Cpe1786/IscR of Clostridium perfringens represses expression of genes involved in Fe-S cluster biogenesis.
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Cpe1786/IscR of Clostridium perfringens, represses expression of genes involved in FeS 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
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 $\sigma^A$-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\textsubscript{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 Scal 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$\rightarrow$G or G$\rightarrow$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 (ΔcymR P(-131 +61)cpe1786$_{Cy}$-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 (Pmel-Ascl fragment treated by Klenow) [13] was cloned into the Nael 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 1 µl 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: $2((Ct_{\text{target}} - Ct_{\text{gyrA}})_{\text{CPIP11}}/(Ct_{\text{target}} - Ct_{\text{gyrA}})_{\text{CPIP01}}$. 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 *trmU* 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 *cpe1786* 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 ΔcymR* mutant or a ΔcymR 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<sub>Cp</sub>. 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<sub>Cp</sub> 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<sub>Cp</sub> 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 28th 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<sub>Cp</sub> 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<sub>Cp</sub> (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<sub>Cp</sub> 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<sub>Cp</sub> 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)$_2$Asn and/or (Man)$_5$(GlcNAc)$_2$Asn 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<sub>Cp</sub>, we first compared the promoter regions of <i>iscR</i> and <i>cpe2093</i>. We identified a conserved sequence upstream of these two promoters (Fig. S3B). In the <i>iscR</i> 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<sub>Cp</sub> of its own transcription in <i>B. subtilis</i> (Table 2). Interestingly, this motif is very similar to the IscR binding sites of the <i>iscR</i> promoter regions of <i>E. coli</i> and <i>T. potens</i> [9, 31] and is conserved in the <i>iscR</i> promoter regions of several clostridia (see discussion). The alignment of the <i>iscR</i> promoters of <i>E. coli</i>, <i>T. potens</i> and <i>C. perfringens</i> and the <i>cpe2093</i> promoter allowed proposing a conserved motif, AWWGTTGACMAWWW<sub>TRMTSGGNWWT</sub> (Fig. 3SB). In all cases, this motif overlaps the -35 boxes of the promoters. To confirm the involvement of this motif in <i>iscR</i><sub>Cp</sub> regulation, two point mutations were introduced in conserved nucleotides in this sequence. The P<i>iscR-lacZ</i> fusions containing mutations were introduced at the <i>amyE</i> locus of a <i>B. subtilis ∆cymR thrC::P<sub>xyLA</sub>-iscR</i><sub>Cp</sub> 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 <i>iscR</i> expression in the presence of cystine while in a double mutant (T-22G/G-19A), the expression of <i>iscR</i> 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 <i>iscR</i><sub>Cp</sub>, a repression mediated by IscR<sub>Cp</sub>. The RegPredict web-server [23] was subsequently used to search for similar DNA motifs and reconstruct candidate IscR regulons in the genomes of 16 <i>Clostridium</i> 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 <i>iscRSU</i> gene cluster in each analyzed genome. We also identified a second potential IscR binding site located downstream of the transcriptional start site of <i>iscR</i><sub>Cp</sub> (Fig. 1 and 3). Additional candidate sites were found upstream of the <i>suf</i> operons in <i>C. acetobutylicum</i> and <i>C. kluwyeri</i>, and of the <i>cysK</i> gene in <i>C. cellulolyticum</i> and <i>C. beijerinckii</i>. Finally, we found additional IscR sites upstream of genes controlled by IscR<sub>Cp</sub> in the obtained <i>C. perfringens</i> transcriptome (Table 3). These include potential IscR binding sites in the promoter regions of <i>cpe0664</i> encoding an A type carrier for Fe-S clusters biogenesis and the lactate dehydrogenase gene <i>ldh</i> (Fig. 3 and Table S2). We mapped the promoters of <i>cpe0664</i> and <i>ldh</i> 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-oxidoreductase (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<sub>Cp</sub> binding motif. However, we fail to detect a clear binding of IscR<sub>Cp</sub> 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<sub>Cp</sub> 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<sub>Cp</sub> 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<sub>Cp</sub>. 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<sub>Cp</sub> 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
for Fe-S biogenesis and the possible existence of a coordinated regulation of the isc genes and the cysK gene might allow supplying for sulfur required for Fe-S production.

