

Conserved *cis*-Acting Elements Upstream of Genes Composing the Chitinolytic System of Streptomyces Are DasR-Responsive Elements

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Key Words

Chitin · *N*-Acetylglucosamine · Streptomyces · Chitinase · Carbon regulation · DasR

Abstract

For soil-dwelling bacteria that usually live in a carbon-rich and nitrogen-poor environment, the ability to utilize chitin – the second most abundant polysaccharide on earth – is a decisive evolving advantage as it is a source for both elements. Streptomyces are high-GC Gram-positive soil bacteria that are equipped with a broad arsenal of chitinase-degrading genes. These genes are induced when the streptomyces sense the presence of chitooligosaccharides. Their expression is repressed as soon as more readily assimilated carbon sources become available. This includes for example glucose or *N*-acetylglucosamine, the monomer subunit of chitin. Historically, the first *cis*-acting elements involved in carbon regulation in streptomyces were found more than a decade ago upstream of chitinase genes, but the transcriptional regulator had so far remained undiscovered. In this work, we show that these *cis*-acting elements consist of inverted repeats with multiple occurrences and are bound by the HutC/GntR type regulator DasR. We have

therefore designated these sites as DasR-responsive elements (*dre*). DasR, which is also the repressor of the genes for the *N*-acetylglucosamine-specific phosphotransferase transport system, should therefore play a critical role in sensing the balance between the monomeric and polymeric forms of *N*-acetylglucosamine. Copyright © 2007 S. Karger AG, Basel

Introduction

Chitin found in the cuticle and the shell of insects and crustaceans or within the cell wall of fungi constitutes the main form of storage of *N*-acetylglucosamine (GlcNAc) in nature [Cohen-Kupiec and Chet, 1998; Keyhani and Roseman, 1999]. For soil-dwelling bacteria, chitin's contribution to primary metabolism 'interests' can easily be deduced from the end products of its complete catabolism. These are acetate, ammonia, and fructose-6-phosphate, which are directed to the tricarboxylic acid cycle, fatty acid metabolism, nitrogen metabolism and glycolysis. Long insoluble molecules of chitin cannot enter the cell. They are degraded prior to their assimilation into chitooligosaccharides and *N,N'*-diacetylchitobiose

(GlcNAc)₂ as major products, and, less abundantly, into GlcNAc by microorganisms that possess the appropriate enzymatic arsenal.

Streptomyces are high-GC Gram-positive soil bacteria that have a selective advantage in colonizing different types of chitin-containing substrates due to their large pattern of chitinases, β -N-acetylglucosaminidases and other chitin-targeting proteins [Saito et al., 2003; Schrempf, 2001]. This genetic predisposition is accompanied by a performing regulatory system that triggers induction of chitin (*chi*) genes in the presence of major chitin degradation products – like (GlcNAc)₂ – and that triggers repression when more readily assimilated sugars (like glucose) are available [Miyashita et al., 2000; Saito et al., 1998, 2000]. The understanding of how the cell coordinates the expression of genes involved in chitin degradation, chitin derivative products sensing, transport, and subsequent catabolism is decisive to interpret the evolving success of streptomyces into their ecological niche.

Previous studies have reported that *chi* genes all share similar *cis*-acting elements in their upstream region, initially described as conserved directed repeats, and, historically, the first DNA motifs found to be involved in carbon regulation in streptomyces. The role of these DNA sequences was reported by Delic et al. [1992] and later by Ni and Westpheling [1997] who showed that point mutations within the conserved sequences upstream of the chitinase gene *chi63* of *Streptomyces plicatus* resulted in the constitutive expression (glucose-resistant and chitin-independent) of a downstream reporter gene. The resulting constitutive expression suggested a negative role exerted by the transcription factor able to bind these sequences.

So far, four regulatory proteins have been identified for their implication in chitinase production, namely the two-component system ChiS/ChiR [Kormanec et al., 2000; Tsujibo et al., 1999], Reg1 [Nguyen et al., 1997; Nguyen, 1999], and Cpb1 [Fujii et al., 2005]. However, none of these seem to interact with the *chi cis*-elements. Interestingly, earlier studies reported that these *cis* sites were similar to the predicted *cis*-acting elements upstream of genes of the GLcNAc-specific phosphotransferase uptake system (PTS^{GlcNAc}), suggesting a common regulator for both systems, and therefore a probable fifth regulatory protein involved in the regulation of the chitinolytic system [Nothaft et al., 2003; Parche et al., 1999; Rigali et al., 2004; Studholme et al., 2004]. We showed recently that the HutC/GntR type regulator DasR (Deficient in aerial mycelium and spore formation) [Seo et al.,

2002] is the repressor of the genes encoding the proteins of the GLcNAc-specific PTS permease complex [Rigali et al., 2004, 2006].

