoproteins to provide mannose and N-Acetyl-glucosamine to *C. perfringens*. Interestingly, an operon encoding a PTS system (Cpe1463 to Cpe1466) belonging to the Mannose/Fructose family of PTS and a gene encoding a phosphomannomutase (Cpe1873) were also up-regulated in the *iscR* mutant. It is tempting to speculate that Cpe0818, the PTS system and the phosphomannomutase are involved in host glycoprotein degradation and in the uptake and utilization of the released sugars and that $IscR_{Cp}$ coordinately regulates the corresponding genes.

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

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

Identification of an $IscR_{Cp}$ binding motif

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

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

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

Discussion

In *E. coli* and several other bacteria, genes involved in Fe-S cluster biogenesis are regulated in response to Fe-S availability through the Fe-S regulatory protein IscR, and are induced during iron starvation and oxidative stress [32]. By contrast, only few data are available concerning the control of Fe-S cluster synthesis in Gram-positive bacteria, in anaerobic bacteria [31] or in response to sulfur availability [11]. In *C. perfringens*, we have previously shown a coordinated regulation in response to cysteine availability of genes involved in Fe-S production (*iscS, iscU, cpe0664*) [1]. We demonstrate in this work that the $IscR_{Cp}$ repressor mediates this control. Other genes encoding transporters (Cpe2093-Cpe2092 and Cpe1371) that might be involved in supplying for sulfur required for Fe-S biogenesis are also under the coordinated control of $IscR_{Cp}$. These regulations may allow *C. perfringens* maintaining its pools of Fe-S clusters, which play a crucial role in the physiology of clostridia lacking the heme synthesis machinery [17]. Interestingly, $IscR_{Cp}$ also controls the expression of genes involved in fermentation pathways (Fig. 2) and we observe accordingly a decrease of butyrate production and a drastic increase of lactate production in the *iscR* mutant. The pyruvate to acetyl-CoA conversion is catalyzed by the pyruvate ferredoxin-oxido-reductase (PFOR). This Fe-S enzyme forms CO_2 and acetyl-CoA by oxidizing pyruvate and reducing a 2Fe-4S ferredoxin. This step implies the utilization of several Fe-S clusters while lactate production by the lactate dehydrogenase does not. So, we propose that during cysteine limitation [1], $IscR_{Cp}$ could reroute the fermentation metabolism increasing *ldh* expression to reoxidize the NADH produced during glycolysis and limiting the utilization of Fe-S clusters. It is intriguing to note that in *C. acetobutylicum*, lactate production increases under conditions of iron limitation [4] while in *C. perfringens ldh* expression increases under conditions of sulfur limitation [1]. In both cases, IscR that is probably able to sense iron and sulfur availability might be involved in this control.

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

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

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

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

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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 α1
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 theses 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
E. coli		
DH5	F-ϕ80 *lacZΔ*M15 *Δ*(*lacZYA-argF*)U169 *deoR recA1 endA1 hsdR17*(r_k^-, m_k^-) *phoA supE44 thi-1 gyrA96 relA1* λ^-	Invitrogen
Top10	F^- *mcr*A D(*mrr-hsd*RMS-*mcr*BC) f80*lac*ZDM15 D*lac*X74 *deo*R, *rec*A1 *ara*D139 D(*ara-leu*)7697 *gal*K *rps*L(StrR) *end*A1 *nup*G	Invitrogen
B. subtilis		
168	*trpC2*	Laboratory stock
BSIP1798	*trpC2 ΔcymR$_{Bs}$ amyE::aphA3 lacZ*	[7]
BSIP1978	*trpC2 ΔcymR$_{Bs}$ amyE::*P(-131 +61) *iscR$_{Cp}$-lacZ cat*	pDIA5820→BSIP1798
BSIP1981	*trpC2 ΔcymR$_{Bs}$ amyE::*P(-131 +61)*iscR$_{Cp}$-lacZ cat thrC::*P*xylA-iscR$_{Cp}$ spc*	pDIA5744→BSIP1978
BSIP2018	*trpC2 ΔcymR$_{Bs}$ amyE::aphA3 lacZ thrC*::P*xylA-iscR$_{Cp}$ spc*	PAC7→BSIP1981
BSIP2020	*trpC2* Δ*cymR*$_{Bs}$ *amyE* ::P(-41,+61) *iscR$_{Cp}$-lacZ thrC*::P*xylA-iscR$_{Cp}$ spc*	PDIA5837→BSIP2018
BSIP2022	*trpC2* Δ*cymR*$_{Bs}$ *amyE* ::P*iscR$_{Cp}$*T-22G-*lacZ cat thrC*::P*xylA-iscR$_{Cp}$ spc*	pDIA5840 →BSIP2018
BSIP2024	*trpC2* Δ*cymR*$_{Bs}$ *amyE* ::P*iscR$_{Cp}$*G-19A-*lacZ cat thrC* ::P*xylA-iscR$_{Cp}$ spc*	pDIA5842 →BSIP2018
BSIP2029	*trpC2* Δ*cymR*$_{Bs}$ *amyE* ::P*iscR$_{Cp}$*T-22G/G-19A-*lacZ cat thrC* ::P*xylA-iscR$_{Cp}$ spc*	pDIA5846 →BSIP2018
C. perfringens		
13	Wild-type	[34]
CPIP01	*cpe1786/iscR::erm*	pMTL007-Cpe1786-28a → strain 13
CPIP11	*cpe1786/iscR::erm* pDIA5928-*iscR*-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-*cpe1786*-28a	Cm^R/Tm^R	This work
pDIA5744	pXT-*iscR$_{Cp}$*	This work
pDIA5820	pAC6 P(-131, +61) *iscR $_{Cp}$-lacZ*	This work
pDIA5837	pAC6 P(-41, +61) *iscR $_{Cp}$-lacZ*	This work
pDIA5840	pAC6 P(-131 +61) T-22G *iscR$_{Cp}$-lacZ*	This work
pDIA5842	pAC6 P(-131 +61) G-19A *iscR$_{Cp}$-lacZ*	This work
pDIA5846	pAC6 P(-131 +61) T-22G / G-19A *iscR$_{Cp}$-lacZ*	This work

