Characterization of the Bacillus subtilis YxdJ response regulator as the inducer of expression for the cognate ABC transporter YxdLM

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The genome of Bacillus subtilis, like those of some other AT-rich Gram-positive bacteria, has the uncommon feature of containing several copies of arrangements in which the genes encoding two-component and cognate ABC transporter systems are adjacent. As the function of one of these systems, the product of the yxd locus, is still unknown, it was analysed further in order to get some clues on the physiological role of the gene products it encodes. The yxdJ gene was shown to encode a DNA-binding protein that directly controls transcription of the neighbouring operon encoding the ABC transporter YxdLM. Primer extension and DNase protection experiments allowed precise definition of the yxdLM transcription start and controlling region. Two putative direct repeats were identified that are proposed to be the YxdJ response regulator binding sites. Whole-cell transcriptome analyses revealed that the YxdJ regulon is extremely restricted. In addition to the yxdJKLMyxeA operon, only a few genes involved in modifications of the bacterial cell wall were shown to be regulated by YxdJ.

INTRODUCTION

In their biotope, bacteria often experience drastic changes in environmental conditions. An appropriate response can be developed by a bacterium that possesses a system ensuring the detection and subsequent transduction of a stimulus generated by modification of the external medium. In many cases, two-component regulatory systems (TCSs), usually composed of a sensor kinase and a response regulator, are involved in this adaptive response (Parkinson & Kofoid, 1992). After detection of the signal, the sensor kinase autophosphorylates on a histidyl residue. The phosphoryl group is then transferred to a conserved aspartyl residue of the cognate response regulator. Once phosphorylated, the regulator can modulate the transcription of specific genes, eliciting an appropriate cellular response to the original stimulus.

Genomic sequencing of several micro-organisms has revealed great diversity of the TCS repertory in many species. This fact reflects the capability of some organisms to respond to a wide range of environmental changes. Bacillus subtilis, a Gram-positive spore-forming soil bacterium, possesses more than 30 such TCSs (36 sensor kinases and 35 response regulators; Fabret et al., 1999). The largest group, the IIIA/OmpR family, comprises 14 systems, to only four of which have known functions been attributed. The PhoP/PhoR and ResD/ResE systems participate in the response to phosphate starvation (Hulett et al., 1994), the latter system playing a central role in aerobic and anaerobic respiration (Sun et al., 1996). The YycF/YycG system is an essential two-component regulator of B. subtilis growth that modulates ftsAZ operon expression (Fukuchi et al., 2000), and the bceRS (formerly ytsAB) system plays a role in resistance to bacitracin (Bernard et al., 2003; Mascher et al., 2003; Ohki et al., 2003).

As exporters or importers of a wide variety of compounds across the membrane (Ames, 1986; Higgins et al., 1986), ABC (ATP-binding cassette) transporters play a key role in the response of bacteria to environmental changes. The prototypic ABC transporter comprises two membrane-spanning domains and two cytoplasmic nucleotide-binding domains (NBDs) that bind and hydrolyse ATP to provide energy for the transport. The inventory and classification of B. subtilis ABC transporters indicated that among the 59
systems predicted as ABC transporters more than 60 % are of unknown function (Quentin et al., 1999).

We recently demonstrated genetic and functional relationship between some members of the IIIA/OmpR family of TCSs and of subfamily 9 of ABC transporters in B. subtilis (Joseph et al., 2002), the TCS structural genes, yxdkJ, yvcPQ and bceRS (formerly ytsAB), controlling the expression of the cognate ABC transporter genes yxdlM, yvcrS and bceAB (formerly ytsCD), respectively. In addition, the BceR/BceS TCS, together with the BceA/BceB ABC transporter, were shown to participate in bacitracin resistance of this bacterium (Bernard et al., 2003; Mascher et al., 2003; Ohki et al., 2003).