Here, we have addressed the question whether DasR also recognizes the related *cis*-acting elements present within the promoter regions of *chi* genes. We show this by a refined in silico analysis and subsequent experimental validation. Hence, the conserved *cis*-acting elements of the *chi* genes are DasR-responsive elements (*dre*). This suggests that the complex regulation of chitin metabolism involves the transcription factor DasR.

Results

Generation of the DasR Weight Matrix

Previous investigations reported sequence similarities between known and predicted *cis*-acting elements that are present in the upstream regions of GLcNAc-related PTS genes and some *chi* genes [Parche et al., 1999; Nothaft et al., 2003]. In order to assess whether both systems could share DasR as a common regulator, we generated a position weight matrix with the DasR-responsive elements (*dre*) already validated by electrophoretic mobility gel shift assays (EMSAs) [Rigali et al., 2004]. These were the *dre* sites of the PTS genes *ptsH*, *crr-ptsI*, *nagE2*, *malX2* and the one between *dasR* and the adjacent *dasABCD* operon (fig. 1). The obtained, refined matrix was used to check for the occurrence of *dre* sites in the upstream region of genes related to the chitinolytic system in the *Streptomyces coelicolor* genome.

Chitin genes with significant *dre* sites (score >5) are listed in table 1. Their consensus (fig. 2A) revealed (i) a signature sequence almost identical to the one of the PTS^{GlcNAc} genes (fig. 2B) and (ii) an almost perfect palindromic structure. The sequence contains also the GT.TA.AC signature motif recognized by DasR-like transcriptional regulators that belong to the HutC/GntR subfamily [Rigali et al., 2002]. Therefore, the *cis*-acting elements upstream of *chi* genes, so far reported as directed repeats [Delic et al., 1992], are in reality inverted repeats with multiple occurrences (up to three) on a single upstream region, as can be seen for example for *chiH* (SCO6012) in table 1. All types of chitin-related genes possess putative *dre*-like sequences. The list in table 1 includes genes encoding chitinases of the family 18 (subfamilies A, B, and C), a chitinase of the family 19, chitin-binding proteins, intracellular and extracellular β -N-acetylglucosaminidases, and the NgcE homologue which is the putative extracellular chitobiose-binding protein