pDIA5852	pAC6 P(-131 +61) T-15C *iscR*$_{Cp}$-*lacZ*	This work
pDIA5925	pGEMTeasy- *iscR*-XFlag	This work
pDIA5928	pGEMTeasy/pCB3 replicon/cat- *iscR*-XFlag	This work

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

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	Δ*cymR*$_{Bs}$ *amyE* ::P(-131,+61)*iscR*$_{Cp}$-*lacZ*	1070+/-90	1025+/-60
BSIP1981	Δ*cymR*$_{Bs}$ *amyE* ::P(-131,+61)*iscR*$_{Cp}$-*lacZ thrC* ::P*xyl*-*iscR*$_{Cp}$	1085+/-2	115+/-20
BSIP2020	Δ*cymR*$_{Bs}$ *amyE* ::P(-41,+61) *iscR* $_{Cp}$-*lacZ thrC* ::P*xyl*-*iscR*$_{Cp}$	730+/-17	58.5+/-1.5
BSIP2022	Δ*cymR*$_{Bs}$ *amyE* ::P(-131,+61) *iscR* $_{Cp}$-T-22G-*lacZ thrC* ::P*xyl*-*iscR*$_{Cp}$	1025+/-30	370+/-20
BSIP2024	Δ*cymR*$_{Bs}$ *amyE* ::P(-131,+61) *iscR*$_{Cp}$-G-19A-*lacZ thrC* ::P*xyl*-*iscR*$_{Cp}$	1070+/-30	380+/-5
BSIP2029	Δ*cymR*$_{Bs}$ *amyE* ::P(-131,+61) *iscR*$_{Cp}$- T-22G/G-19A-*lacZ thrC* ::P*xyl*-*iscR*$_{Cp}$	1050+/-24	515+/-10

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

Table 3. Genes differentially expressed between strain 13 of *C. perfringens* and a *iscR*$_{Cp}$ mutant.

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

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

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

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

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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: *Staphyloccoccus 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

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

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

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

Table S1. List of oligonucleotides

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

Figure

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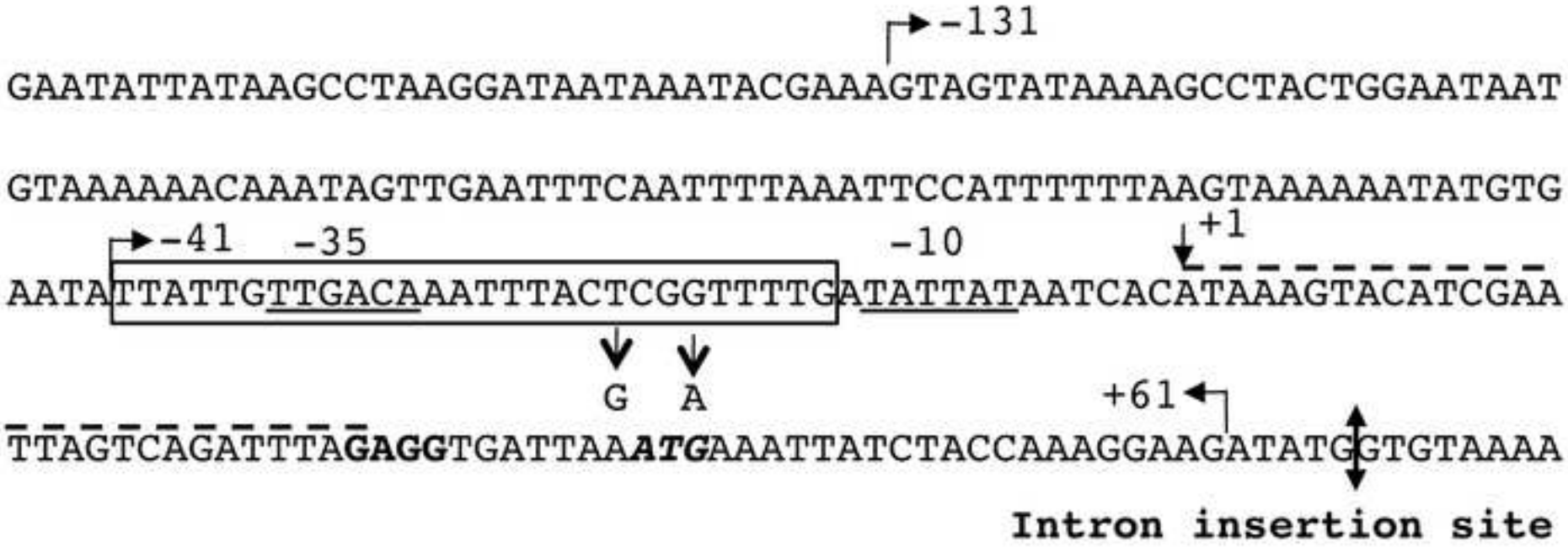