We have focused our study on the yxd locus. The operon encoding these ABC transporter structural genes contains an additional gene, yxeA, which encodes an 80 aa peptide conserved in several bacteria of the Bacillus/Clostridium group. The goal of the present work was to characterize the promoter region of the yxdlMyxeA operon and to identify other genes regulated by YxdJ. We showed that YxdJ directly interacts with DNA upstream of the yxdL gene, but this is the only strongly regulated transcript which we detected.

**METHODS**

**General molecular biology techniques.** All molecular biology procedures not presented in detail were carried out as described by Sambrook & Russell (2001). DNA-modifying enzymes were used as recommended by the manufacturer (New England Biolabs). DNA fragments were purified using either Microcon-30 (Millipore) or Qiaquick nucleotide removal kit (Qiagen). DNA cloning was done using T4 polynucleotide kinase (Amersham Pharmacia Biotech) and [c-32P]ATP (Amersham Pharmacia Biotech). Primer extension reactions were carried out with 5–50 µg of total RNA, 10 pmol of one of the 32P end-labelled primers YxdL_EA1 or YxdL_EA2, and 200 units of SuperScript II RNaseH

**RNA isolation.** Culture aliquots (2 ml) were harvested and submitted to a brief centrifugation (13 000 r.p.m. for 3 min). The bacterial pellet was then frozen immediately and stored at −20 °C. Total RNA was isolated using the High Pure RNA isolation kit (Roche) according to the supplier’s recommendation. To avoid genomic DNA contamination, which was tested by PCR with each RNA preparation, two DNase treatments and column purifications were done instead of one.

**Primer extension analysis.** Primer extension reactions were carried out with 5–50 µg of total RNA, 10 pmol of one of the 32P end-labelled primers YxdL_EA1 or YxdL_EA2, and 200 units of SuperScript II RNaseH

**Table 1. Primers**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>YxdKL_left_A</td>
<td>GTGCTTGAAGTCAAAGATTACGG</td>
</tr>
<tr>
<td>YxdKL_left_B</td>
<td>GGCATCAGTTCAGTTTCTATT</td>
</tr>
<tr>
<td>YxdKL_left_C</td>
<td>TACAAAAATGTAAGGCTGCGTAAAG</td>
</tr>
<tr>
<td>YxdKL_left_D</td>
<td>CGCTTGTGTCTCCTGATAACATGG</td>
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<tr>
<td>YxdKL_right</td>
<td>CTATCAAGCCTTTAAAGGCCGTAGAGGACACT</td>
</tr>
<tr>
<td>YxdKL_F</td>
<td>CTACGGGAAATCGCCCTCCATC</td>
</tr>
<tr>
<td>pET_his_1</td>
<td>TATGACCACTACATCATCATCAGTGGG</td>
</tr>
<tr>
<td>pET_his_2</td>
<td>AATTCACGTATGATGATGATGCTGCA</td>
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<tr>
<td>YxdL1</td>
<td>TAGAAGAAACGCGAAGTCCAGGG</td>
</tr>
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<td>YxdL2</td>
<td>GCGATGCCGTGCAGTTTTTCTTC</td>
</tr>
<tr>
<td>148licatg_yxdJ</td>
<td>AAGGAGGAAGGAGGAGGTAATAAATAATCATATGATGTGGAAGACAG</td>
</tr>
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<td>148licstop_yxdJ</td>
<td>GACAGCGCAGGCTGCTGAGGAGCCGAGAAAAACGC</td>
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<td>YxdL_EA1</td>
<td>TTGTTGACCTCAAAGTGCTCGCA</td>
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<td>YxdL_EA2</td>
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</tr>
<tr>
<td>IolL_1</td>
<td>CGTAAAGAAACAAAAATATGCAGCC</td>
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<tr>
<td>YxdJ_dir</td>
<td>ATTTAATCATATGATGCTGGAAGACAG</td>
</tr>
<tr>
<td>YxdJ_rev</td>
<td>GAGCCGCGCCTGAGAGGAGAAAAACGAGAAATCAG</td>
</tr>
<tr>
<td>PBS-X</td>
<td>CTGAACTGAGTGATCCCC</td>
</tr>
<tr>
<td>YxdKL_right_1</td>
<td>GGGAGCTTCAACATAAAAATCC</td>
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</table>
Deletion analysis of the yxdLMyxeA promoter. DNA fragments were PCR-amplified using genomic *B. subtilis* DNA as template and were cloned upstream of lacZ in plasmid pGE593 (Eraso & Weinstock, 1992). The PCR products were generated using a common primer (YxdKL_right) and one of the following specific primers: for fragment A (−281, 159), YxdKL_left_A; for fragment B (−94, 159), YxdKL_left_B; for fragment C (−69, 159), YxdKL_left_C; and for fragment D (−30, 159), YxdKL_left_D. yxdJ was amplified with Yxdkl_dir and Yxdkl_rev and then cloned in pBAD33 (Guzman et al., 1995) under the control of an arabinose-inducible promoter. Both recombinant plasmids were introduced into *E. coli* BL21/DE3 strains and the YxdaL_right primer with the YxdaL_right_1 and YxdaL_F primers was cloned into pBAD33 (Amersham Pharmacia Biotech). After migration the gel was soaked in an aqueous ethidium bromide solution (0.5 μg ml⁻¹) for 5 min and then observed on a UV transilluminator.

