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Regulation of anaerobic arginine catabolism in *Bacillus licheniformis* by a protein of the Crp/Fnr family

Abdelouahid Maghnouj [a], Atif A.W. Abu-Bakr [c], Simon Baumberg [c], Victor Stalon [a,b], Corinne Vander Wauven [b,*]

[a] *Laboratoire de Microbiologie, Université Libre de Bruxelles, Brussels, Belgium*
[b] *Institut de Recherches Microbiologiques Jean-Marie Wiame, Av. E. Gryson, 1-B 1070 Brussels, Belgium*
[c] *School of Biology, University of Leeds, Leeds, UK*

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Abstract

Arginine anaerobic catabolism occurs in *Bacillus licheniformis* through the arginine deiminase pathway, encoded by the gene cluster *arcABDC*. We report here the involvement of a new protein, ArcR, in the regulation of the pathway. ArcR is a protein of the Crp/Fnr family encoded by a gene located 109 bp downstream from *arcC*. It binds to a palindromic sequence, very similar to an *Escherichia coli* Crp binding site, located upstream from *arcA*. Residues in the C-terminal domain of Crp that form the DNA binding motif, in particular residues Arg-180 and Glu-181 that make specific bonds with DNA, are conserved in ArcR, suggesting that the complexes formed with DNA by Crp and ArcR are similar. Moreover, the pattern of DNase I hypersensitivity sites induced by the binding of ArcR suggests that ArcR bends the DNA in the same way as Crp. From the absence of anaerobic induction following inactivation of *arcR* and from the existence of a binding site upstream of the *arcA* transcription start point, it can be inferred that ArcR is an activator of the arginine deiminase pathway.

Keywords: Arginine deiminase; Anaerobic regulation; Crp/Fnr family; *Bacillus licheniformis*

1. Introduction

Under anaerobic conditions, *Bacillus licheniformis* catabolizes L-arginine through the arginine deiminase pathway. This anaerobic pathway allows the use of arginine as an energy source in the absence of respiration, by coupling the phosphorylation of 1 mol of ADP into ATP per mol of arginine taken up from the medium (Fig. 1) [3]. The four genes encoding the deiminase pathway form an operon-like cluster *arcABDC* [16]. The pathway is induced by arginine only in oxygen-depleted media. The presence of an electron acceptor (oxygen or nitrate) prevents induction [3]. Induction by arginine is mediated by ArgR, the repressor of arginine biosynthesis and a homologue of the *Bacillus subtilis* AhrC arginine regulator. In the presence of arginine, ArgR binds to a single ARG box (the accepted term for an ArgR/AhrC binding sequence: [12]) located 107 bp upstream from the transcription start point of *arcA* (Fig. 4) – an unusually promoter-distal position for an ARG box – and activates the expression of the *arc* genes [16]. Nothing is known so far of the mechanism of anaerobic induction. Sequence analysis has revealed the existence of a region of imperfect dyad symmetry, centered at −65.5, containing a sequence similar to the *Escherichia coli* Crp binding consensus site [16]. In *Pseudomonas aeruginosa*, anaerobic induction of the arginine deiminase pathway occurs in the absence of arginine and is mediated by a protein of the Crp family, Anr, acting as an anaerobic activator [9]. As the Crp/Fnr family is a widely distributed family of homologous proteins binding to well conserved DNA sites, this suggested that in *B. licheniformis* too, a Crp/Anr-like protein could be involved in the regulation of the arginine deiminase genes. We report here the existence in *B. licheniformis* of a gene *arcR*, lying downstream of the *arcABDC* cluster, predicted to encode a protein resembling members of the Crp/Fnr family and functioning as an activator of the *arcABDC* genes.

* Corresponding author. Tel.: +32 (2) 526 7272; Fax: +32 (2) 526 7273; E-mail: cvwauven@dbm.ulb.ac.be

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2. Materials and methods

2.1. Bacterial strains and plasmids

Bacterial strains and plasmids used or created during this study are listed in Table 1. *E. coli* transformants were selected on LB plates supplemented with ampicillin (Ap) at 100 μg ml^{-1}. *Bacillus* transformants were selected on enriched or minimal media supplemented with erythromycin (Em), kanamycin (Km) or chloramphenicol (Cm) at 1, 3–5, or 3–10 μg ml^{-1}.

