Identification of iron-regulated genes of *Bifidobacterium breve* UCC2003 as a basis for controlled gene expression

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Iron is an essential growth factor for virtually all organisms. However, iron is not readily available in most environments and microorganisms have evolved specialized mechanisms, such as the use of siderophores and high-affinity transport systems, to acquire iron when confronted with iron-limiting conditions. In general these systems are tightly regulated to prevent iron-induced toxicity and because they are quite costly to the microbe. Because of this tight regulation we chose to explore the response of *Bifidobacterium breve* UCC2003 to iron limitation. Through microarray and complementation analyses we identified and characterized a presumed ferrous iron uptake system, encoded by *bfeUOB*, from *B. breve* UCC2003 and exploited its regulated transcription to develop an inducible expression system for use in bifidobacteria.

Introduction

Under normal physiological conditions, iron exists in one of two interconvertible redox states: the oxidized ferric (Fe^{3+}) or its more reduced ferrous (Fe^{2+}) form.[1] Iron is involved in important biological processes, where its functionality is largely dependent upon its incorporation into proteins, either as a mono- or binuclear species, or in a more complex form as part of iron-sulfur clusters or haem groups (see ref. 2 and references therein). It serves as a cofactor for several electron-transport proteins involved in vital metabolic processes such as aerobic and anaerobic ATP biosynthesis, making iron an essential element for most living organisms.[1] The latter fact is exploited by certain higher organisms, which sequester nutrient iron to limit the growth of pathogenic organisms as a form of nutritional immunity.[3] However, iron may also impose oxygen toxicity because it is a promoting factor of the Fenton reaction.[4] Therefore, strict regulation of iron metabolism and associated defenses against oxidative damage is essential for aerobic life.[4] Appropriate iron homeostasis requires an effective iron scavenging system to ensure adequate supplies, coupled to a delicate management of cellular, free iron levels in order to prevent iron-mediated toxicity.

Bacteria employ various physiological approaches to achieve iron homeostasis through high- and low-affinity iron transport systems, as well as to tightly control intracellular iron stores in case external supplies become exhausted.[5] Furthermore, under iron-restricted conditions the expression of iron-requiring proteins can be downregulated.[6]

Several iron uptake mechanisms in Gram-negative bacteria have been described. For example, *Escherichia coli* can employ, depending on the strain investigated, more than 10 different iron-uptake routes.[7] Bacterial iron uptake frequently involves so-called siderophores, which are low molecular-weight, extracellular, high-affinity iron-scavenging molecules capable of binding specific receptors in the bacterial outer membrane when complexed with iron.[8] Gram-negative pathogens may acquire iron from host-derived, iron-containing compounds, e.g., transferrin, lactoferrin, hemoglobin, haem and hemopexin. Often bacteria contain multiple ferric-complex uptake systems (six defined for *Escherichia coli* K-12) targeting a variety of iron sources, including siderophores produced by other organisms.[5] Bacteria can also acquire the soluble, ferrous form of iron via the anaerobic-microaerophilic FeoAB pathway.[9] In addition, ferrous iron can be taken up by NRAMP (natural resistance-associated macrophage protein)-like transporters (e.g., MntH of *E. coli*), metal-type ABC transporters (e.g., SitABCD of *Salmonella*) and ZIP transporters (e.g., ZupT of *E. coli*).[10]

The acquisition of iron from host proteins has been studied in just a handful of Gram-positive microbes. Some species employ surface receptors for proteins, such as transferrin or lactoferrin,[11,12]

while other Gram-positive bacteria, for example *Bacillus subtilis*, utilize siderophores to obtain ferric iron.[13] The use of haem or heamo proteins mediated through dedicated surface receptors has also been reported.[14,15] However, the principal machinery involved in the uptake of free or complex iron in Gram-positive bacteria involves the use of ABC-type transporters.[16] A summary of known iron transporters of common Gram-positive pathogens is presented in **Table S1**.

Iron is essential not only for pathogenic but also for commensal bacteria. Indeed, it has been hypothesized that one of the beneficial actions of health-promoting or probiotic bacteria is to sequester iron, thus making it less available to pathogens, thus representing another form of nutritional immunity.[17] A further benefit of iron sequestration is that it would abate free radical production in the gastrointestinal tract (GIT) or at sites of inflammation, where OH-free radicals are produced by the action of neutrophils or other means.[18] Iron-induced free radical damage to DNA appears to promote the development of cancer, while cancer cells are also known to grow rapidly in response to iron.[19]

Bifidobacterium, Gram-positive, non-motile, anaerobic bacteria, first described by Tissier in 1900 are among the most dominant organisms present in the breast-fed infant gut.[20] Various bifidobacterial strains are widely used as probiotic bacteria in functional foods.[21] Uptake of iron by Bifidobacterium was first described by Bezkorovainy[22] who demonstrated that *Bifidobacterium bifidum* possesses a membrane-bound ferri-reductase, which converts ferric iron to its ferrous state at the cell surface.[22] Recently, *B. longum* DJ010A has been reported to bacteriostatically inhibit other bacteria through the production of a siderophore,[23] although this characteristic seems rare among bifidobacteria and strain-dependent.[24] Bifidobacteria grow under anaerobic, reducing and/or acidifying

conditions, where the iron equilibrium is expected to shift from the ferric to the ferrous form, thereby allowing permeases of different protein families to take up iron without the need for a diffusible iron-sequestering compound. Iron uptake systems present in bifidobacteria have not been characterized at the genetic level and with the availability of bifidobacterial genome sequences this has now become feasible.[25] Global gene expression of *Bifidobacterium breve* UCC2003 under conditions of iron deficiency enabled us to identify genes whose transcription is iron dependent, among which the *bfeUOB* gene cluster, whose encoded proteins are similar to the EfeUOB ferrous iron transporter from *E. coli*.[26] The iron-responsive promoter region of the *bfeUOB* cluster was used for the construction of an inducible promoter system for bifidobacteria.