Overexpression and purification of His-tagged YxdJ protein from *B. subtilis*. yxdJ was amplified from *B. subtilis* genomic DNA as template using Yxdkl_dir and YxdaL_rev primers. The DNA fragment obtained was purified and cut with *Eco*RI. It was then cloned in a modified version of pET22b+ (Novagen) linearized with PmlI. To get the modified pET22b+, an adapter, obtained by hybridization of the two primers pET_his_1 and pET_his_2, was cloned between the *NdeI*/EcoRI sites of pET22b+. This construction introduces a PmlI cloning site into this vector and also allows addition of several codons at the 5’ end of the cloned gene, creating a His-tag at the N-terminus of the protein to be produced. The resulting plasmid was introduced into *E. coli* BL21/DE3. The recombinant His-tagged YxdJ protein was thus overproduced and purified on Ni-NTA resin (Qiagen). Resin-bound His-tagged YxdJ was washed with buffer A (HEPES 10 mM, NaCl 150 mM, pH 7.4) containing 30 mM imidazole. The protein was eluted with buffer B containing 300 mM imidazole. After dialysis against buffer A containing 10% (v/v) glycerol, the tagged protein was stored at −80°C.

Gel mobility shift assay. DNA fragments were obtained by PCR amplification using one of the Yxdkl_left primers (A, B, C or D) and the Yxdkl_right primer with *B. subtilis* genomic DNA as template. A nonspecific DNA fragment, chosen within the yxdJ gene, was PCR-amplified with primers Yxdl and Yxdl2. Fifty nanograms each of DNA fragment (A, B, C or D) and nonspecific DNA fragment were mixed together with variable amount of purified His-tagged YxdJ protein in 50 mM Tris/HC1 (pH 8), 1–25 mM EDTA, 0.25 M sucrose and 0.025% bromophenol blue. The mixture (4 μl final volume) was incubated for 30 min at room temperature and loaded on a native 12.5% acrylamide Phast gel (PhastSystem from Amersham Pharmacia Biotech). After migration the gel was soaked in an aqueous ethidium bromide solution (0.5 μg ml⁻¹) for 5 min and then observed on a UV transilluminator.