2.2. Enzyme assays

All cultures intended for catabolic ornithine carbamoyltransferase (OCT) assay were grown up in minimal medium 154 [23] supplemented with thiamine at 1 μg ml^{-1}; carbon and nitrogen sources were added to the medium to the final concentration of 20 mM, except for arginine which in oxygen-limited cultures was present at 40 mM. Well-aerated cultures and oxygen-limited cultures were obtained as in [16]. OCT assays were performed at 37°C. OCT activities were estimated from the amount of citrulline formed in a reaction mixture containing 50 mM EDTA–NaOH buffer pH 8.5, 50 mM ornithine, 5 mM carbamoyl phosphate, and sonicated cell extract, according to [4]. Specific activities are expressed in μmol product formed h^{-1} (mg protein)$^{-1}$. β-Galactosidase assays were carried out for *B. subtilis* cultures as described in [14].

2.3. DNA manipulations

Plasmid construction and control restrictions were carried out according to standard procedures. Plasmid DNA was purified on Nucleobond AX PC100 columns (Macherey-Nagel) or by use of Wizard resin (Promega). Genomic DNA to be used as template in PCR was purified on Qiagen RNA/DNA maxi kit or Promega Wizard mini columns according to the manufacturer's instructions.

Bacillus plasmids were constructed as follows. pAB161: the predicted *arcR* sequence has a *Bam*HI site at 205–210 and a *Bcl*I site at 650–655 (taking the initiating ATG as positions 1–3; the terminating TGA is then at 714–716). This internal *Bam*HI–*Bcl*I fragment, which lacks a *Pst*I site, was cloned into the *Bam*HI site of pUC19 to yield pAB160. pAB160 and pE194ts were then cut at their unique *Pst*I sites and ligated together to yield pAB161. pAB200: a fragment extending from a *Cla*I site 18 bp upstream from the predicted translational start of *arcR* to an *Eco*RI site 27 bp downstream from its predicted translational termination site was cloned in pBluescript-IISK. This resulting construct was cut with *Sal*I and *Eco*RI, and the resulting fragment cloned into similarly cut pRB374. The latter carries the powerful constitutive *B. subtilis veg* promoter, followed by *Sal*I and *Eco*RI sites [6], so that the procedure described provides *arcR* transcription from the *veg* promoter. pAB190:pDG268, a *B. subtilis* integrative vector, carries separated front and back parts of the *B. subtilis amy* gene (encoding α-amylase) with, between them, a cassette comprising the *cat* gene (conferring Cm resistance) and a promoter-less *E. coli lacZ* gene preceded by a cloning site. A 272-bp fragment extending from 264 bp upstream to 8 bp downstream of the translation start of *arcA*, and therefore likely to contain its promoter and associated regulatory regions, was cloned into pDG268 upstream of *lacZ*. A construct shown by restriction analysis (via a convenient *Bcl*I site overlapping the *arcA* translation start) to have the presumed promoter pointing towards *lacZ*, was termed pAB190.

2.4. Transformation of, and strain construction in, B. licheniformis and B. subtilis

B. licheniformis and *B. subtilis* were transformed as in

Table 1
Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype and relevant markers	Source
B. licheniformis		
ATCC14580	Wild-type	ATCC
B. subtilis		
EMG50	Prototrophic version of transformable 168 strain	Laboratory stock
E. coli		
HMS174(DE3)plys S	F^- *recA* r_K^- m_K^+ Rif^r(DE3)	Novagen
XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac*/F′[*proAB*+*lacI*^{q} *lacZ*Δ*M15, Tn10*]	Stratagene
Plasmids		
pUC19	Standard cloning vector; Ap^r	Laboratory stock
pCR2.1	T overhang vector for PCR product cloning; Ap^r, Km^r	Invitrogen
pET-3a	T7 expression plasmid; Ap^r	Promega
pET-3a.*arcR*	expression vector for the production of ArcR; Ap^r	This work
pHin4a	pGEM-7Zf(+)::10 kb *Hin*dIII fragment containing *B. licheniformis arc* genes; Ap^r	[16]
pE194ts	Gram-positive plasmid able to replicate in *B. licheniformis*; temperature-sensitive for replication; MLS^r	[8], F.G. Priest
pRB374	Gram-positive plasmid expression vector carrying strong constitutive *veg* promoter	[6]
pDG268	*B. subtilis* integrative vector (pBR322-based replicon) with (i) 'front' and 'back' halves of *B. subtilis amy* gene, (ii) promoter-less *lacZ*; Cm^r	[22], P. Stragier