Results

Growth of bifidobacterial strains under iron-limiting conditions. In order to assess the growth inhibitory effect of the ferrous iron chelator 2'2-dipyridyl, growth was assessed for a collection of bifidobacterial strains. The strains were inoculated at 1% in CDM media and 2'2-dipyridyl was added at a final concentration that ranged from 0 to 5 µM. Following 15 h anaerobic incubation at 37°C, the OD_{600nm} was recorded. Growth, as assessed by the OD_{600nm} measurements, of almost all strains was inhibited by the presence of the chelator except for that of *B. longum* JCM7052. An inverse concentration-dependent correlation between the added amount of chelator and final optical density reached was shown following 15 h of growth (**Table 1**).

Genome response of B. breve UCC2003 to iron limitation. To investigate differences in global gene expression of *B. breve*

Table 1. Growth of bifidobacterial strains in response to increasing concentrations of 2'2 dipyridyl*

Species	Strain	0 µM Dipyridyl	0.5 µM Dipyridyl	1 µM Dipyridyl	5 µM Dipyridyl
B. adolescentis	CIP 64.61	++	+	-	-
B. adolescentis	NCFB 2229	++	+	-	-
B. animalis	JCM 20097	++	+	-	-
B. animalis	DSM 20105	++	+	-	-
B. bifidum	NCIMB8810	++	+	-	-
B. bifidum	LMG 11041	++	+	-	-
B. breve	UCC2003	++	+	-	-
B. breve	JCM 7017	++	+	-	-
B. dentium	NCFB 2843	++	+	-	-
B. longum	CIP 64.63	++	++	+	+
B. longum	JCM 7052	++	++	++	++
B. infantis	NCFB 2205	++	++	+	-
B.pseudocatenulatum	NCIMB 8811	++	+	-	-
B. pseudolongum	NCIMB 2244	++	+	+	-
B. globosum	JCM 7092	++	+	-	-
B. thermophilum	JCM 7027	++	+	-	-

Note: *Following 24 h growth in CDM medium, each strain of Bifidobacterium was subcultured to CDM containing a range of 2'2 dipyridyl from 0 to 5 µM and incubated for a further 15 h anaerobically at 37°C. The OD_{600nm} was established and is represented as (++) when $OD_{600nm} > 1.0$; (+) when $OD_{600nm} > 0.05$ and < 0.5; and (-) when $OD_{600nm} < 0.05$.

UCC2003 when grown at limiting concentrations of iron, global transcriptional changes were analyzed using DNA microarrays probed with cDNA from *B. breve* UCC2003 grown under iron-limiting conditions (see Materials and Methods). Because iron omission is likely to cause a significant impact on cell physiology, which would make it difficult to differentiate between genes directly influenced by iron-limitation from those affected by reduced metabolism, we exposed *B. breve* UCC2003 to 3 μM of the ferrous-iron specific chelator 2'2-dipyridyl. This resulted in the upregulation of 24 genes and the downregulation of 18 genes (fold change > 3.0 or < 0.25, p < 0.001) (**Table 2**). The upregulated genes were presumed to be involved in (high affinity) iron transport in *B. breve* UCC2003 and were subjected to further scrutiny.

Genes differentially regulated by iron-chelation. Two clusters of genes upregulated following exposure to dipyridyl were identified: the Bbr_0885–0887 gene cluster, whose products are annotated as an uncharacterized ABC transport system, and Bbr_0221–0227 (**Table 3**), encoding conserved hypothetical proteins with homology to the FTR1 system from *Saccharomyces cerevisiae* and to the EfeUOB transport system from *E. coli*, both ABC transporter systems responsible for iron uptake.[26,27] Comparative analysis shows that the latter FTR1/EfeU-like transport system is present in virtually all kingdoms of life (**Fig. 1**) and form a subgroup of the iron/lead transporter (ILT) superfamily[28] renamed as oxidase-dependent iron transporters (OFeT) (TC.9.A.10.1).[28]

The EfeUOB operon is conserved in *E. coli* and the dental pathogen *Treponema denticola*. Furthermore, a homolog of EfeU and part of the transport system are found in the surfactin siderophore cluster of the Gram-positive organism *B. subtilis*, which is responsible for high-affinity ferric iron uptake.[29] A feature of all OFeT proteins is the occurrence of two REXXE amino acid residue motifs.[27,30] On examination of the BfeU amino acid sequence two corresponding REGLE motifs were identified (**Fig. S1**).

Because of the high level of similarity to EfeUOB, we designated the Bbr_0221-Bbr_0223-encoded proteins BfeUOB. The iron-dependent transcriptional induction of *bfeUOB* in *B. breve* UCC2003, as observed using microarray analysis, was confirmed by QRT-PCR experiments, where *bfeU*, *bfeO* and *bfeB* were induced 64-, 30- and 69-fold, respectively, under iron-chelating conditions as compared with iron-replete conditions. This convergent expression pattern suggests that *bfeUOB* is controlled by a single, iron-dependent promoter.