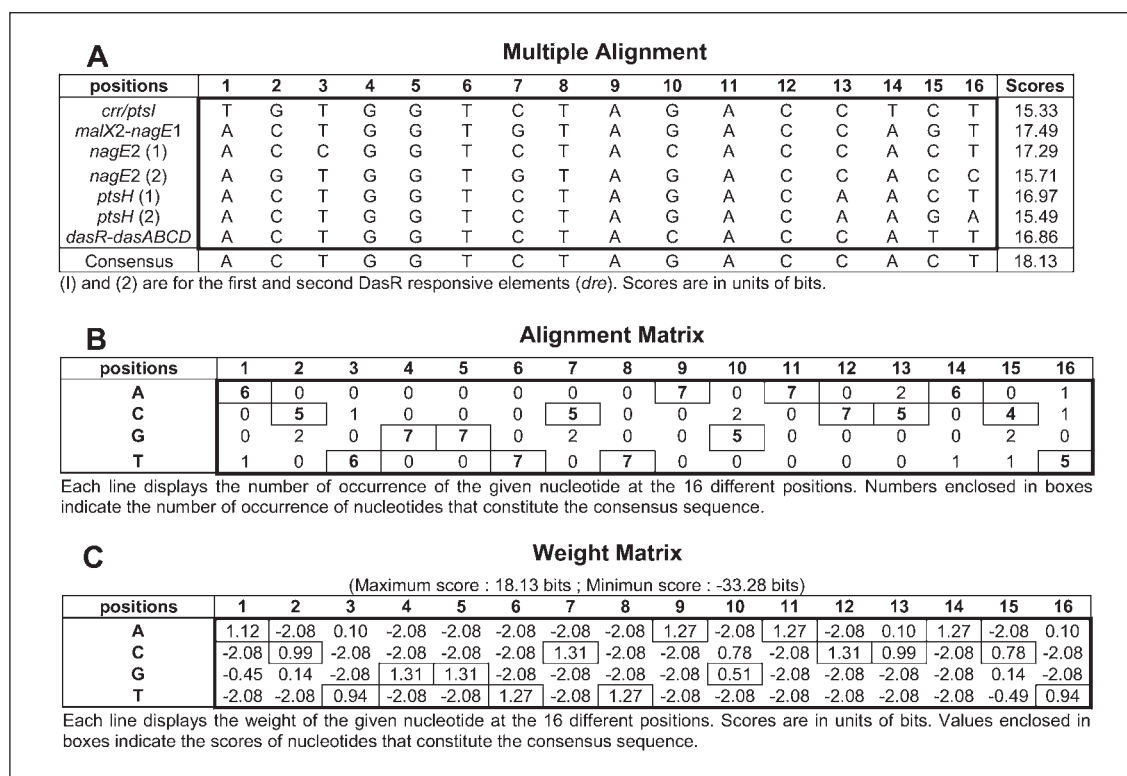


Fig. 1. Alignment and weight matrices deduced from experimentally validated *dre* sites.

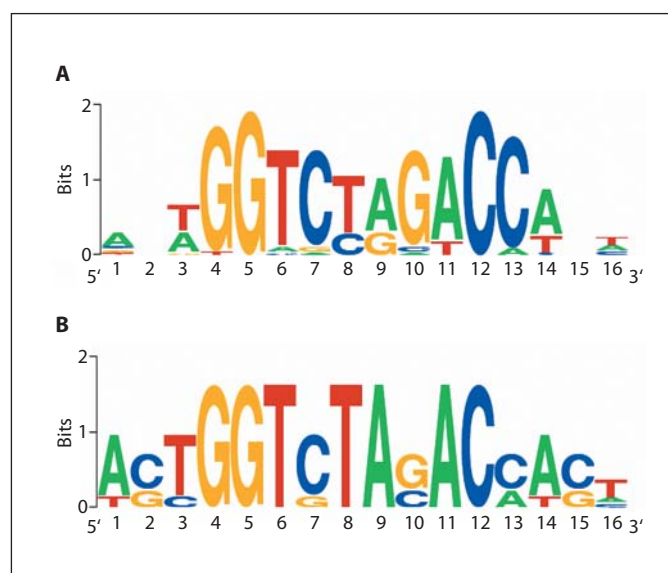


Fig. 2. Consensus of *cis*-acting elements upstream of *chi* (A) and $\text{PTS}^{\text{GlcNAc}}$ genes (B). Sequences used to build the chitinase and PTS consensus are listed in table 1 and in figure 1A. Consensus sequences were built with the WebLogo program available at <http://weblogo.berkeley.edu/logo.cgi>.

of the NgcFG ABC-transporter [Bertram et al., 2004; Saito et al., 2003]. The distribution of *dre* sites is thus apparently not limited to a category of *chi* genes but has emerged within the upstream region of genes with different evolving origins to weave a coordinated regulatory network.

cis/trans Relationship between DasR and chi Genes

In order to validate our computational predictions, EMSA experiments were performed using pure histidine-tagged DasR and labeled DNA fragments of five *chi* gene upstream regions including single or multiple predicted *dre* sites. The upstream regions tested were those of *chiD* (SCO1429, one *dre*), *chiI* (SCO1444, one *dre*), *chiH* (SCO6012, three *dres*), SCO6345 (one *dre*), and SCO7225 (three *dres*) (table 1, fig. 3A). For these five, we used longer PCR fragments (170 and 313 nt) that contained the upstream regions with the predicted *dre* sites. Positive DNA-DasR interactions were observed for all tested *dre*-containing promoter regions (fig. 3A). We then tested one of the *dre* sites, the one from SCO6345 (putative chitin-binding protein) promoter, where we took a 30-bp

Table 1. DasR-responsive elements (*dre*) predicted upstream of chitin metabolic genes. Positions of matching sequences from the starting codon were calculated from the most distal nucleotide of the putative *cis*-acting sequence