[19] and [5] respectively. The transformation frequency of the strain used here, ATCC14580, was appreciably lower than that of strain ATCC9800 (kindly provided by F.G. Priest) used in [19], but was adequate. Disruption of the *B. licheniformis arcR* gene was achieved as follows. pAB161 was transformed into *B. licheniformis*; to confirm the presence of the autonomous plasmid, it was necessary, since the low copy number precluded visualization by ordinary gel methods, to prepare DNA from transformants, transform this into *E. coli* and characterize plasmid DNA from the resulting transformants by restriction analysis. *B. licheniformis* carrying pAB161 was grown at 30°C, shifted to 42°C in mid-exponential growth, and allowed to grow for another 2 h. Dilutions were plated on Em medium at 42°C. Large colonies were checked for the presence of autonomous plasmid as before, in all cases proving negative. To confirm the presence of pAB161 in integrated form, an isolate was grown at 28°C and streaked on antibiotic-free medium, the resulting colonies then being tested. Most had lost antibiotic resistance completely, while the few that remained Em resistant showed the presence of pAB161 as before. As a further test, total DNA was isolated from the putative integrate and subjected to PCR using two primers (arcRF and arcRR) corresponding to the start and end of *arcR*, and two (blaF and blaR) corresponding to internal sites within the *bla* gene of pUC19. The products produced with the pairs arcRF–blaR and arcRR–blaF were as predicted for a single crossover integrating pAB161 into the chromosome at the *arcR* gene (results not shown). Integration of pAB190 into the *B. subtilis* chromosome was achieved by linearizing this plasmid with *Pst*I (for which it has a single site) and transforming into competent *B. subtilis* cells. Transformants were selected on Cm medium, and were then tested on 1% starch medium subsequently flooded with I_2/KI, whereby the *amy*$^-$ colonies sought were revealed by the absence of clear zones produced by amylase action.

2.5. DNA sequencing and sequence analysis

DNA sequences were read on both strands of plasmid DNA by the enzymatic chain termination method, using the T7 polymerase Sequencing™ kit (Pharmacia) and [α-^{35}S]dATP (Amersham). Part of the sequence of *arcR* was obtained during the sequencing of a *Hin*dIII–*Bam*HI fragment carrying the *arcABDC* gene cluster [16]. The remainder of the gene was sequenced on plasmid pHin4a carrying the 10-kb *Hin*dIII–*Hin*dIII fragment initially cloned, using a primer walking strategy with synthetic oligonucleotides. Database searches were performed using the BLAST program, and multiple alignments of protein sequences using CLUSTAL W. The nucleotide sequence of *arcR* has been submitted to the DDBJ/EMBL/GenBank databases under accession number Y17554.

2.6. Overproduction of ArcR protein and in vitro binding to DNA

The *arcR* gene was amplified from genomic DNA by PCR with the oligoprobes Nd-Fn+10 (5′-AGGGTGGTCCATATGAAGAACAAAGAC-3′) and Nd-Fn-11 (5′-CGCATATGCGGTTTGAAACAGAGGATTTC-3′). The PCR was performed in a Techne thermocycler (Progene) with *Taq* polymerase, in 100 μl reaction mixture of the composition recommended by the manufacturer of the polymerase (Boehringer Mannheim). The procedure consisted of 30 cycles (30 s at 94°C, 30 s at 50°C, and 2 min at 72°C), preceded by 3 min at 94°C and followed by 10 min at 72°C. The amplified fragment was cloned in plasmid pCR2.1. The sequence of the insert was verified. The *Nde*I sites present on the oligoprobes used for the amplification allowed the excision of the fragment and its cloning into the corresponding cloning site of the expression vector pET-3a. The correct orientation of the insert was confirmed by the *Bam*HI restriction pattern. The recombinant plasmid was transformed into the *E. coli* strain HMS174(DE3)pLysS. For production of ArcR, the strain was grown at 37°C in minimal medium 132 [11] supplemented with 20 mM glucose to mid-exponential phase. Overexpression was then triggered by addition of IPTG (1 mM) and allowed to proceed for another 3 h before harvesting. The cells were sonicated in 10 mM Tris–HCl buffer pH 8, and the production of ArcR was monitored by SDS-PAGE on a Phast System (Pharmacia), using 10–15 gradient gels. A large fraction of the protein was produced as insoluble inclusions; nevertheless, when the overexpression was carried out in minimal medium, a significant fraction of the ArcR protein was soluble. Such extracts containing a good proportion of soluble proteins were used in the binding assays, after centrifugation and concentration, as well as control extracts of the same recombinant strain where induction by IPTG was omitted.

For in vitro binding studies, a 272-bp fragment spanning nucleotides −221 to +51 with respect to the transcription start point upstream from *arcA* was produced by PCR and labelled at one end with [γ-^{32}P]ATP (Amersham) and T4 polynucleotide kinase as in [16]. DNase I footprinting was performed on this fragment with *E. coli* HMS174(DE3)pLysS pET-3a.*arcR* cellular extract containing the overproduced ArcR protein. The conditions of the footprinting were as in [16], except that arginine was omitted from the binding mixture.