DNase I protection assay. The DNA fragment obtained by PCR amplification of *B. subtilis* genomic DNA with primers (Yxdkl_right_1 and Yxdkl_F) was cloned into Smal-linearized pBluescript KS (Promega) and the sequences of the recombinant clones were verified. Labelling of the DNA fragment used for DNase I footprinting was done as follows. PCR-amplified fragment obtained with primers Yxdkl_F and PBS-X was 5’-end-labelled with [γ⁻³²P]ATP (4000 Ci mmol⁻¹, 150 TBq mmol⁻¹; NEN) and T4 polynucleotide kinase (Promega). Unincorporated nucleotides were removed using Nucleotide Removal Kit (Qiagen) following the recommendation of the manufacturer. Once purified, the labelled DNA fragment was digested with *Ban*HI and subjected to treatment with the Qiaquick PCR purification kit (Qiagen). Then 5 × 10⁴ c.p.m. purified labelled DNA fragment diluted to a concentration of 1–5 mM was incubated with His-tagged YxdJ for 30 min at room temperature in 50 μl binding buffer containing 10 mM Tris/HCl pH 7.4, 50 mM NaCl, 2.5 mM MgCl₂, 0.5 mM dithiothreitol, 4% glycerol and 1–5 μg ml⁻¹ poly(dI-dC): poly(dI-dC). The DNA–protein complexes were treated with 1 unit DNase I (Amersham Pharmacia Biotech).
Biotech) for 1 min at room temperature. The reaction was stopped by addition of a solution containing 192 mM sodium acetate, 32 mM EDTA, 0-14 % SDS and 64 μg yeast RNA ml⁻¹. The samples were extracted by a phenol/chloroform treatment and, after ethanol precipitation, they were resuspended in conventional loading buffer. After denaturation, the samples were loaded on a 6 M urea/8 % polyacrylamide gel together with a G+A Maxam and Gilbert reaction done on the same labelled DNA fragment.

**Global transcriptional analysis.** Fluorescently labelled cDNA was synthesized during reverse transcription of BSmrs112 and BSmrs139 RNA (10 μg) using Cyanine-modified dCTP. The reaction mixture contained (in 40 μl): 20 μg random primers (GibcoBRL); 1 × first-strand buffer (GibcoBRL); 10 mM dithiothreitol (Amersham Pharmacia Biotech); 100 μM (each) dATP, dTTP and dGTP; 50 μM dCTP; 25 μM Cy (3 or 5)-dCTP (Amersham Pharmacia Biotech) and 200 units Superscript II (GibcoBRL). cDNA synthesis was carried out at 42 °C and 200 units Superscript II. RNA was subjected to alkaline hydrolysis by adding NaOH to a final concentration of 32 mM EDTA, 0 % denatured for 5 min at 95 °C in 25 μl Dige Easy buffer (Roche) containing 10 μg salmon sperm DNA (Sigma) previously denatured for 5 min at 95 °C. Hybridizations were done in a final volume of 25 μl containing a mixture of the labelled cDNA at a final concentration of 1 μg ml⁻¹. Each slide was incubated in water-bath using a waterproof hybridization chamber (Corning). Slides were scanned on a ScanArray 4000 (Packard Bioscience) and hybridization signals were quantified with QuantArray software version 2.1 (Packard Bioscience).

**RESULTS**

**Localization of the yxdLMyxexA transcriptional start point**

Transcriptional fusions of yxdK and yxdL to the *E. coli* lacZ gene, integrated into the *B. subtilis* chromosome, were tested for expression of β-galactosidase. Regardless of whether cells were grown in Luria–Bertani medium or in Spizizen defined medium, the β-galactosidase activity never exceeded 5 or 0.5 Miller units for yxdK or yxdL, respectively (data not shown). Because of low expression, primer extension experiments were done with variable amount of RNAs failed to generate detectable products.

We therefore used the strain BSmrs112, in which the overproduction of the YxdJ response regulator mimics the unknown stimulus of the YxdJ/Yxdk TCS and triggers the expression of the *yxdLMyxexA* operon (Joseph et al., 2002). Using an RNA preparation from this strain, the *yxdLMyxexA* transcription start site was identified; it corresponds to an adenine located 87 bp upstream from the putative transcription initiation codon of *yxdL* (Fig. 1). Upstream of this transcription start, the sequences corresponding to an extended σ^70-binding site (TGXTAATAT), and a −35 region (Helmann, 1995; Jarmer et al., 2001) were found.