Putative iron-dependent regulatory sequences. An in silico sequence analysis was performed to determine whether the genes

Table 2. Microarray fold alteration in gene expression following exposure to 3 μM of dipyridyl for 180 min. Genes significantly up or downregulated (fold change > 3.0, p < 0.001)

Locus	Gene	Description	Dipyridyl Array	qRT-PCR	Regulator/Motif
Bbr_0221	**BfeU**	OFeT family high affinity iron permease	**9.6**	64.33	Unknown: AAAATCAAGACTGTTGTT
Bbr_0222	**BfeO**	Secreted protein with iron binding domain	**9.6**	30.9	
Bbr_0223	**BfeB**	Membrane protein	**4.3**	69.76	
Bbr_0224		ABC transporter	4.4	3.33	
Bbr_0225		ABC transporter	4.3	ND	
Bbr_0226		ABC transporter	3.7	ND	
Bbr_0227		Lipoprotein	3.1	ND	
Bbr_0573	FUR	Ferric uptake regulation protein	- 1.7	-1.0	Fur: GATAATGAATATCATTTGT
Bbr_0574		Conserved hypothetical	1.2	ND	
Bbr_0575		ABC transporter	1.0	ND	
Bbr_0576		Hypothetical protein	**3.3**	ND	
Bbr_0577	RpmE1	LSU ribosomal protein	2.6	ND	
Bbr_0885		Membrane protein	**5.7**	36.3	Unknown: AAAATCAAGACTGTTGTT
Bbr_0886		ABC transport permease	**6.1**	52.5	
Bbr_0887		ABC transport binding protein (similar Bbr0226)	**5.4**	32.12	
Bbr_1364	GroEL	Chaperone	**3.0**	146.2	
Bbr_1523	MerR	MerR regulatory protein	**4.5**	27.8	
Bbr_1898	NrdF	Ribonucleoside diphosphatereductase-β	**3.1**	127.2	
Bbr_1899	NrdE	Ribonucleoside diphosphatereductase-α	2.4	16.4	

Note: Underlined is the first member of the operon. Significantly regulated genes are shown in bold. ND is not determined.
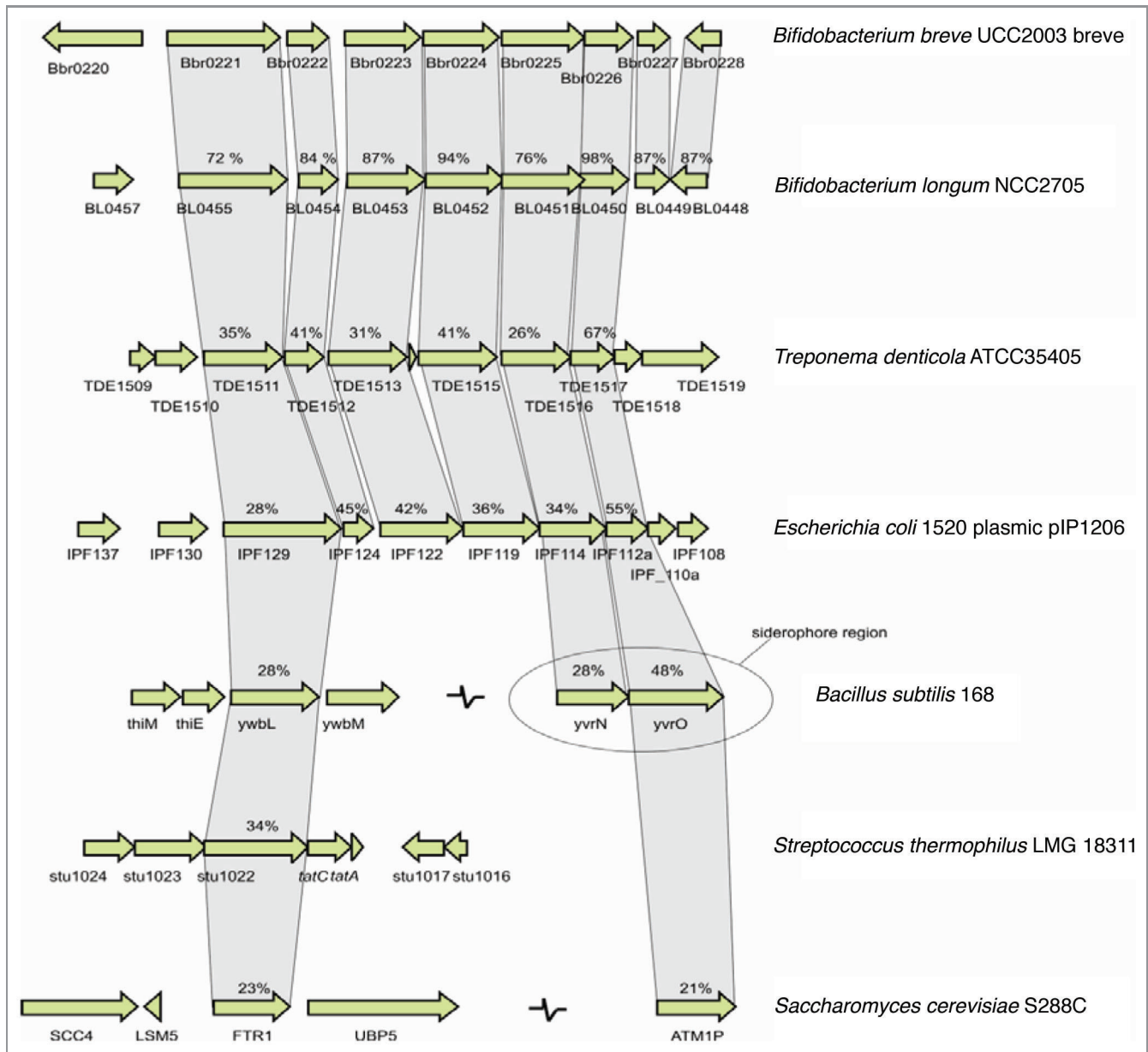
Figure 1. Comparison of the bfeU (Bbr_0221) locus in *B. breve* UCC 2003 with the corresponding loci in various bacteria. Each arrow indicates an ORF. Corresponding genes are indicated by the vertical links between the arrows. The levels of amino acid identity to the *B. breve* UCC 2003 sequence, expressed as percentages, are indicated. The siderophore locus in *B. subtilis* 29 is indicated.

upregulated by exposure to dipyridyl in *B. breve* UCC2003 contain conserved regulatory sequences in their putative promoter regions. We assumed that these genes were under direct control of one or more iron-responsive regulators, which elicit this control through binding to specific sequences in their target promoter regions. Data sets of sequences containing 400 bp upstream of the putative translation start sites of the upregulated genes were generated. In this way, a data set of five sequences was examined for the occurrence of common elements with a length between 10 bp and 30 bp using the MEME algorithm.[31] Nearly identical imperfect inverted repeat (IR) sequences, AAAATCAAGACT-GTTGTT and AAAAACAAGACTTGTGTT, respectively, were

present in the putative promoter region of Bbr_0221 and Bbr_0885, which are both strongly upregulated (9.6 and 5.7 fold by micro array analysis, respectively, see **Table 2**) when *B. breve* UCC2003 is exposed to dipyridyl.