3. Results

3.1. Identification of a gene arcR, encoding a protein of the Crp/Fnr family, in the vicinity of the arcABDC cluster

Sequence analysis revealed the existence, 109 bp downstream from *arcC*, of a 678-bp open reading frame (ORF)

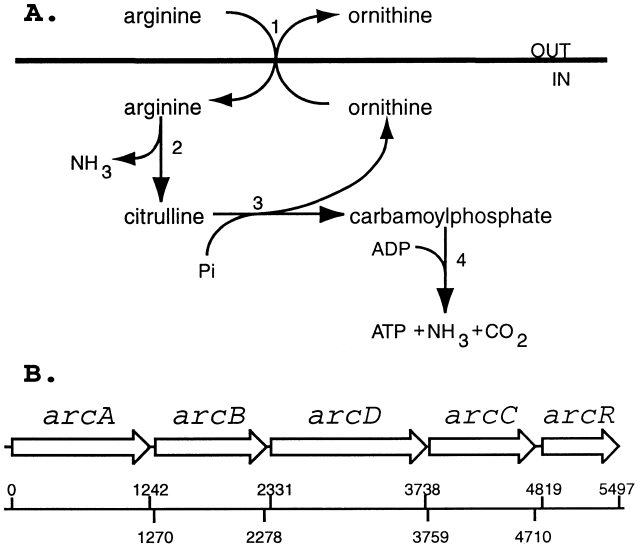

Fig. 1. The arginine deiminase pathway. A: the pathway. B: Organization of the genes. 1: arginine–ornithine antiporter (*arcD*), 2: arginine deiminase (*arcA*), 3: catabolic OCT (*arcB*), 4: carbamate kinase (*arcC*).

in the same orientation as the *arcABDC* cluster (Fig. 1). This ORF would encode a 225-residue polypeptide with a molecular mass of 28 kDa. Sequence database searches suggested that the protein could be a member of the Crp/Fnr family: among the 10 proteins with the highest similarity score, nine were attributed to this family. The most similar proteins included: a nitrite and nitric oxide reductase regulator of *Rhodobacter sphaeroides* (accession no U62403), several NtcA nitrogen regulators from Cyanobacteria (*Anacystis nidulans*, Q05061: 28% identical residues; *Cyanothece* sp., U80855: 29% identity; *Synechocystis* sp., P33779: 26% identity; *Synechocystis* sp., P29283: 25% identity), a Fnr-like protein from *Haemophilus influenzae* (P45199: 25% identity), Fnr of *B. subtilis* (P46908: 25% identity), and the regulatory protein HlyX from *Actinobacillus pleuropneumoniae* (P23619: 22% identity). The highest similarity (35% identity) was found with the product of unknown function (5′ SAGP, accession number JH0364) of a gene located in the vicinity of the arginine deiminase gene in *Streptococcus pyogenes* [13]. This suggested that the gene product could be a regulatory protein involved in the regulation of the *arc* genes; the gene was therefore named *arcR*.

Multiple sequence alignment between ArcR and the most studied representatives of different groups of the Crp/Fnr family showed that identical residues and conservative substitutions are found throughout the entire sequence of ArcR (Fig. 2). The percentage of identical residues shared by ArcR and each of the three proteins aligned in Fig. 2 is not large: 21% with *E. coli* Crp, and 19% with NtcA of *Anabaena* sp. strain PCC 7120. However, ArcR shows a higher similarity with Crp than with any other representative of the family if the C-terminus alone is taken into consideration: 27% of the residues are conserved in that part of the ArcR protein that would correspond to the C-terminal domain of Crp. Within this small domain, conservation is even higher at the level of the DNA recognition element, where 14 residues within the 24-residue helix–turn–helix motif are identical in ArcR and Crp. Mutagenesis and crystallography have shown that Arg-180 and Glu-181 in Crp (Arg-181 and Glu-182 in Fig. 2) make specific contacts with bases within the DNA site ([18] and references therein). Arg-180 and Glu-181 as well as their context are conserved in ArcR. ArcR and Crp are different in their N-terminal domain, with ArcR lacking the cAMP binding residues identified by crystallographic studies (Fig. 2; [20]). Interestingly, the similarity of ArcR to anaerobic regulators of the Fnr type is restricted: none of the cysteine residues (C20, C23, C29