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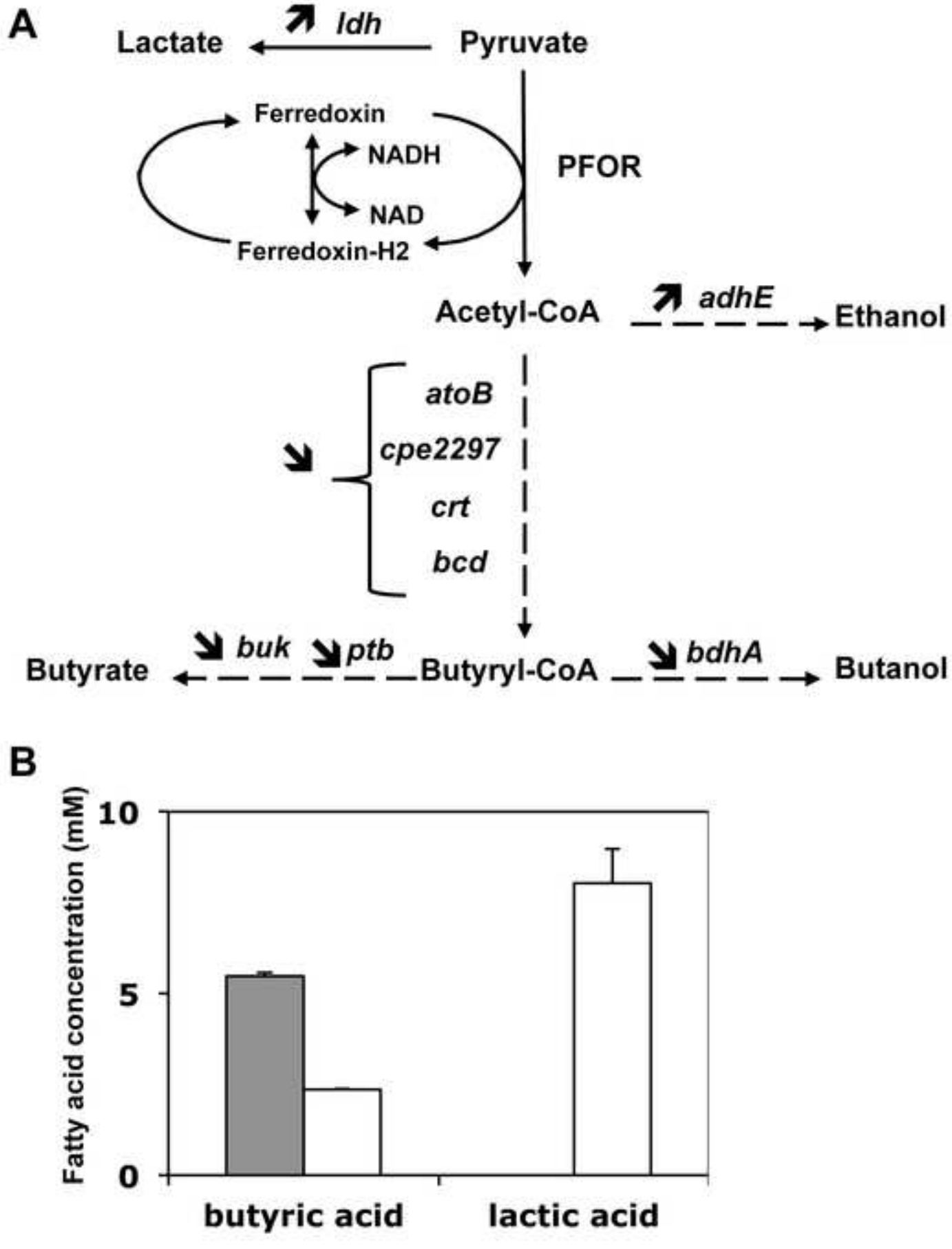