A screen of putative promoter regions of all currently available bifidobacterial genomes for the sequence AAAAWCAAGACT-NNTGTT (**Fig. 2A**) revealed that the putative promoter regions of bifidobacterial homologs of Bbr_0221 and Bbr_0885 are the only sequence regions to contain this motif (**Table 3**). These findings suggest that this inverted repeat is involved in mediating the regulatory response to iron limitation, for instance as a binding site for a regulatory protein, although further studies need
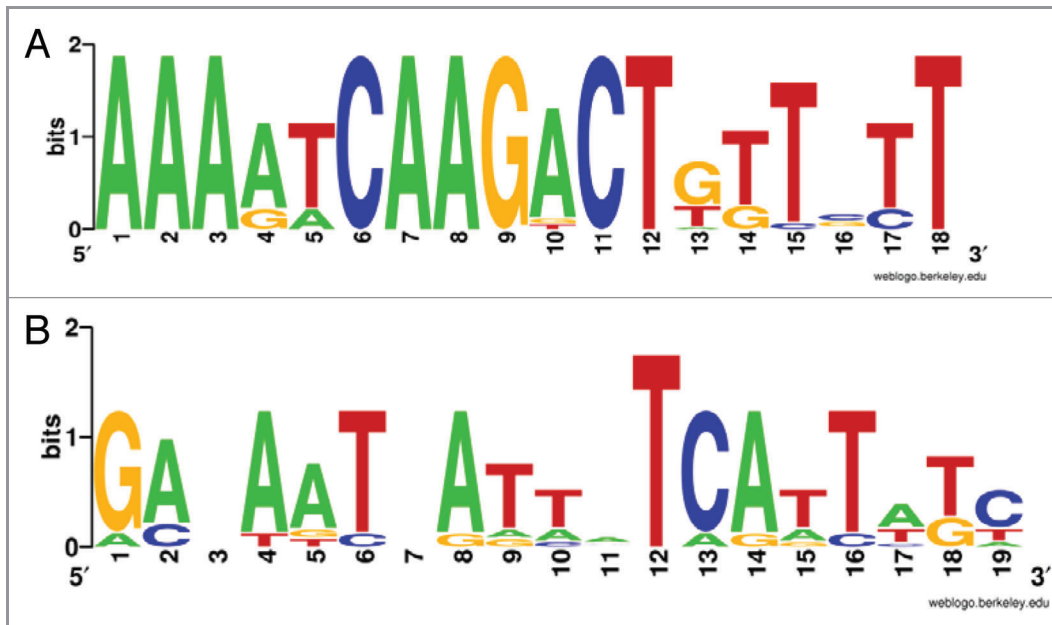
Figure 2. (A) Weblogo depiction of a motif based on the alignment of bifidobacterial sequences from the promoter regions of Bbr_0221 and Bbr_0885 homologs. (B) Weblogo depiction of FUR motif as detected in the *B. breve* UCC2003 genome.

to be performed to verify this possibility and to identify the regulator involved.

The influence of the ferric uptake regulation (FUR) protein in the response by *B. breve* UCC2003 to iron chelation by dipyridyl is less likely. Although a putative FUR protein (Bbr_0573) has been annotated on the genome of UCC2003, screening with a model based on the FUR motif of this gene (**Fig. 2B**) using HMMSEARCH[32] detected no FUR motifs in any of the genes upregulated by dipyridyl-mediated iron chelation (results not shown).

Expression of UCC2003 bfeUOB improves growth of an iron-uptake mutant of *E. coli* K12 (W3110). In order to

establish the functionality of the *bfeUOB* operon in iron-uptake we cloned *bfeU*, *bfeUO* and *bfeUOB* separately in the low-copy vector pWSK29, creating pBfeU, pBfeUO and pBfeUOB, respectively, as described in Materials and Methods. These plasmids were introduced into the *E. coli* K12 (W3110)-derived mutant, designated GR536 (D*fecABCDE*::*kan*D*zupT*::*cat* D*mntH*D*feoABC*D*entC*),[7] in order to assess plasmid-mediated effects on growth under iron limiting conditions. The GR536 mutant grows well in Luria–Bertani (LB) medium, but poorly in Tris-mineral salts medium even when iron is supplied (**Fig. 3**). As a positive control the *E. coli* Nissle strain which contains a functional EfeUOB operon[33] was included in our analysis.

Table 3. Sequence analysis of the currently available bifidobacterial genomes for the motif identified upstream of Bbr_0221 and Bbr_0885