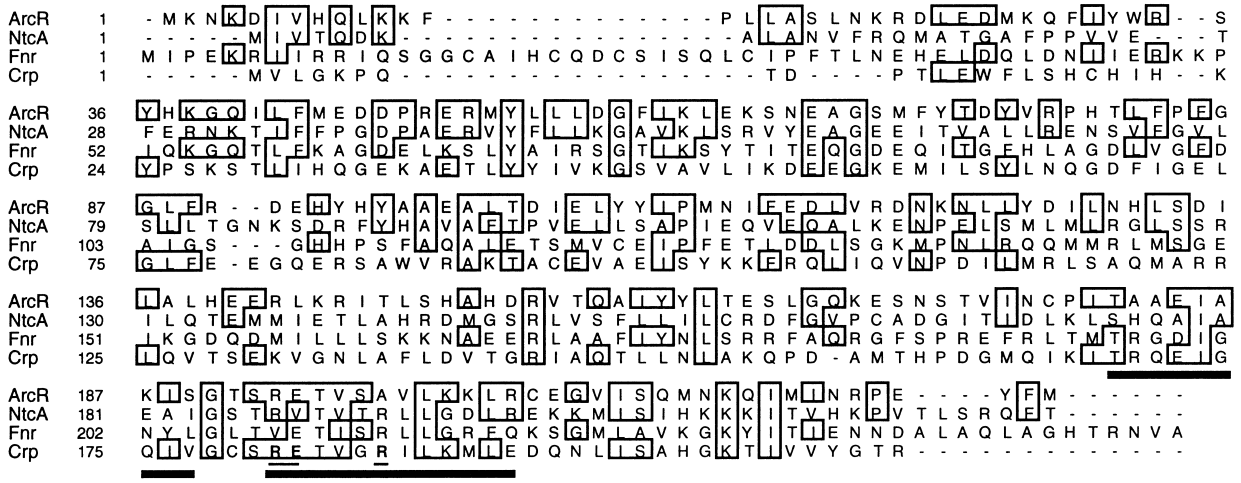

Fig. 2. Alignment of ArcR with proteins representative of the Crp/Fnr family: NtcA from *Anabaena* sp (accession number Q05061), Fnr from *E. coli* (accession number M18458), Crp from *E. coli* (accession number P03020). Residues conserved in ArcR are boxed. Parts of the sequences that correspond to the helices of the helix–turn–helix motif in Crp are underlined. The Arg-180, Glu-181 and Arg-185 residues, that make specific bounds with DNA in Crp, are printed in bold and are underlined.

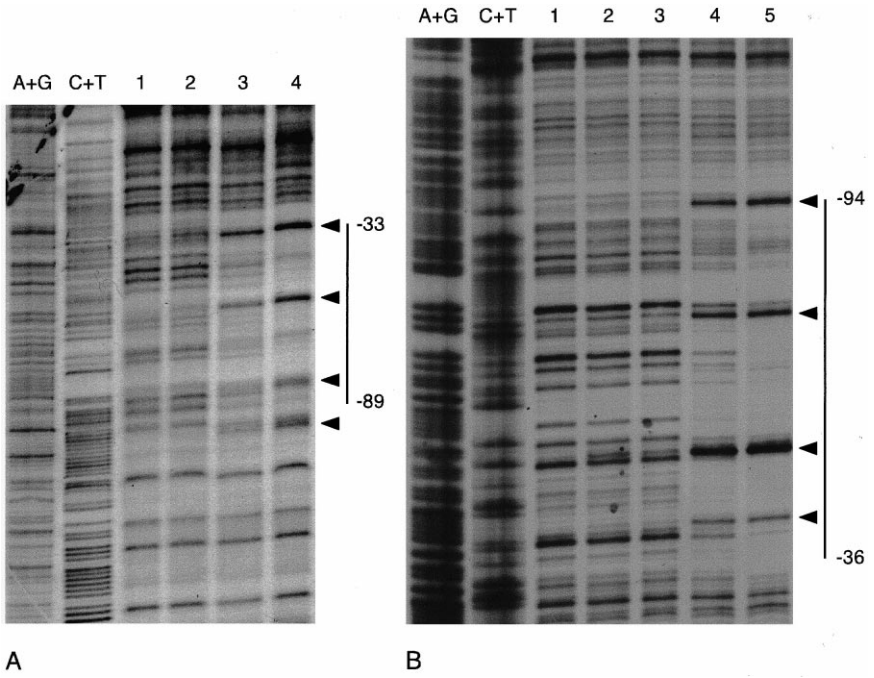

Fig. 3. DNase I footprinting of ArcR binding to the *arcA* promoter region. A 272-bp fragment (spanning from −221 to +51, with respect to the transcription start point) was incubated with increasing amounts of cellular extract containing overproduced ArcR. The upper strand is shown in A, and the lower strand in B. Reference ladders (A+G and C+T) were generated by sequencing (by chain termination in A, and by Maxim and Gilbert in B). The regions protected against DNase I digestion are indicated by vertical lines. Sites hypersensitive to DNase I are marked by arrows. Protein additions are: A: lane 1: no addition, 2: 25 μg total proteins of cellular extract containing overproduced ArcR, 3 and 4: 50 μg of the same cellular extract. B: 1: no addition of protein, 2: 50 μg total protein of cellular extract of non-induced cells, 3: 10 μg total proteins of a cellular extract containing overproduced ArcR, 4: 25 μg total proteins of the same induced cells, 5: 50 μg.