Figure 3

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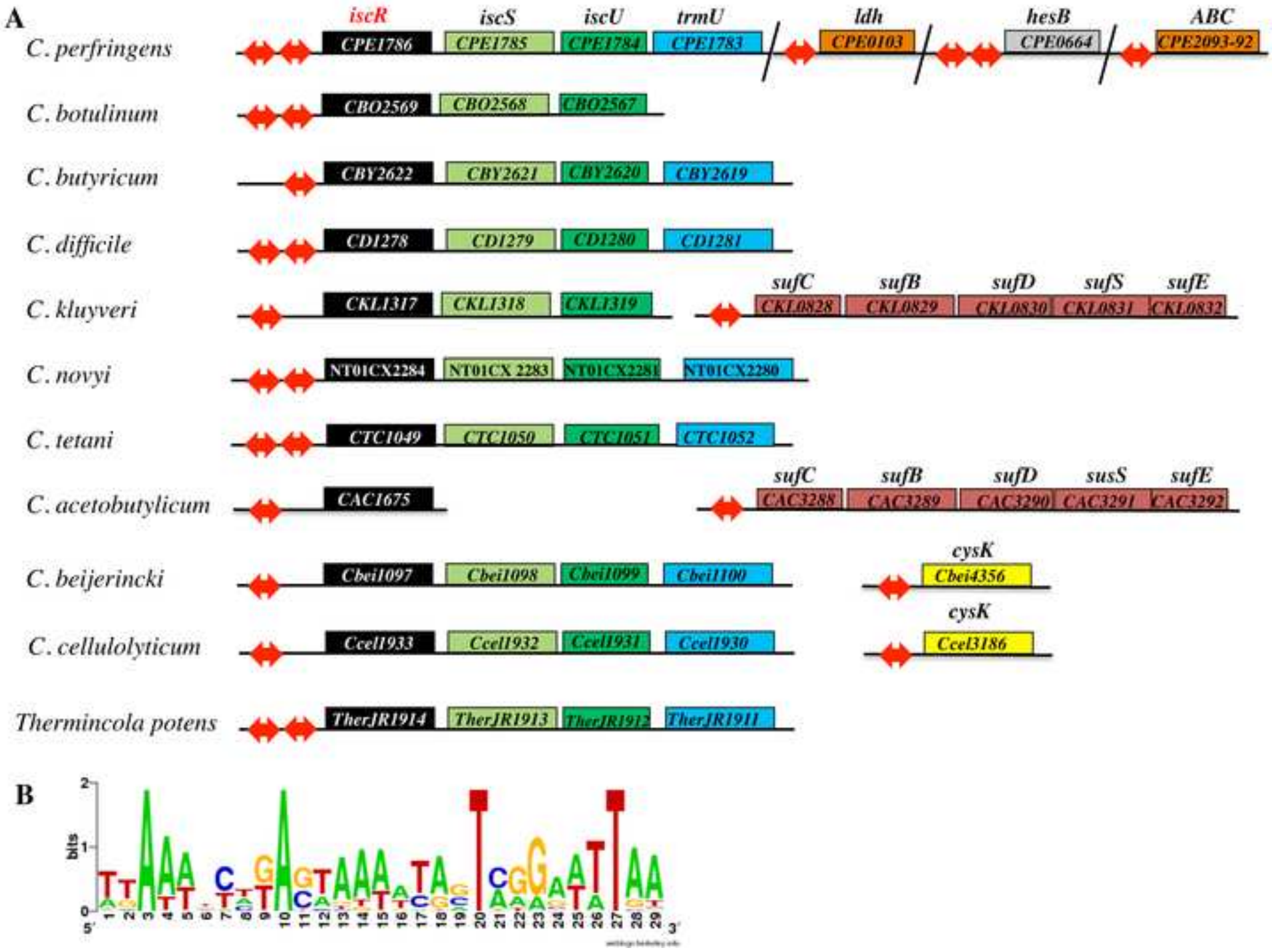


Figure S1

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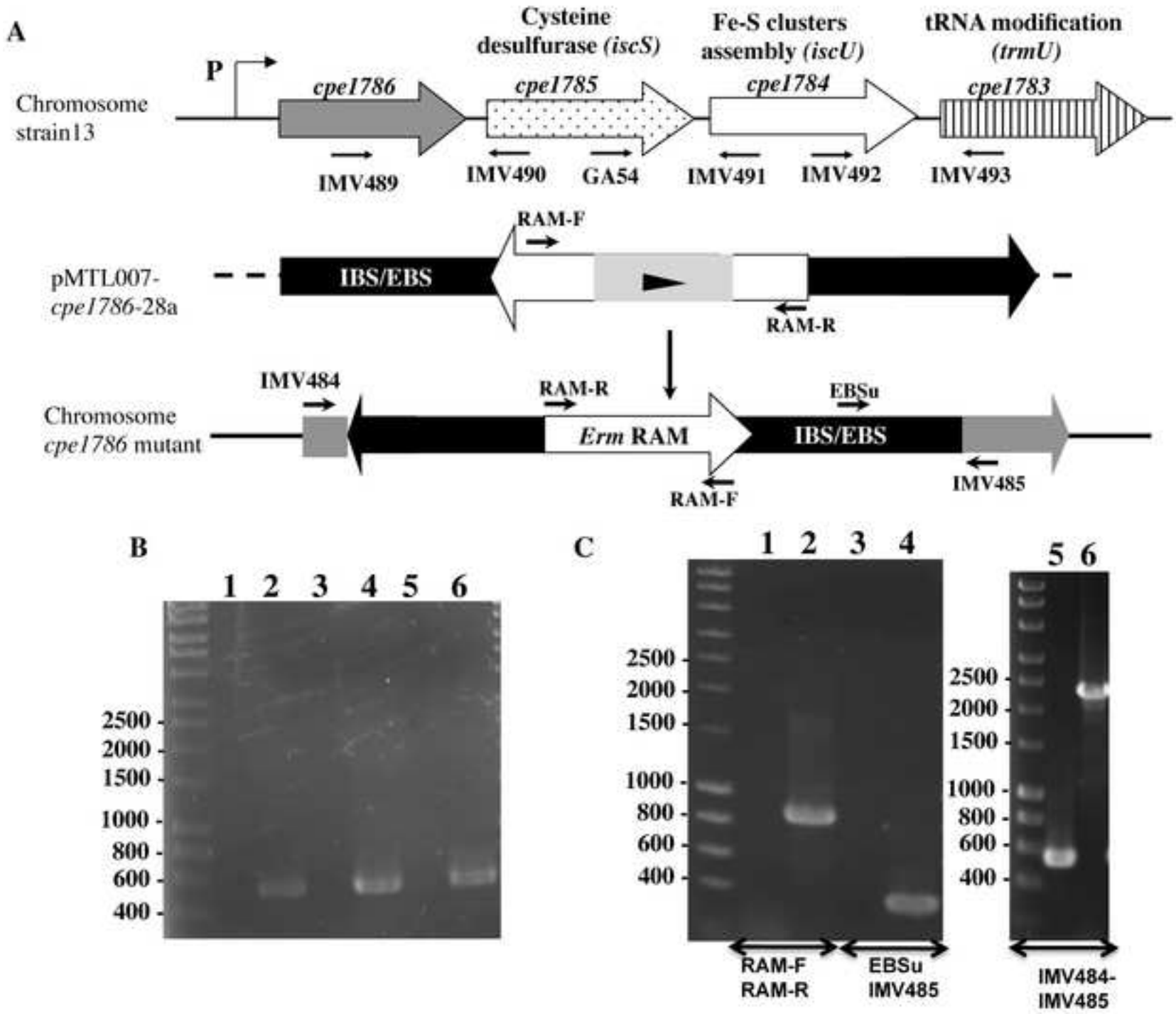