Strain	Bbr_0221 Homolog	Motif	Bbr_0885 Homolog	Motif
B. breve DSM 20213	BIFBRE_01998	AAAATCAAGACTGTTGTT	BIFBRE_01208	AAAAACAAGACTTGTGTT
*B. infantis*ATCC15697	Blon_0196	AAAATCAAGACTGTTGTT	Blon_1615	AAAAACAAGACTTGTGTT
B. longum DJO10A	BLD_1255	AAAATCAAGACTGTTCCT		
B. longum infantis ATCC 55813	HMPREF0175_0087	AAAATCAAGACTGTTCCT		
B. longum NCC2705	BL0455	AAAATCAAGACTGTTCCT	BL0889	AAAAACAAGACTTGTGTT
B. longum infantis CCUG 52486	BLIG_00474	AAAATCAAGACTGTTCCT		
B. bifidum NCIMB 41171	BbifN4_010100006928	AAAGTCAAGACTGTTCTT	BbifN4_010100003655	AAAGTCAAGGCTTGTGTT
B. dentium ATCC 27678	BIFDEN_00651	AAAATCAAGACTGTTCTT	BIFDEN_02200	AAAGTCAAGACTTTTCTT
B. angulatum DSM 20098	BIFANG_02052	AAAATCAAGACTGTTGTT	BIFANG_02981	AAGTCAAGTCTTGTTTTT
B.pseudocatenulatum DSM 20438	BIFPSEUDO_04117	AAAATCAAGACTGTTCTT		
B. catenulatum DSM 16992	BIFCAT_00140	AAAATCAAGACTGTTCTT	BIFCAT_00981	AAAAACAAGACTATCTTT
B. adolescentis ATCC 15703	BAD_0097	AAAATCAAGACTGTTATT		
B. adolescentis L2–32	BIFADO_00280	AAAATCAAGACTGTTATT		

Heterologous expression of bfeU or bfeUO in the pWSK29 plasmid in *E. coli* GR536 provided a modest increase in cellular growth yield in the presence of dipyridyl (ranging from 0–100 µM) when compared with a vector-only control (**Fig. 3**). However, strain GR536 carrying pBfeUOB showed a significant increase in cellular growth (**Fig. 3**) and was essentially unaffected by the presence of low concentrations of dipyridyl and only ceased growing at concentrations above 100 µM. These results clearly demonstrate that expression of functional *bfeUOB* is advantageous for *E. coli* under conditions of iron depletion.

Expression of a p272bfeU-gusA fusion is regulated by iron in multicopy plasmids. Plasmid pDIP1-gusA was constructed to investigate the putative promoter region upstream of *bfeU* and to establish if this promoter could be employed as an iron-dependent inducible gene expression system in *B. breve* UCC2003. Initial assays in CDM broth showed a 2-fold increase in GUS activity of UCC2003 harboring pDIP1-gusA when exposed to 1.5 µM dipyridyl in comparison to either the control strain (UCC2003 with plasmid pNZ272) or UCC2003 containing plasmid pDIP1-gusA in CDM (no chelator) as illustrated in **Figure 4**.

However, a marked improvement was observed when the media was changed to MRS broth (**Fig. 4**), with an at least 5-fold increase in the GUS activity of UCC2003 pDIP1-gusA on exposure to 1.5 µM dipyridyl in comparison to either UCC2003 pNZ272 or UCC2003 pDIP1-gusA in MRS (no chelator).

Discussion

In order to assess the inhibitory effect of iron limitation on bifidobacterial strains we studied the growth of a collection of bifidobacterial strains under these conditions. In addition we imposed iron limitation through the use of the ferrous-iron chelator,[34] 2'2-dipirydyl, and performed global transcriptional analysis on *B. breve* UCC2003. A chelator-dependent variation in the expression of iron-regulated outer membrane proteins in *E. coli* has previously been reported.[35] The authors concluded that the nature of the chelator used to restrict iron supply influences the synthesis of iron-regulated proteins. Lim et al.[36] have exploited this strategy for the development of an expression vector based on the PentC promoter for *E. coli* that is induced by iron chelation. Similarly for the actinomycetes the iron-regulated *desA* promoter serves as an inducible system for expression of genes in *Streptomyces pilosus* following iron removal with dipyridyl.[37] Apart from *B. longum* JCM7052, growth of all other Bifidobacterium strains tested showed an inverse concentration-dependent correlation between the added amount of chelator and final optical density
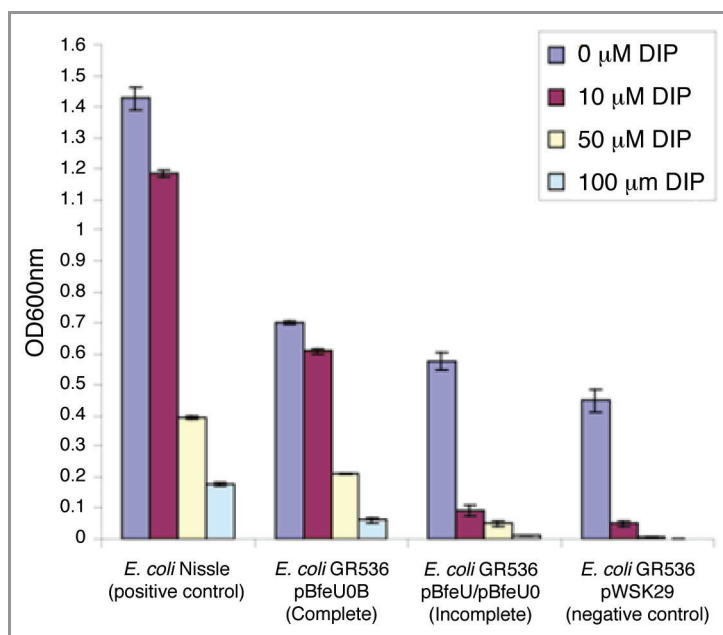

Figure 3. Complementation analysis of *E. coli* GR536 with the complete bfeUOB operon (pBfeUOB) in comparison to empty vector (pWSK29) or plasmids containing just bfeU (pBfeU) or bfeUO (pBfeUO) (illustrated jointly as incomplete fragments of the operon) monitored as OD600nm following 12 h of growth under increasing iron chelating conditions, dipyridyl (DIP) range 0–100 µM. *E. coli* Nissle containing a functional EfeUOB operon serves as a positive control.