Finally, the control by IscR<sub>Cp</sub> of genes encoding proteins involved in host compound degradation suggests that IscR<sub>Cp</sub> might play a role during the host colonization or the infection by C. perfringens. This is in agreement with recent works showing that in several pathogenic Gram-negative bacteria, IscR controls expression of factors involved in virulence or allowing adaptation to hostile conditions encounters in the host [18, 28].

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Figure legends

Figure 1. C. perfringens cpe1786/iscR<sub>Cp</sub> promoter region.

The initiation of transcription start site « +1 » identified by 5’RACE is indicated by an arrow. The -10 and -35 boxes of the σ<sup>70</sup>-dependent promoter are underlined. The ribosome-binding site is indicated in bold while the translational start site of cpe1786 is indicated in bold and italic. In the genome of C. perfringens 13, the translational start codon of cpe1786 is a GTG corresponding to a valine leading to a truncation of 25 amino acids and the absence of the α1 helix of the wHTH motif (Fig. S2). We rather proposed the ATG preceded by a ribosome binding site (GAGG). This start codon corresponds to the start of CPF_2040, the homologs of Cpe1786 in the genome of C. perfringens ATCC 13124 [19]. Broken arrows indicate the end points of the iscR-lacZ fusions. The conserved motif present upstream of the iscR genes in clostridia is boxed. Point mutations obtained in this motif are indicated. A second potential IscR binding motif (see Table S2) is over-lined.

Figure 2. Effect of iscR<sub>Cp</sub> gene inactivation on fermentation pathways. (A) Genes involved in fermentation pathways differentially expressed in an iscR mutant compared to strain 13. Arrows and <strong>▽</strong> indicated genes whose expression increased or decreased in an iscR mutant compared to strain 13. (B) Changes in metabolic end products in the C. perfringens strain 13 and the iscR mutant. Chromatography analysis from these two strains after 48 h of growth in minimal medium containing cystine was performed. The mean and standard error of two experiments are shown.
Figure 3. Candidate IscR regulons reconstructed by comparative genomics approach in **Clostridia spp.** (A) Chromosomal clusters of candidate IscR-regulon genes in Clostridia and *Thermincola potens*. Homologous genes are marked by the same color. Potential IscR-binding sites are shown by red arrows. (B) Consensus sequence logo of the predicted IscR-binding sites in Clostridia. The logo was generated from the sequence alignments with WebLogo (http://weblogo.berkeley.edu).