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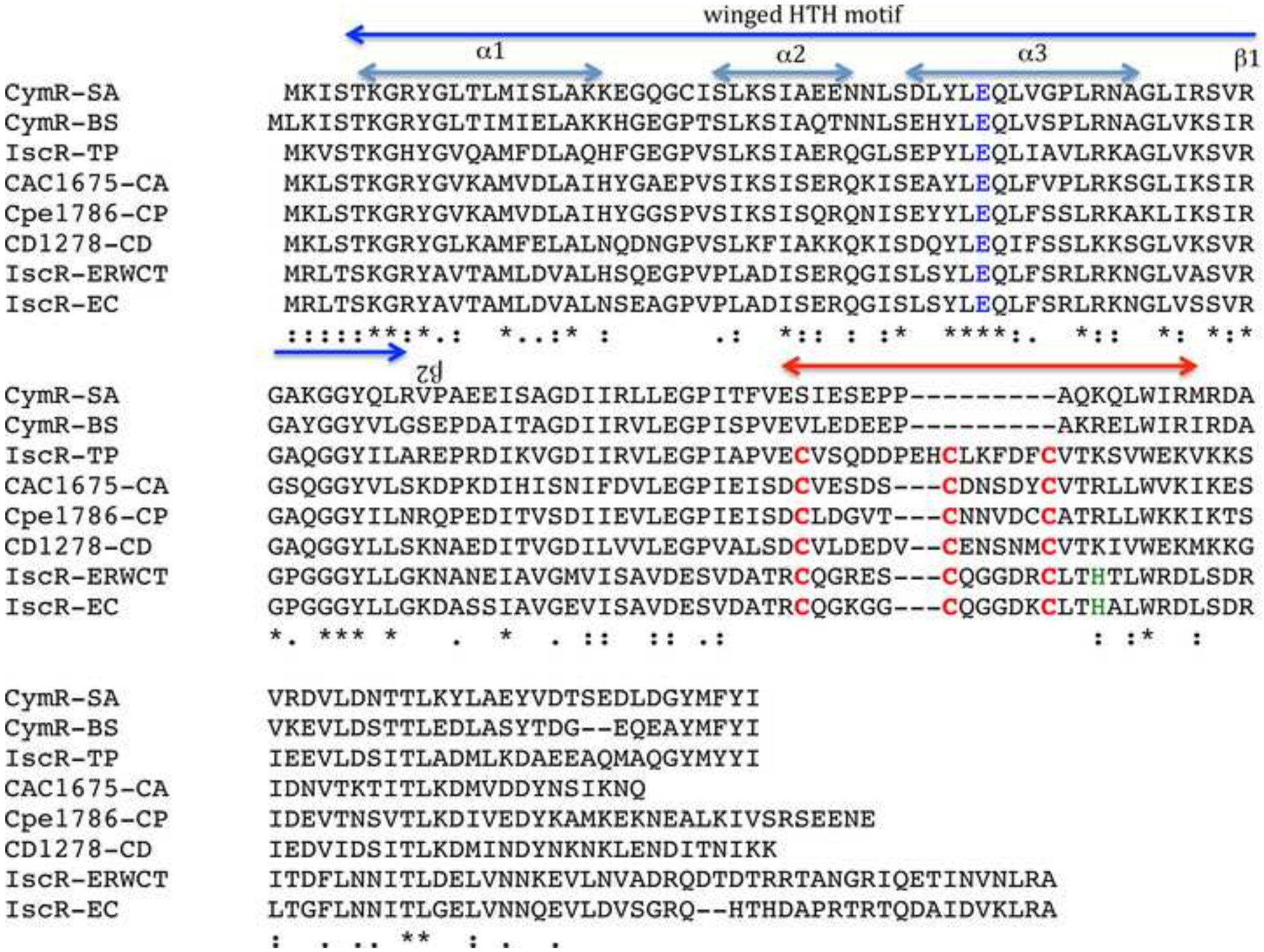