No.	Gene	Function	<i>dre</i>	Position	Score	Reference
1	SCO0481, <i>chb3</i>	chitin-binding protein	TATGGTCTAGTCCAAC	-201	7.76	Saito et al., 2001
2	SCO1226c	chitinase	CTTGGTCCAGACCTGT	-187	7.79	Bentley et al., 2002
3	SCO1429, <i>chiD</i>	chitinase (family 18-A)	ACTGGTCTAGTCCTCC	-96	10.61	Saito et al., 1999
4	SCO1444, <i>chiI</i>	chitinase (family 18-A)	ACTGGTCTAGTCCTCT	-53	12.18	CAZy database** Bentley et al., 2002; Saito et al., 2003
5	SCO2503, <i>chiJ</i>	chitinase (family 18-B)	AAAGGTCTGGACCACA	-78	9.76	CAZy database
			CTTGGTCCAGACCTCT	-99	6.94	Bentley et al., 2002; Saito et al., 2003
6	SCO2786c	β -N-acetylglucosaminidase	ACGGGTGCGGACCACT	-70	6.59	Bentley et al., 2002
7	SCO2943c*	β -N-acetylglucosaminidase	AGAGGTCTGAACCAAT	-112	7.37	Bentley et al., 2002
8	SCO2833c, <i>chb</i>	secreted chitin-binding protein	GCAGGTCTAGACCAAG	-70	9.57	Bentley et al., 2002
9	SCO5003c, <i>chiA</i>	chitinase (family 18-B)	GGTGGTCCAGACCAAT	-77	9.23	Saito et al., 1999
10	SCO5235*, <i>dasD</i>	intracellular β -N-acetylglucosaminidase	ACTGGTCTACACCATT	-106	16.86	Bentley et al., 2002
			CTTGGTCTAGTCCATA	-322	6.13	
11	SCO5376, <i>chiC</i>	chitinase (family 18-A)	AAAGGTCTGGACCATA	-88	8.49	Saito et al., 1999
			ATAGGTCTGGACCAAT	-109	7.74	
12	SCO5673, <i>chiB</i>	chitinase (family 18-B)	ATTGGTCTGGACCAAA	-63	7.74	Saito et al., 1999
13	SCO5954, <i>chiE</i>	chitinase (family 18-A)	ATTGGTCCAGACCTTC	-95	6.17	Bentley et al., 2002;
			ACAGGCGCAGACCACC	8	5.02	Saito et al., 2003
14	SCO6005, <i>ngcE</i>	(GlcNAc) ₂ ABC-type, sugar-binding component	AGTGGACTATACCTGT	-334	10.28	Bentley et al., 2002
15	SCO6012c, <i>chiH</i>	chitinase (family 18-C)	AATGGTCTGGACCAGA	-111	10.04	Saito et al., 2000
			GGTGGACTGGACCACC	-184	5.72	
			ATGGGACTAGACCAAT	-127	5.56	
16	SCO6032c	β -N-acetylhexosaminidase	CTTGGTCTAGTCCATT	-154	6.97	Bentley et al., 2002
17	SCO6300c	secreted β -N-acetylglucosaminidase	AGAGGTCTAGACAAAA	-116	11.58	Bentley et al., 2002
			ATAGGTCTAGACAAAA	-131	9.36	
18	SCO6345	chitin-binding protein	TAAGGTCTAGACCTGC	-114	9.99	Bentley et al., 2002
			GTAGGTCTAGACCTGC	-94	6.22	
19	SCO7069c	chitinase	ACAGGTCCGGACCAAT	-61	7.46	Bentley et al., 2002
20	SCO7070	chitosanase	ACAGGTCCGGACCAAT	-61	7.46	CAZy database Bentley et al., 2002
21	SCO7225	secreted chitin-binding protein	TCAGGTCTAGACCTGT	-34	12.19	Bentley et al., 2002
			TATGGTCTAGACCTGA	-55	11.71	
			CCTTGTCTAGACCAAT	-168	10.24	
22	SCO7263, <i>chiF</i>	chitinase (family 19)	ACTGGTCTACACCCTT	-172	13.51	Saito et al., 1999
			ACTGGTACAGACCAAA	-155	7.42	

* The position of the *dre* has been calculated from the translation start of the first *orf* of an operon; scores are expressed in units of bits.

** CAZy (Carbohydrate Active Enzymes) database, <http://www.cazy.org/CAZY/> [Coutinho and Henrissat, 1999].

long fragment with the *dre* site in the center, to demonstrate the DasR-*dre* interaction in a more specific manner (fig. 3B). Finally another five *dre* sites from diverse *chi* genes were chosen and examined in the same way. These were from the chitinase genes *chiE* and *chiA*, the chitin-binding protein genes *chb3* and SCO2833, and the putative intracellular β -N-acetylglucosaminidase encoded by SCO2943. All targets showed binding to DasR in vitro,

thus highlighting DasR as a common DNA-binding protein of both PTS and *chi* genes. As controls, we observed similar electrophoretic retardation with the upstream region of the *crr-ptsI* operon encoding for enzyme IIA (IIA^{Crr}) and enzyme I of the sugar phosphotransferase system, while no binding of DasR was observed to the upstream regions of *xlnA* and *crp*.