and C122 in *E. coli*) that bind the oxygen-sensing Fe–S cluster in Fnr [21] are present in ArcR (Fig. 2). The cysteine-rich C-terminal extension present in the Fnr homologues in *B. subtilis* [7] and *B. licheniformis* [15] is also absent in ArcR

3.2. Identification of the DNA binding site of ArcR

The *arcA* transcription start point is preceded by a long stretch of non-coding sequence [16]. Footprinting experiments were performed to test the possibility that ArcR could bind in the vicinity of the *arcA* promoter. A fragment spanning 221 bp upstream to 51 bp downstream from the transcription start point was used in these experiments. We have so far been unable to purify ArcR. For this reason, in vitro binding was attempted with a cellular extract of the *E. coli* strain HMS174(DE3) plysS pET-3a.*arcR*, containing overproduced ArcR protein. A single stretch of DNA was protected on each strand against DNase I digestion in the presence of ArcR (Fig. 3). No protection was observed with comparable amounts of extract from non-induced cells of the same strain. The region protected in the presence of ArcR was located just downstream from the binding site of ArgR [16] (Fig. 4); it made contact with the -35 region of the promoter, and covered a region of imperfect dyad symmetry centered at −65.5, as revealed by sequence analysis [16] (Fig. 4). The sequence 5′-TGTGAAATATATCACG-3′ in the middle of the symmetrical region is similar to the Crp consensus binding site (Fig. 4) [2]. It is a nearly perfect palindrome: a 6-bp spacer separates two core motifs, consisting of the 5-bp sequence TGTGA (the core motif in the Crp consensus binding site) for the left arm, and CGTGA, a slightly degenerate form of this, for the right arm (Fig. 4). In the complex formed with DNA by Crp, each subunit of the Crp dimer makes specific contacts with a few bases in one half-site: Arg-180 in one subunit makes one hydrogen bond with G-7, and Glu-181 two with C-5′ at one half-site, while Arg-180 and Glu-181 in the second subunit make the symmetrically related bonds with G-7′ and C-5 (positions within the DNA site are numbered from the symmetry axis) [18,20]. As those bases are conserved at the ArcR site, and Arg-180 and Glu-181 are conserved in ArcR (residues 193 and 194), it seems that ArcR binds to DNA in the same way as Crp. Crp sharply bends DNA [20]; most of the DNA bend angle is accounted for by the primary kinks induced between nucleotides pairs −6 and −5 in the first half-complex and 5 and 6 in the second half-complex [18,20]. Binding of ArcR results in the appearance of several points of hypersensitivity to DNase I within or at the boundary of the footprint (Figs. 3 and 4). Hypersensitivity is thought to arise when DNA bends towards the major groove, enhancing DNA cleavage at the 3′ end of the regulator-induced kink [1]. The hypersensitive points appearing at C-7′ and

A-6 could thus indicate that ArcR introduces a kink at the very place where Crp induces a major kink. Moreover the pattern of hypersensitive bands reveals that secondary kinks are induced by ArcR in A/T-rich segments (Fig. 3), in the same way as Crp [10].

The length of the footprint (57 nucleotides on one strand and 59 on the other) is unusual for proteins of the Crp/Fnr family: all proteins of this family for which footprints have been obtained protect about 22 bp against DNase I digestion. The symmetry at the ArcR site spans 38 bp, far more than the usual 22 bp at a Crp site. The region contains A/T-rich segments that would add to the bendability of the DNA, favoring bending towards the protein when they are facing the major groove [10]. These A/T-rich regions could allow wrapping of a longer segment of DNA around ArcR than around Crp, which would result in a larger footprint. However, as the footprints have been obtained with a crude cellular extract, it is impossible to exclude the possibility that the large footprint is the consequence of additional binding by *E. coli* proteins in the presence of ArcR.