Figure S3

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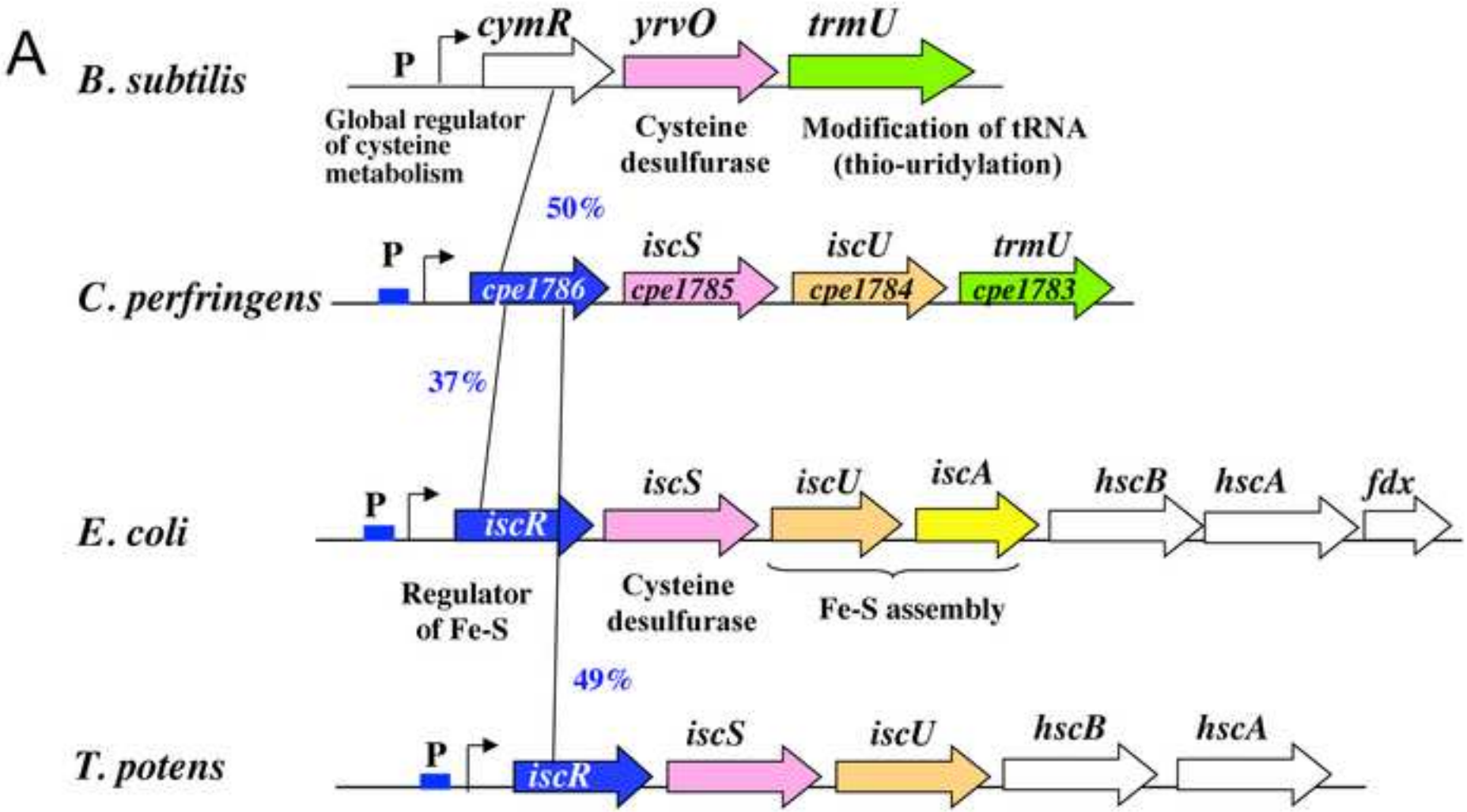


Figure S4

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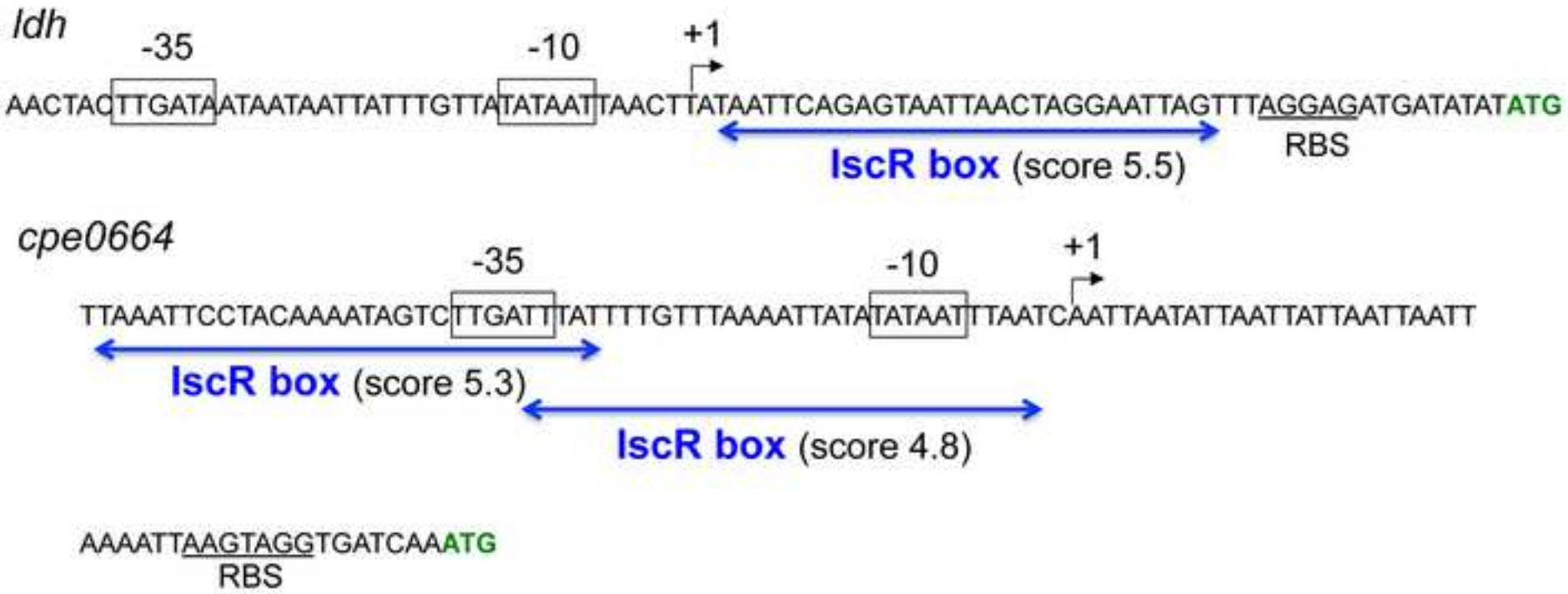


Table S1. Oligonucleotides used in this study.