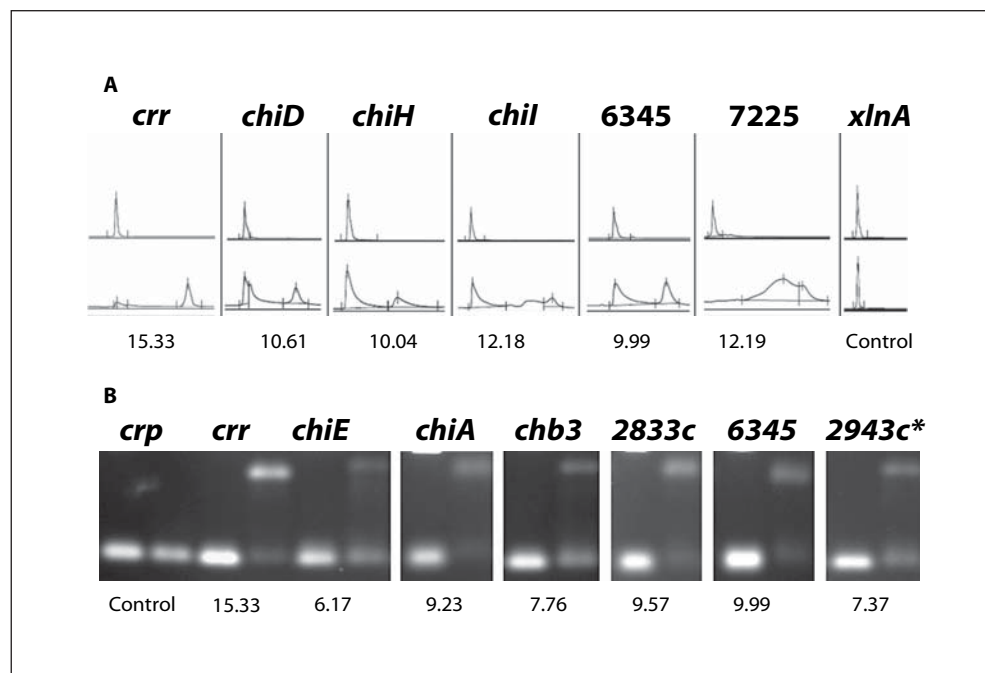


Fig. 3. Validation of DasR-*chi* genes *cis-trans* relationship. **A** EMSAs with pure histidine-tagged DasR and labeled DNA probes corresponding to the upstream regions of *chiH* (SCO6012), *chiD* (SCO1429), *chiI* (SCO1444), SCO6345, and SCO7225. The upstream regions of the *crr-ptsI* operon and of *xlnA* were used as positive and negative controls. Each plot shows the labeled upstream regions without DasR (top) and with DasR (bottom). EMSAs were performed using fluorescent probes (10 nM), purified DasR (3 μ M), and 1,000-fold excess of non-specific DNA. **B** A 1% agarose gel stained with ethidium bromide is shown that displays the double-stranded oligonucleotide at the bottom and a

signal band corresponding to the DasR-DNA complex at the top. Oligonucleotides (25 pmol, 36 bp length) with the *crp* *cis*-element and the *crr dre* site served as negative and positive controls. Oligonucleotides (25 pmol) of 30 bp length containing the *dre* sites of *chiE*, *chiA*, *chb3*, SCO2833c (c, indicates opposite direction of transcription), SCO6435, and SCO2943c* (* indicates that the *dre* site lies in front of the first gene, SCO2946c, of the operon) were used in the absence (first lane) and in the presence of 1 μ g purified DasR (second lane). The predicted scores for the *dre* sites are shown below each figure.

Discussion

In this communication, we have defined a consensus sequence (A.TGGTCTAGACCA.T) for the *dre* sites of chitin-metabolic genes and have demonstrated that DasR binds to these *cis*-acting elements that occur as single or multiple inverted repeats in the promoter regions of 22 different *chi* genes in the genome of *S. coelicolor* A3(2). Thus, we have identified with DasR the transcription factor that was originally detected by Ni and Westpheling [1997].