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

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DH5</td>
<td>F-φ80 lacZΔM15 Δ(lacZYA-argF)U169 deoR recA1 endA1 hsdR17(k^R,m^R) phoA supE44 thi-1 gyrA96 relA1 λ^-</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>Top10</td>
<td>F- mcrA D(mrr-hsdRMS-merBC) b80lacZDM15 DlacX74 deoR, recA1 araD139 D(ara-leu)7697 galK rpsL(StrR) endA1 nupG</td>
<td>Invitrogen</td>
</tr>
<tr>
<td><em>B. subtilis</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>168</td>
<td>trpC2</td>
<td>Laboratory stock</td>
</tr>
<tr>
<td>BSIP1798</td>
<td>trpC2 ΔcymR88, amyE::aphA3 lacZ</td>
<td>[7]</td>
</tr>
<tr>
<td>BSIP1978</td>
<td>trpC2 ΔcymR88, amyE::P(-131 +61) iscR_Cp-lacZ cat</td>
<td>pDIA5820→BSIP1798</td>
</tr>
<tr>
<td>BSIP2020</td>
<td>trpC2 ΔcymR88, amyE::P(-41,+61)iscR_Cp-lacZ thrC::PxyLA-iscR_Cp,spc</td>
<td>PDIA5837→BSIP2018</td>
</tr>
<tr>
<td><em>C. perfringens</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>Wild-type</td>
<td>[34]</td>
</tr>
<tr>
<td>CPIP01</td>
<td>cpe1786/iscR::erm</td>
<td>pMTL007-Cpe1786-28a</td>
</tr>
<tr>
<td>CPIP11</td>
<td>cpe1786/iscR::erm pDIA5928-iscR-Xflag</td>
<td>pDIA5928→CPIP01</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
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<td></td>
</tr>
<tr>
<td>pMTL007</td>
<td>group II intron, ErmBdRAM2 and ltrA ORF from pMTL20lacZTTErmBdRAM2, Cm^R/Tm^k</td>
<td>[12]</td>
</tr>
<tr>
<td>pMTL83151</td>
<td>pCB102 replicon, Cm^k</td>
<td>[13]</td>
</tr>
<tr>
<td>pAC6</td>
<td></td>
<td>[38]</td>
</tr>
<tr>
<td>pMTL007-cpe1786-28a</td>
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<td></td>
</tr>
<tr>
<td>pDIA5744</td>
<td>pXT-iscR_Cp</td>
<td>This work</td>
</tr>
<tr>
<td>pDIA5820</td>
<td>pAC6 P(-131, +61) iscR_Cp-lacZ</td>
<td>This work</td>
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<tr>
<td>pDIA5837</td>
<td>pAC6 P(-41, +61) iscR_Cp-lacZ</td>
<td>This work</td>
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<tr>
<td>pDIA5840</td>
<td>pAC6 P(-131 +61) T-22G iscR_Cp-lacZ</td>
<td>This work</td>
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<tr>
<td>pDIA5842</td>
<td>pAC6 P(-131 +61) G-19A iscR_Cp-lacZ</td>
<td>This work</td>
</tr>
<tr>
<td>pDIA5846</td>
<td>pAC6 P(-131 +61) T-22G / G-19A iscR_Cp-lacZ</td>
<td>This work</td>
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</table>
Table 2. Regulation of \textit{iscR}_{\text{Cp}} expression by \textit{IscR}_{\text{Cp}} in \textit{B. subtilis}.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>(\beta)-galactosidase activity (nmol ONP/min/mg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Methionine</td>
<td>Cystine</td>
</tr>
<tr>
<td>BSIP1978</td>
<td>(\Delta\text{cymR}<em>{\text{Bs}}) \text{amyE ::P(-131,+61)}iscR</em>{\text{Cp}}-lacZ</td>
<td>1070+/−90</td>
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<tr>
<td>BSIP1981</td>
<td>(\Delta\text{cymR}<em>{\text{Bs}}) \text{amyE ::P(-131,+61)}iscR</em>{\text{Cp}}-lacZ \text{thrC ::P}<em>{\text{Xyl}}-\text{iscR}</em>{\text{Cp}}</td>
<td>1085+/−2</td>
</tr>
<tr>
<td>BSIP2020</td>
<td>(\Delta\text{cymR}<em>{\text{Bs}}) \text{amyE ::P(-41,+61)}iscR</em>{\text{Cp}}-lacZ \text{thrC ::P}<em>{\text{Xyl}}-\text{iscR}</em>{\text{Cp}}</td>
<td>730+/−17</td>
</tr>
<tr>
<td>BSIP2022</td>
<td>(\Delta\text{cymR}<em>{\text{Bs}}) \text{amyE ::P(-131,+61)}iscR</em>{\text{Cp}}-T-22G-lacZ \text{thrC ::P}<em>{\text{Xyl}}-\text{iscR}</em>{\text{Cp}}</td>
<td>1025+/−30</td>
</tr>
<tr>
<td>BSIP2024</td>
<td>(\Delta\text{cymR}<em>{\text{Bs}}) \text{amyE ::P(-131,+61)}iscR</em>{\text{Cp}}-G-19A-lacZ \text{thrC ::P}<em>{\text{Xyl}}-\text{iscR}</em>{\text{Cp}}</td>
<td>1070+/−30</td>
</tr>
<tr>
<td>BSIP2029</td>
<td>(\Delta\text{cymR}<em>{\text{Bs}}) \text{amyE ::P(-131,+61)}iscR</em>{\text{Cp}}-T-22G/G-19A-lacZ \text{thrC ::P}<em>{\text{Xyl}}-\text{iscR}</em>{\text{Cp}}</td>
<td>1050+/−24</td>
</tr>
</tbody>
</table>