3.3. *In vivo properties of an arcR loss of function mutant*

In order to be certain that *arcR* was in fact a regulatory gene for the *arcABDC* cluster, it was necessary to observe the phenotype of a *B. licheniformis arcR* mutant. This was produced by cloning an internal fragment of *arcR* in the temperature-sensitive Gram-positive vector pE194ts and forcing recombination via a single crossover by selection at elevated temperature of the pE194ts-encoded Em resistance. pAB161 consists of an *arcR* internal *Bam*HI–*Bcl*I fragment cloned into pUC19, the combination then being ligated with pE194ts (see Section 2). pAB161 was transformed into *B. licheniformis* as in [19]. The resulting strain was grown at 30°C, shifted to 42°C, and then plated on Em medium at 42°C as described in Section 2. Large colonies resulting from this procedure were expected to possess chromosomally integrated pAB161 because of the temperature sensitivity for replication of pE194ts. The absence of autonomous plasmid in them was confirmed, and in addition in one such isolate, BLA14–10, the presence of the integrated pE194ts genome was demonstrated by PCR, both as described below. BLA14-10 was grown in oxygen-limited and aerated cultures, with and without arginine. In oxygen-limited cultures on arginine-glutamine medium, the catabolic OCT specific activities were less than 5 for BLA14-10, as opposed to 5200 for the wild-type; under aerobic conditions, specific activities of OCT were similar, and responded similarly to the presence or absence of arginine, in BLA14-10 and the wild-type (data not shown). It may therefore be concluded that ArcR is required for anaerobic expression of *arcB*, and by extension probably of the *arcABDC* cluster.

3.4. *Effects of overproduction of ArcR in vivo in B. licheniformis and B. subtilis*

It was of interest to observe whether expression of *arcR* at a high constitutive level in *B. licheniformis* was capable of overcoming the need for anaerobiosis or arginine for induction of the *arc* operon. pAB200, consisting of a *Cla*I–*Eco*RI fragment containing *arcR* cloned into the expression vector pRB374, was transformed into *B. licheniformis* as before. Under aerobic conditions, in glucose-arginine medium, the strain carrying pAB200 gave an OCT specific

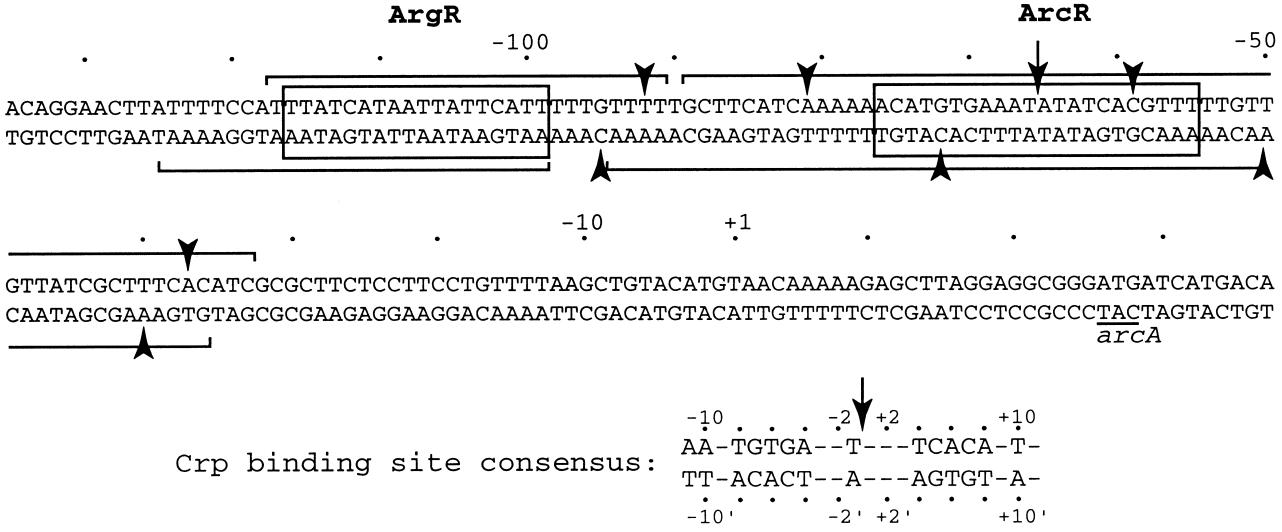

Fig. 4. Schematic representation of the *arcA* upstream region spanning the transcription start and the ArcR and ArgR binding sites. The transcription start point is located at +1. The protected regions are indicated on each strand by horizontal brackets. Arrowheads show the sites of increased sensitivity to DNase I. The Arg box and the sequence resembling a Crp binding site are boxed. The vertical arrow indicates the center of dyad symmetry in the ArcR binding site. The Crp consensus binding site [2] is given for comparison. Data concerning the transcription start point and binding of ArgR are taken from [16].

activity of 1400, as opposed to the wild-type's 2. In glucose–ammonium medium, the values were 34 and 8 respectively, showing that induction by arginine is still required.