There are several other regulatory proteins involved in *chi* gene regulation. These are the two-component system proteins ChiS/ChiR [Kormanec et al., 2000; Tsujibo et al., 1999], Reg1 of the LacI/GalR family [Nguyen et al., 1997], and Cpb1 [Fujii et al., 2005]. *chiR* and *chiS* genes were studied in *Streptomyces thermoviolaceus*, where overexpression of ChiR and ChiS led to an increase the chitinase

40 activity. Disruption of *chiR* caused a reduced expression of *chiC* as observed in *S. coelicolor*. The suggested model says that ChiS is autophosphorylated at a conserved histidine residue (H1199), which would happen in response to chitobiose or a related chitin breakdown product. The phosphoryl group is then transferred to a conserved aspartate (D54) of ChiR, which then binds to the *chi* promoter regions to activate *chi* genes expression. However, pure ChiR does not seem to be able to interact alone with the conserved *dre* sites, suggesting another target site for this system.

More recently, Cpb1 (Chitinase promoter binding) has been isolated from crude extracts of *Streptomyces lividans* for its natural affinity to the promoter of *chiA* [Fujii et al., 2005]. Cpb1 binds to the promoter of *chiA* but its gene disruption only showed a slight increase of the total chitinase activity under glucose repression conditions.

The authors suggest that Cbp1 might work as an auxiliary factor in chitinase gene expression by supporting or modifying the function of other major regulators [Fujii et al., 2005].

Eventually, Reg1 of *S. lividans*, which is identical to MalR (the maltose operon regulator) of *S. coelicolor* [van Wezel et al., 1997], was proposed to be involved in *chi* gene regulation. Chitinase activity measurements in the *S. lividans reg1* null mutant revealed that induction of chitinase production was lost and relieved from carbon catabolite repression by glucose [Nguyen, 1999]. DNA-binding experiments showed that Reg1 bound to the upstream region of *chi40* [Nguyen, 1999].

In the light of the available information, the transcriptional control of the chitinolytic system seems exceedingly elaborated in streptomycetes, far from the initial idea of a relatively straightforward operator-repressor concept, but rather with many DNA-binding proteins each having its own specificity and timing. This multilevel control system suggests a cooperative mode of action between the various protagonists, in which DasR may serve as a global regulator that operates in concert with several *chi* gene-specific transcription factors. The characterization and comparison of the global chitinase activity and expression analysis of individual *chi* genes in strains that are deficient in *dasR*, *chiSR*, *reg1*, and *cbp1* or that overproduce these factors should provide decisive answers to understand the complex network of transcriptional control of the chitinolytic system in streptomycetes.

Experimental Procedures

Bacterial Strains, Growth Conditions and DasR Production

E. coli BL21(DE3) was used for DasR overexpression experiments (Novagen, UK). Cultures were grown in Luria-Bertani

broth (LB) at 37°C. Production and purification of recombinant histidine-tagged DasR were performed as described [Rigali et al., 2004].

Electrophoretic Mobility Gel Shift Assays (EMSAs)

DNA-binding studies were conducted with the use of an ALF express sequencer [Filee et al., 2001]. The fluorescent double-stranded promoters containing the DasR *cis*-acting element(s) were generated by PCR using oligonucleotides listed in supplementary table 1. Genomic DNA of *S. coelicolor* served as a template for DNA amplification. PCR products were purified using the GFX PCR DNA and Gel Band Purification kit (Amersham). The assay comprised 10 nM of fluorescent probes, 3 µM of pure DasR and a 1,000-fold excess of non-specific DNA. Alternatively, two complementary oligonucleotides of 30 nucleotides (supplementary table 1) were hybridized and used in the EMSAs as described [Rigali et al., 2004].

Construction of DasR Weight Matrices and Prediction of DasR-Binding Sites

Multiple alignment, alignment and weight matrices were generated as described previously [Rigali et al., 2004]. For this purpose, we used *dre* sequences upstream of PTS^{GlcNAc} genes already validated as DasR-dependent. These were from the promoter regions of *crr-ptsI*, *malX2-nagE1*, *nagE2*, *ptsH*, and between *dasR* and *dasABCD* (fig. 1). Sites within regulatory regions (350 nt upstream of and 15 nt downstream from the beginning of each open reading frame) with scores comprised between 5 and 18 bits were recovered from total predictions.

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