reached following 15 h of growth. The lack of growth inhibition of *B. longum* JCM7052 when exposed to dipyridyl may indicate, similar to the findings reported for *B. longum* DJO10A,[23] that this strain produces a siderophore that endows it with a very efficient iron scavenging ability.
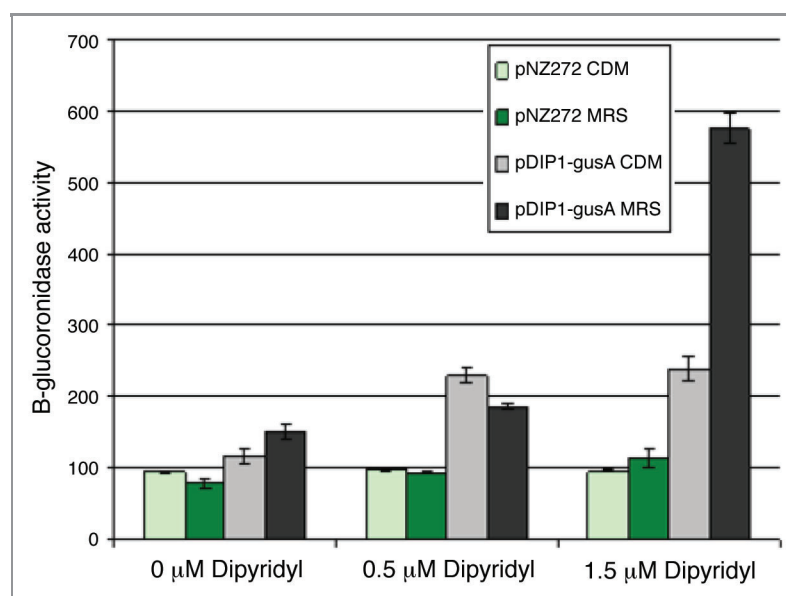

Figure 4. β-glucuronidase assay of *B. breve* UCC2003 containing pNZ272 or pDIP1-gusA in defined (CDM) or complex (MRS) media with increasing concentrations of 2'2-dipyridyl from 0 to 1.5 µM.

Following chelation of ferrous iron, *B. breve* UCC2003 evokes a stress-like response demonstrated by the upregulation of the chaperone GroEL (Bbr_1364).[38,39] Iron-chelation was also shown to upregulate transcription of *nrdF* (Bbr_1898), encoding a predicted ribonucleotide reductase. The latter enzyme is known in *E. coli* to catalyze the reduction of ribonucleotides to deoxyribonucleotides as a first step in the pathway for DNA replication, where synthesis of *nrd* mRNA increases when DNA synthesis is inhibited.[40] The expression of *nrdF* in *E. coli* was found to be growth-phase dependent, and growth conditions that result in a decreased DNA/mass ratio result in increased levels of ribonucleotide reductase.[41] We propose that this is the reason for the upregulation of *nrdF* (Bbr_1898) in *B. breve* UCC2003 in response to iron-chelation as an obvious reduction in growth rate is observed on inclusion of dipyridyl in the growth media.

Among the genes, whose expression was repressed by iron-chelation, are those that encode transport systems, as well as a number of hypothetical proteins with predicted membrane association. Of interest is the downregulation of genes annotated to play a role in pyroxidine biosynthesis (Bbr_0549) and aspartate carbamoyltransferase (Bbr_0973–0975). Aspartate carbamoyltransferase (ACTase) catalyzes the first committed step in de novo pyrimidine biosynthesis in bacteria, and the acquisition of nucleotides is a vital process of all living cells.[42] Previous work has shown that ACTase activity in *H. pylori* is highly regulated,[43] and it is probably a major point of control of de novo pyrimidine biosynthesis pathway in this bacterium. In *E. coli* a transient repression of ACTase has previously been used as an early indicator of a stress response,[44] and a similar effect may thus be applicable in *B. breve* UCC2003.

The most significantly upregulated gene-cluster in *B. breve* UCC2003 following iron-chelation is *bfeUOB*. Homologs of *bfeUOB* are present in many sequenced bacteria and their protein products form a subgroup of the ILT superfamily[28] renamed as oxidase-dependent iron transporters (OFeT) (TC.9.A.10.1).[28] The first functional report on a bacterial OFeT transporter, YwbL, came from *Bacillus subtilis*,[45] which was shown to be necessary for growth in defined medium under conditions of low iron availability and when citrate as a siderophore is absent. The identification of the homologous system in *E. coli* was complicated by the fact that the commonly studied laboratory strains such as K12 contain a truncated and therefore non-functional permease (YcdN). In *E. coli* strains other than K12, full-size *ycdN* genes can be identified, which are therefore presumed to contain a functional YcdN-dependent iron-uptake system. Grosse et al. (2006) reported that the iron permease YcdN from the probiotic *E. coli* strain Nissle 1917 is encoded as part of a Fur-regulated tricistronic operon and renamed YcdN to EfeU (elemental ferrous iron uptake), in contrast to siderophore-chelated ferric iron-uptake systems.[7] A striking feature of the OFeT family is the occurrence of two REXXE amino acid residue motifs that were found to be essential for Ftr1p function in yeast.[27,30] Two corresponding REGLE motifs were identified in BfeU. In *E. coli* the importance of this motif was highlighted by mutation of the first glutamate residue of each motif into an alanine, which failed to improve growth of strain GR536, an *E. coli* K12(W3110)-derived mutant, (D*fecABCDE*::*kan*D*zupT*::*cat* D*mntH*D*feoABC*D*entC*), which carries deletions and/or insertions in all known iron acquisition systems,[46] suggesting that each motif is necessary for its biological function.