Name	sequence	function
Quantitative RT-PCR		
GA99	TGCAGGATTTGTTGGTTCAA	5' *ldh*
GA100	GCACCTTGTGCTAGGTCCAT	3' *ldh*
GA103	GGTAGCAATGAGCGATGATG	5' *cpe0664*
GA104	TCCGTTCCCACCAAAGTTTA	3' *cpe0664*
GA105	CCTGGCTCTTTTGAAGGTGT	5' *cpe1371*
GA106	ACAACCATACCGCATCCATT	3' *cpe1371*
GA108	TCCCAATGTTACCCTTTTTGA	5' *cpe2092*
GA109	TTGCAAAGCTCATTTCATGG	3' *cpe2092*
GA110	CTCCTTACAAGGCACCAGGA	5' *cpe0818*
GA111	CCCTTTGGCTACTGGAACAC	3' *cpe0818*
GA112	ATGCCTGAAACAATGCCTTC	5' *cpe0866*
GA113	TTGATCCCAATCCCAGAAAG	3'*cpe0866*
GA114	TGGTACTGCTCCAACTTTCG	5' *cpe1031*
GA115	TTCCACCAACAACTACAGCATC	3' *cpe1031*
GA124	GAGGGAAACATTGGGGTTTT	5' *crt*
GA125	ATCAGCACCTGCAACAAATG	3' *crt*
GA126	TCAAAAGCAAGCTGAGTGGA	5' *nagL*
GA127	CATTGTTGCATGTCCTGTCC	3' *nagL*
GA30	TGCCAGAATAGTTGGGGAAG	5' *gyrA*
GA31	TACCATGTCCGTCAACAAGC	3' *gyrA*
5'RACE		
GA130	TTCATAGTCGCTCATTAATGGAT	5'RACE *cpe0664*
GA131	AGAAGAATCAATAACAAAAGTTAA	5'RACE *cpe0664* int
GA132	TACTAAAAGTATTGAGTTTGGGC	5'RACE *ldh*
GA133	GTTATTATAACTATGTCAGAG	5'RACE *ldh* int
GA51	TCCTCCTTGAGCACCTCTAA	5'RACE *cpe1786*
GA62	GTCTAGTCGCACAACAA	5'RACE *cpe1786* int
Plasmid constructions		
pMTL007-F	TTAAGGAGGTGTATTTCATATGACCATGATTACG	intron variable region
pMTL007-R	AGGGTATCCCCAGTTAGTGTTAAGTCTTGG	intron variable region
RAM-F	ACGCGTTATATTGATAAAAATAATAATAGTGGG	Erm cassette
RAM-R	ACGCGTGCGACTCATAGAATTATTTCCTCCCG	Erm cassette
EBSu	CGAAATTAGAAACTTGCGTTCAGTAAAC	intron internal
TBD043	AAAAAAGCTTATAATTATCCTTAGGATACGATACTGTGCGC CCAGATAGGGTG	IBS *cpe1786*
TBD044	CAGATTGTACAAATGTGGTGATAACAGATAAGTCGATACT GGTAACTTACCTTTCTTTGT	EBS1d *cpe1786*
TBD045	TGAACGCAAGTTTCTAATTTCGATTTATCCTCGATAGAGGA AAGTGTCT	EBS2 *cpe1786*
IMV484	GACAAATTTACTCGGTTTTGAT	5'*cpe1786*
IMV485	CTCTATTATTCATTTTCTTCACTC	3' *cpe1786*
IMV390	GGCGAATTCGTAGTATAAAAGCCTACTGG	*cpe1786*-P-131 EcoRI
IMV391	GGCGAATTCTTATTGTTGACAAATTTACT	*cpe1786* P-41 EcoRI
IMV394	GGCGGATCCCTTCCTTTGGTAGATAA	*cpe1786* P+61 BamHI
IMV439	TTGTTGACAAATTTACGCGGTTTTGATATTATA	T-22G P-*cpe1786*
IMV440	TATAATATCAAAACCGCGTAAATTTGTCAACAA	T-22G P-*cpe1786*
IMV441	TTGACAAATTTACTCGATTTTGATATTATAATC	G-19A P-*cpe1786*
IMV442	GATTATAATATCAAAATCGAGTAAATTTGTCAA	G-19A P-*cpe1786*
IMV455	TTGTTGACAAATTTACGCGATTTTGATATTATAATC	T-22G/G-19A P-*cpe1786*
IMV456	GATTATAATATCAAAATCGCGTAAATTTGTCAACAA	T-22G G-19A P-*cpe1786*
EH18	AAATCTAGATCA**CTA**CTTG*T*CA*T*CGTCATCCTTGTAGTCGATGTCATGATCT TTATAATCACCGTCATGGTCTTTGTAGTCTTCATTTTCTTCACTCCTACT	Fusion of a XFlag at the end of *cpe1786*
ChIP-PCR		
IMV524	ACGAAATAAAAATCAAAGTTGACAAT	*cpe2093* ChIP-PCR