\(\beta\)-galactosidase activity was measured on crude extracts from strains grown in minimal medium in the presence of 1 mM methionine or 1 mM cystine. \(\text{iscR}_{\text{Cp}}\) = \text{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 \textit{C. perfringens} and a \textit{iscR}_{\text{Cp}} mutant.

<table>
<thead>
<tr>
<th>gene (Synonym)</th>
<th>Functions/similarities</th>
<th>Transcriptome quantative RT-PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(\text{iscR}_{\text{Cp}})/13</td>
</tr>
<tr>
<td><strong>Fe-S clusters and redox functions</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>\text{cpe0664}</td>
<td>HesB-like protein (114 aa)</td>
<td>12.5</td>
</tr>
<tr>
<td>\text{cpe1785 (iscS)}</td>
<td>Cysteine desulfurase, Fe-S clusters biosynthesis</td>
<td>2.1</td>
</tr>
<tr>
<td>\text{cpe1784 (iscU)}</td>
<td>Fe-S clusters assembly</td>
<td>2</td>
</tr>
<tr>
<td>\text{cpe1783 (trmU)}</td>
<td>Methylaminomethyl-2-Thiouridylate-Methyltransferase</td>
<td>2</td>
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<tr>
<td>\text{cpe2511 (fer)}</td>
<td>Ferredoxin [3Fe-4S]</td>
<td>0.29</td>
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<tr>
<td>\text{cpe0855 (rubY)}</td>
<td>Ruberythrin</td>
<td>0.17</td>
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<td><strong>Transporters, membrane or exported proteins</strong></td>
<td></td>
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<tr>
<td>\text{cpe2092}</td>
<td>Amino acid ABC transporter, ATP binding cassette</td>
<td>10.2</td>
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<td>\text{cpe2093}</td>
<td>Amino acid ABC transporter, permease</td>
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<td>\text{cpe2295 (lepW)}</td>
<td>Type I Signal peptidase</td>
<td>6.6</td>
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<td>\text{cpe1371}</td>
<td>Na\textsuperscript+ dependent symporter</td>
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<tr>
<td>\text{cpe1621}</td>
<td>putative cation efflux protein</td>
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<td>\text{cpe1343}</td>
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<td>\text{cpe1341}</td>
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<td><strong>Regulators</strong></td>
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<tr>
<td>\text{cpe1031}</td>
<td>ArsR-SmtB family regulator</td>
<td>7.8</td>
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<tr>
<td>Gene ID</td>
<td>Gene Name</td>
<td>Function</td>
</tr>
<tr>
<td>---------</td>
<td>-----------</td>
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<tr>
<td>cpe2304</td>
<td>ArsR-SmtB family regulator</td>
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<tr>
<td>cpe0103 (ldh)</td>
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<tr>
<td>cpe2351 (adhE)</td>
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<tr>
<td>cpe2195 (atoB)</td>
<td>Acetyl-CoA acetyltransferase</td>
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<tr>
<td>cpe2297</td>
<td>3-hydroxybutyryl-CoA dehydrogenase</td>
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<td>cpe2298 (fixB)</td>
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<tr>
<td>cpe2299 (etfB)</td>
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<tr>
<td>cpe2300 (bcd)</td>
<td>Acyl-CoA dehydrogenase</td>
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<tr>
<td>cpe2301 (crt)</td>
<td>3-hydroxybutyryl-CoA dehydratase</td>
<td>0.33 0.5</td>
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<tr>
<td>cpe0892</td>
<td>Butanol dehydrogenase NADPH dependent</td>
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<tr>
<td>cpe2347 (buk)</td>
<td>Butyrate kinase</td>
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<td>cpe2348 (ptb)</td>
<td>Phosphotransbutyrylase</td>
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<td>cpe1185 (pjk)</td>
<td>6-phosphofructokinase</td>
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<td>cpe1299 (eno)</td>
<td>Enolase</td>
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<td>cpe2149 (pykA)</td>
<td>Pyruvate kinase</td>
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<td>cpe2267</td>
<td>Glucose-6-phosphate isomerase</td>
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<tr>
<td>cpe1463</td>
<td>PTS system IID component (IIDMan)</td>
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<td>1.45</td>
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<tr>
<td>cpe1465</td>
<td>PTS system IIB component (IIDMan)</td>
<td>1.53</td>
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<tr>
<td>cpe1466</td>
<td>PTS system IIA component (IIAMan)</td>
<td>2.65</td>
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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\textsubscript{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.