The involvement of *B. breve bfeUOB* in iron metabolism was demonstrated through complementation analysis: when *bfeUOB* was present on a low copy-number plasmid (pBfeUOB) it allowed increased growth of the iron transport-debilitated *E. coli* strain GR536 grown in the presence of the iron-chelating agent dipyridyl but provided no major advantage under iron proficient conditions, and no advantage to a "wild-type" strain. Interestingly, progressive removal of *bfeO* and *bfeB* from the complementation-vector negated the growth advantage, further confirming the functionality of *bfeUOB* to specify a multi-component iron-uptake system. Based on its sequence, the presence of the functional motifs (REGLE) and its conferred growth advantage when heterologously expressed in the iron-uptake mutant GR536, *bfeU* can be classified as encoding an OFeT-family ferrous iron permease. Although the precise functions of BfeU and BfeO are unknown,[47] we speculate that they are involved in the presentation of Fe^{2+} to the transporter.

A primary goal of this work was to identify a promoter which is directly affected by the presence of the chelator and which can therefore be exploited to create an inducible protein expression system in *B. breve* UCC2003. The putative promoter region upstream of *bfeU* was selected and cloned into pNZ8048, thereby replacing the nisin-inducible promoter, followed by the insertion of a β-glucuronidase reporter gene (pDIP1-gusA). The actual induction level measured using this dipyridyl-inducible promoter is likely to be higher than the observed 5-fold, although it is comparable to the (2–7 fold) increase observed with the *desA*-amylase dipyridyl-inducible system developed for *Streptomyces pilosus*.[37] However, the control plasmid produces a significant level of background GUS activity, which is probably due to transcription originating from elsewhere on the pNZ-derived plasmid. This inducible system could therefore be improved by the inclusion of a transcriptional "silencer" just upstream of the *bfeU*-derived promoter. Significantly, these results do represent a dose-dependent response to the presence of an inducer, in this case the chelator 2'2-dipyridyl. Regulation of the response to ferrous iron chelation by *B. breve* UCC2003 is likely to be FUR-independent and presumably involves a novel but as yet unidentified regulator. Unraveling the precise mechanism of control should allow us to improve our inducible system with the goal of creating an improved genetic tool for use in bifidobacteria for controlled expression of proteins that become toxic or insoluble when induced at high levels.

Methods

Bacterial strains and culture conditions. The various bacterial strains and plasmids used in this study are listed in **Table 4**. *E. coli* strain DH10B (Invitrogen) was used as a cloning host and was grown aerobically at 37°C in LB medium (Sigma). For iron limitation experiments *E. coli* cultures were grown overnight in LB at 37°C, diluted 1:400 in Tris-buffered mineral salts medium

containing 2 ml of glycerol and 3 g/L of casamino acids and grown overnight. Cultures were inoculated (as a 1:400 dilution) in fresh Tris-buffered mineral salts medium,[48] and after 2 h early log cultures were diluted 1:400 in fresh medium to which a varying amount of 2'2-dipyridyl was added, and growth ability was recorded spectrophotometrically at OD_{600nm}. B. breve UCC2003 was routinely grown at 37°C in reinforced clostridial medium (Oxoid). For iron-limitation tests involving B. breve UCC2003 we used a chemically defined medium, which was developed from first principles (designated as chemically defined medium or CDM)[49] and which was supplemented with 0.05% (w/v) cysteine-HCl. Anaerobic conditions were established and maintained using an anaerobic chamber [Mac500, Don Whitley Scientific, (atmosphere 10% H_2, 10% CO_2, 80% N_2)]. Varying amounts of 2'2-dipyridyl and/or various antibiotics [chloramphenicol (15–20 $\mu g\ ml^{-1}$), kanamycin (25 $\mu g ml^{-1}$), ampicillin (100 $\mu g\ ml^{-1}$)] were added to growth media where appropriate.

DNA techniques. Plasmid DNA was isolated from E. coli using a QIAprep Spin Miniprep kit according to the manufacturer's instructions (QIAGEN). Genomic DNA isolation from B. breve UCC2003 was performed as described previously.[50] Standard procedures were used for DNA manipulation in E. coli. Plasmid DNA was introduced into B. breve by electrotransformation as previously described.[51] Restriction endonucleases (Roche Diagnostics), T4 DNA ligase (Roche), and 2 × PCR mixture (Promega) were used as recommended by the manufacturers. Primers were purchased from MWG and are listed in **Table S1**. PCR products that needed to be cloned were generated using KOD hot-start high-fidelity DNA polymerase (Merck) and subsequently sequenced to verify sequence integrity.

DNA-microarray and QRT-PCR experimental procedures. DNA-microarrays of B. breve UCC2003 were obtained from Agilent Technologies.[52] An overnight culture of B. breve UCC2003 in CDM was inoculated at 1% into fresh CDM and incubated at 37°C until an OD at 600 nm of 0.5 was

Table 4. Bacterial strains and plasmids used in this study

Bacterial species	Strain Details	Reference
B. breve	UCC2003 Source of bfeUOB,[50]	University College Cork Culture Collection
B. longum	JCM 7052 Encodes a potential siderophore	Japan Collection of Microorganisms
B. adolescentis	CIP 64.61	Pasteur Institute Collection, France
B. adolescentis	NCFB 2229	National Collection of Food Bacteria, Reading
B. animalis	JCM 20097	Japan Collection of Microorganisms
B. animalis	DSM 20105	Deutsche Sammlung von Mikroorganismen GmbH, Germany
B. bifidum	NCIMB 8810	National Collection of Industrial, Food and Marine Bacteria, Aberdeen
B. bifidum	LMG 11041	Laboratorium voor Microbiologie, Universiteit Gent, Gent, Belgium
B. breve	JCM 7017	Japan Collection of Microorganisms
B. dentium	NCFB 2843	National Collection of Food Bacteria, Reading
B. longum	CIP 64.63	Pasteur Institute Collection, France
B. infantis	NCFB 2205	National Collection of Food Bacteria, Reading
B. pseudocatenulatum	NCIMB 8811	National Collection of Industrial, Food and Marine Bacteria, Aberdeen
B. pseudolongum	NCIMB 2244	National Collection of Industrial, Food and Marine Bacteria, Aberdeen
B. globosum	JCM 7092	Japan Collection of Microorganisms
B. thermophilum	JCM 7027	Japan Collection of Microorganisms
E. coli	DH10B, Cloning host	Invitrogen, Paisley, United Kingdom
E. coli Nissle 1917	DSM 6601	Deutsche Sammlung von Mikroorganismen, GmbH, Germany
E. coli	GR536	7
Plasmid Name	**Description**	**Source**
pWSK29	Amp[r] low copy vector	59
pNZ272	Promoterless gusA,Cm[r], 4.6 kb	61
pDIP1-gusA	Transcriptional fusion between bfeU promoter region and gusA reporter.	This study
pBfeU	pWSK29 containing bfeU only	This study
pBfeUOB	pWSK29 containing bfeUOB entire operon	This study
pBfeUO	pWSK29 containing bfeUO and a truncated non-functional bfeB.	This study