IMV525	TTGCTACAACCAACTAATACCATCA	*cpe2093* ChIP-PCR
IMV520	GAAATTTCCATTTTAAATTCCTACTAAAA	*cpe0664* ChIP-PCR
IMV521	AGCATCATCGCTCATTGCTA	*cpe0664* ChIP-PCR
EH40	TTGACAAATTTACTCGGTTTTGA	*cpe1786* ChIP-PCR
EH41	ATCAACCATGGCTTTTACAC	*cpe1786* ChIP-PCR
EH44	GAGGTGGCTTATGGAAGATAAAA	*cpe1031* ChIP-PCR
EH45	CTAGCTATTCCCTTTGCACACA	*cpe1031* ChIP-PCR
EH50	GTGTTGGTGCCGCTTTAGG	*cpe1371* ChIP-PCR
EH51	GACCTGTAGTTCCAAGTATAAAAACAA	*cpe1371* ChIP-PCR

Table

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

Genome	First gene locus tag	regulated operon	Site Position	Site Score	Candidate IscR-binding site
Clostridium perfringens str. 13	CPE1786	*iscRSU-trmU*	-80	5.8	TTATTGTTGACAAATTTACTCGGTTTTGA
			-39	5	ATAAAGTACATCGAATTAGTCAGATTTAG
	CPE0103	*ldh*	-46	5.5	ATAATTCAGAGTAATTAACTAGGAATTAG
	CPE0664	*hesB*	-101	5.3	TAAATTCCTACTAAAATAGTCTTGATTTA
			-76	4.8	TTTATTTTGTTTAAAATTATATAATTTAA
	CPE2093	*yxeONM*	-69	5.3	TCAAAGTTGACAATTTTGATGGGAATTAA
Clostridium acetobutylicum ATCC 82	CAC1675	*iscR*	-41	5.8	ACAAAGTAGAGTATTATTGTCGGAATTAA
	CAC3288	*sufCBDSE*	-58	5.4	TGAATTCCGACTTAAATGGTCAAGATTAA
Clostridium novyi NT	NT01CX_228	*iscRSU-trmU*	-82	5.9	TTAATGTTGACAAAAACACTCGGATTTGA
			-41	5.6	AGAATTCATAGTATAACACTCGGATTTAA
Clostridium kluyveri DSM 555	CKL_1317	*iscRSU*	-71	5.8	TTATTGTTGACAAGTTTACTCTGATTTGA
	CKL_0828	*sufCBDSE*	-41	5.1	TTAAATATGAGTCAAATACTCGGATTTAA
Clostridium butyricum 5521	CBY_2622	*iscRSU-trmU*	-61	5.3	CAATAGCCGAGTATTTTGATAAGATATAA
Clostridium beijerincki NCIMB 8052	Cbei_1097	*iscRSU-trmU*	-105	5.7	CAAAAGCCGAGTATTTTGGTAAGATATAA
	Cbei_4356	*cysK*	-55	5.2	TTATAACATAGTAAACTGATAAGAATTAT
Clostridium tetani E88	CTC01049	*iscRSU-trmU*	-92	5.2	TTATAGTTGACAGGAATACTCGGATTTGT
			-52	5.5	TGAAAGTTTAGTAAAGCAGTAGACTTTCA
Clostridium botulinum A str. ATCC 35	CBO2569	*iscRSU*	-82	4.8	ATATTGTTGACAGGTTTAGTCTGATTTGC
			-43	6	ATAAATCATAGTAAAGTGGTCAACATTAA
Clostridium bartlettii DSM 16795	CLOBAR_021	*iscRSU-trmU*	-113	6.6	TTAATTCTGACTAAAATAGTCAAAATTAA
			-88	5.7	TTAATGTTGACAAAAACACTCAAGTATAA
			-41	4.7	AGAAATCCTACTAAAAAAGTAGGAATAAG
Clostridium hiranonis DSM 13275	CLOHIR_011	*iscRSU-trmU*	-103	5.9	ACAAATCATACTAAAATAGTAGGAAATAA
			-77	5.7	TAATACTTGACAAAAATAGTCGGATATAA
			-43	6	TTAAACCATAGTAAAACAGTAGGAATTAA
Clostridium difficil e 630	CD1278	*iscRSU-trmU*	-98	6	TTAATCTTGACAAAATTAGTAGGGTATAA
			-53	5.4	GTAATTCCTACTAAAACAGTATGAATTAG
Clostridium sp. OhILAs	Clos_1671	*iscRSU*	1	5.2	TGAATGTTGACAAAAATAGTAGGGTATAC
	Clos_0333	*cysK*	-43	5	ATAAAACCTATCGGTTTACTATGAATTAA
Clostridium cellulolyticum H10	Ccel_1933	*iscRSU-trmU*	-93	6.1	TTAAATCATAGTAATTTAATTGGAATTAA
	Ccel_3186	*cysK*	-106	5.2	TTAATTCATACTAATTAGATAGGAAATAT
Clostridium nexile DSM 1787	CLONEX_030	*iscRSU-trmU*	-51	5.9	ATAAACCCTAGTAATTCACTAGGAATTAA
Clostridium scindens ATCC 35704	CLOSCI_0139	*iscRSU-trmU*	18	5.7	AGAAATCCTAGCAAATTGCTAGGAATTAA
Clostridium sp. SS2/1	CLOSS21_00	*iscRSU-trmU*	-38	5.4	TGAAATCCGAGTTATATAGTCGGGATTGA
Thermincola potens JR	TherJR_1914	*iscRSU-trmU*	-120	5.2	AAAAATCTTAGTATTTTAGTTGGAATTTT
			-50	4.8	CCATAACCGAGTGCAACACTAGGAATTAA