References


**Supplementary data**

**Figure S1.** Genetic organization of the cpe1786/iscR locus and inactivation of the cpe1786 gene. The ClosTron delivery system (A) encoded on plasmid pMTL007 consists of a group II intron (black arrow) with an internal Retrotransposition-Activated Marker (RAM) conferring erythromycin resistance (white arrow) that is itself interrupted by the td group I intron (hatched box) [12]. The group II intron is retargeted to the cpe1786 gene (grey arrow) by altering the sequence of the IBS/EBS region using overlap PCR. Splicing of the group II intron into cpe1786 disrupts it and the splicing out of the td group I intron from the erm RAM restores a functional ermB gene allowing positive selection of cpe1786 mutants. B) Analysis of genetic organization of the cpe1786 locus by RT-PCR experiments. cDNA were synthesized using RNA extracted from cells grown in the presence of homocysteine. Couples of primers used for PCRs are indicated in panel A. Agarose gel corresponds to the migration of the different PCR fragments obtained from RNA without (lane 1,3,5) or with (lane 2,4,6) a reverse transcriptase treatment. Lane 1 and 2: IMV489-IMV490, lane 3 and 4: GA54-IMV491, lane 5 and 6: IMV492-493.

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

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

Click here to download high resolution image

GAATATTATAAGCCCTAAGGGATAATAATATACGAAAGTATGTAATAAAAGCCTACTGGAATAAT

GTAAAAAACAAATAGTTGAATTTCATTTTAAATTTCTCATTTTTTAAAGTAAAAATATGTG

AATATTATTGTGGACAAATTACTCGGTGTTTGATATTATAATCACATAAAGTACATCGAA

TTAGTCAGATTGAGGTTGATTAATGAAATTATATCTACCAGGAAGATATATGTGTTAAAA

Intron insertion site
**Figure S3**

**A**

- **B. subtilis**
  - Global regulator of cysteine metabolism
  - Cysteine desulfurase
  - Modification of tRNA (thio-uridylation)

- **C. perfringens**
  - Cpe1786
  - Cpe1785
  - Cpe1784
  - Cpe1783

- **E. coli**
  - IscR
  - Cysteine desulfurase
  - Fe-S assembly

- **T. potens**
  - IscR

**B**

- **iscR**
  - E.C: ATAGTTGAACCAATTATTACTCGGAAT
  - T.P: ATAGTTGAACCAATTATTACTCGGAAT
  - C.P: ATGGTTGAACAAATTTTACTCGGTTTT

- **cpe2093**
  - AAGGTGACACTTTTGATGGGAATT
  - AWWGTTGACMAWWWTRMTSGGNWWT
Table S1. Oligonucleotides used in this study.

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

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