reached and then divided into individual subcultures. Two subsequent subcultures were exposed to 3 μM of the iron-chelator 2'2-dipyridyl for 180 min (DIP⁺) while a further two acted as controls, i.e., growth at 37°C for 180 min (DIP⁻). In all cases, cells were harvested by centrifugation at 8,000 × g for 1 min at room temperature and immediately frozen in liquid nitrogen prior to RNA isolation.

Methods for cell disruption, RNA isolation, RNA quality control, cDNA synthesis and indirect labeling were performed as described previously.[53] Labeled cDNA was hybridized using the Agilent Gene Expression hybridization kit (part number 5188–5242) as described in the Agilent Two-Color Microarray-Based Gene Expression Analysis v4.0 manual (G4140–90050). Following hybridization, all microarrays were washed as described in the manual and scanned using Agilent's DNA microarray scanner G2565A. The scans were converted to data files with Agilent's Feature Extraction software (Version 9.5). DNA-microarray data were processed as previously described.[53-55] Differential expression tests were performed with the Cyber-T implementation of a variant of the t-test.[56] A gene was considered to exhibit a significantly different expression level relative to the control when $p <$ 0.001 and an expression ratio of $>$ 3 or $<$ 0.25. Final data represents the average of at least two independent array experiments.

The QRT-PCR amplifications were performed on an ABIPRISM 7000 using SYBR®Green I dye assay chemistry. A 12.5 μl PCR assay for each gene of interest consisted of 6.25 μl of 2X RT PCR master mix (Biogene, Kimbolton, UK), 0.625 μl 1/3000 SYBR green dye (Biogene), 4.225 μl of H_2O, 0.2 μl (3 μmol) of forward and 0.2 μl (3 μmol) of reverse primers, and 1 μl (10 ng) of cDNA template. All QRT-PCR's were run in triplicate; three biological replicates were taken for both iron restricted and non-restricted cultures, resulting in nine measurements per gene for each environmental condition. No amplification was observed for the QRT-PCR controls (no reverse transcriptase and no template; data not shown). Cycling conditions used for all amplifications were one cycle of 95°C for 10 min and 40 cycles of 95°C for 15 sec and 58°C for 1 min. From the QRT-PCR data, an average cycle threshold (Ct) value was calculated from the triplicate reactions. Averaged Ct values were then normalized (to adjust for varying amounts of cDNA for each reaction) relative to the control gene, *rnpA*, which was selected because the average gene expression did not appear to change in previously published microarray studies performed on *B. breve* UCC2003.[52,57] The average fold ratio differences from the biological triplicates of the genes from iron restricted and non-restricted samples were determined as described previously.[58]

Plasmid constructions. Plasmid pBfeU was constructed as follows; the *bfeU*gene (Bbr_0221) was amplified by PCR from chromosomal DNA of *B. breve*UCC2003 using primers bfe1 and bfe2and cloned as a KpnI-BamHI insert into the vector pWSK29.[59] Plasmid pBfeUOB (Bbr_0221-0223) was constructed by cloning a DNA fragment encompassing the entire *bfeUOB*gene cluster including the presumptive promoter region of *bfeU* (500 bp immediately upstream of *bfeU*). This *bfeUOB*-containing fragment was obtained by PCR using primers bfe3 and bfe4, and chromosomal DNA of *B. breve* UCC2003 as a template and cloned into pWSK29 as a SacI-BamHI insert. Plasmid pBfeUO was constructed by restricting plasmid pBfeUOB with BstB1 and BamHI, which removes 282 bp from the 3' end of *bfeB*. Following digestion, the larger DNA fragment, which contains the pWSK29 vector plus the *bfeUOB* gene cluster with a truncated *bfeB* gene, was isolated, treated with Klenow (New England Biolabs) to polish single strand overhangs and re-ligated. The dipyridyl-inducible plasmid pDIP1 was constructed by cloning the presumed promoter region of *bfeU*, which was amplified by PCR from chromosomal DNA of *B. breve* UCC2003 using primers PbfeUfw and PbfeUrev, as a BglII-Nco I insert into the corresponding sites of vector pNZ8048, thereby replacing the nisin-inducible promoter. Plasmid pDIP1-gusA was subsequently constructed by inserting the *gusA* gene from plasmid pNZ272 (66), amplified by the primers gusAfw and gusArev as an Nco I–Hind III insert in the corresponding sites in pDIP1, thereby creating a transcriptional fusion of the presumed iron-responsive promoter of *bfeU* and *gusA*.

β-glucuronidase activity. Following overnight growth *B. breve* UCC2003 containing plasmid pNZ272 (control) and *B. breve* UCC2003 containing plasmid pDIP1-gusA(test) were subcultured and grown until the OD_{600nm} had reached approximately 0.5, at which point the cultures were split and exposed to increasing amounts of 2'2-dipyridyl, the final concentration of which ranged from 0 to 5 μM. Following 180 min incubation with this chelator the cells were harvested and the levels of β-glucuronidase (GUS) activity determined as described previously.[60]

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