A similar experiment was carried out with *B. subtilis*. pDG268 is a *B. subtilis* integrative vector with cloning sites upstream of a promoter-less *lacZ* gene. A fragment expected to contain the *arcA* promoter was cloned into pDG268 to generate pAB190, which was then cut and transformed into wild-type *B. subtilis* such that a cassette carrying a selectable *cat* marker plus the *arc* promoter–*lacZ* fusion was introduced by recombination into the *amy* gene. β-Galactosidase specific activities were determined in both BSA 50-1 and the wild-type strain after transformation with the *arcR*-expressing plasmid pAB2000, on glucose-ammonium and glucose-arginine medium. Only in the case of BSA50-1 carrying pAB200 were measurable activities found, namely 250 in glucose-arginine and 25 in glucose-ammonium medium.

It is therefore clear that overexpression of *arcR* permits activation by arginine under aerobic conditions of the promoter presumed to lie upstream of *arcA*, whether in its natural location or fused to *lacZ*. The implications of this are discussed below.

4. Discussion

We have reported here the involvement of a new protein, ArcR, in the regulation of the arginine deiminase pathway in *B. licheniformis*: ArcR is the product of a gene located 209 bp downstream from *arcC*, the final member of the operon-like cluster *arcABDC* encoding the arginine deiminase pathway. From (i) the absence of anaerobic induction of the pathway following inactivation of *arcR* and (ii) the existence of a binding site upstream from the *arcA* transcription start point, it can be inferred that ArcR is an activator of the arginine deiminase pathway, up-regulating the transcription of the *arc* genes when bound to its target upstream of *arcA*. The arginine repressor ArgR is also an activator of the arginine deiminase pathway [16]. Aerobic induction of the pathway in the *arcR*-overexpressing strain is still dependent on the presence of arginine; it seems therefore that both ArgR and ArcR have to be bound to their targets for activation to take place.

Sequence similarities allowed us to identify ArcR as a member of the Crp/Fnr family of transcriptional regulators. Although none of the distinctive features of the Fnr- and Crp-like proteins are conserved in the N-terminal domain, in the C-terminus good similarity is observed in that part of the sequence that corresponds to the DNA binding motif in *E. coli* Crp. Crp is a dimer of identical subunits, each subunit interacting with one half of the DNA site. The two residues known to make specific contact with bases in the DNA site, Arg-180 and Glu-181, are conserved in ArcR; they would be able to form the same hydrogen bonds as in Crp, since the target sequence is similar to a Crp binding site and contains the bases bound by Crp. This suggests that the DNA–protein complexes formed by ArcR and Crp are very similar. Moreover the pattern of DNase I hypersensitivity sites induced by the binding of ArcR suggests that ArcR bends the DNA in the same way as Crp.

Arginine and anaerobiosis are the two stimuli promoting induction of the arginine deiminase pathway. As ArgR activation is modulated by the availability of arginine, it is tempting to see ArcR as the anaerobic regulator. An anaerobic regulator, Fnr has already been described in *B. licheniformis*. The protein, a member of the Crp/Fnr family, is characterized by a cysteine-rich C-terminus [15] and is expected to be, like its homologue in *B. subtilis* [7], a regulator of anaerobic nitrate respiration. Data from *B. subtilis* suggest that the target site of the *Bacillus* Fnr could be very similar to a Crp binding site. However, under the growth conditions tested here, Fnr is evidently unable to compensate for the loss of a functional ArcR in the *arcR* mutant. Our results therefore clearly indicate that ArcR is an activator of the *arc* system.

In *B. subtilis*, an Fnr-independent promoter is located immediately upstream of the *fnr* gene [7]. Transcription from this promoter is up-regulated by anaerobiosis, probably through a cascade involving the two-component system encoded by *resDE* [17]. The similarity between the *narK-fnr* intergenic regions in *B. subtilis* and *B. licheniformis* suggests the existence of a similar regulation in the latter. When the *B. licheniformis narK-fnr* and *arcC-arcR* intergenic regions were compared, similarities were observed only between the 50 bp immediately upstream from the first ATG, which share 23 identical nucleotides; this DNA stretch contains a putative ribosome binding site (−12 to −9 upstream from the ATG) and a possible −10 promoter element centered at −36.5. No significant similarity between the two intergenic regions was observed further upstream. If the transcription of *arcR* is anaerobically regulated, it is therefore likely to be in a manner different to *fnr*. Also, there is no recognizable ArcR binding site in this intergenic region. Clearly, more work has to be done to unravel the properties of ArcR, and the way its synthesis is regulated, in order to determine upon which signal, anaerobiosis or some alternative, ArcR comes to bind to its target site and trigger activation.

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