**Deletion analysis of the yxdLMyxexA promoter region**

As YxdJ positively controls *yxdLM* expression, it was of interest to precisely delineate the cis region of the *yxdLMyxexA* promoter required for this activation. For this purpose, fragments of various lengths of the *yxdL* regulatory region were cloned in plasmid pGE593 (Eraso & Weinstock, 1992), creating a series of transcriptional fusions with the *lacZ* reporter gene (plasmids pGE-A, pGE-B, pGE-C and pGE-D). Each of the recombinant plasmids was introduced into a ΔlacZ *E. coli* strain (DH5α) bearing the compatible plasmid pBAD-βgal plasmid that contains the *yxdL* gene under the control of an arabinose-inducible promoter. Each recombinant strain was grown in medium containing either glucose or arabinose and samples were taken to measure the β-galactosidase activity. As shown in Fig. 2(a), in cells grown in the presence of glucose, the four DNA fragments tested for their promoter activity gave β-galactosidase activities of the same order of magnitude, which did not exceed 50 Miller units. In the presence of arabinose, a strong increase of β-galactosidase activity was observed for fragments A and B, with an induction ratio above 20 in both cases. In contrast, a much lower level of induction was observed with fragment C and no induction could be detected with fragment D. These results indicated that the −69 to −94 region upstream of fragment C contains information required for the YxdJ protein to induce the transcription of the *yxdL* promoter.
RNA synthesis from the promoter upstream of the yxdLMyxeA operon.

Direct interaction of YxdJ with the yxdMLyxeA promoter

The observation that YxdJ positively controls the expression of the yxdLMyxeA operon promoter in E. coli suggested that this response regulator binds directly to this region. To check this, we purified His-tagged YxdJ by nickel affinity chromatography and used it in a mobility shift assay with various yxdL promoter fragments. We first verified that the in vivo activity of YxdJ bearing a His-tag at the amino terminus is similar to that of the wild-type regulators. Indeed, both were shown to give similar levels of β-galactosidase activity when overexpressed in B. subtilis bearing the yxdL–lacZ transcriptional fusion (data not shown). As indicated in Fig. 2(b), a change of mobility was observed with DNA fragments A (441 bp) and B (254 bp) using increasing amounts of YxdJ. When fragment C was used, no band shift could be detected (data not shown). This result confirms the importance of the –69 to –94 region not only for a full stimulation of the yxdLMyxeA operon (Fig. 2a) but also for efficient binding of the YxdJ regulator.

Characterization of the YxdJ binding site by DNase I protection assay

To define the YxdJ binding site more precisely, DNase I footprint experiments were performed using a fragment corresponding to positions –178 to +160 with respect to

Fig. 2. Analysis of the yxdLMyxeA operon regulatory region. (a) Effects of deletion in the yxdL promoter region on the in vivo expression of lacZ. The DNA fragments A, B, C and D were cloned upstream of lacZ in pDE593. yxdJ was cloned in pBAD33 under the control of an arabinose-inducible promoter. Both recombinant plasmids were introduced into E. coli DH5α. Positions of the DNA fragments are indicated with respect to the yxdL transcription start. The yxdK and yxdL genes are indicated by large hatched arrows and –10 and –35 motifs are indicated by white boxes. E. coli cells were grown in the presence of glucose (white bars in histogram) or arabinose (grey bars), both at 0.2% final concentration. β-Galactosidase activities are in Miller units and represent the mean of three experiments ± sd. The ratios correspond to the activities obtained in the presence of arabinose divided by those obtained in the presence of glucose. (b) Gel shift analysis of binding of the purified His-tagged YxdJ to the yxdL promoter: 0.24 pmol of DNA fragment A (441 bp) or 0.33 pmol of DNA fragment B (254 bp) and 0.2 pmol of control DNA fragment (326 bp from within the yxdL gene) were incubated in the presence of various amounts of purified His-tagged YxdJ (lanes 1 and 5, none; lanes 2 and 6, 0.75 pmol; lane 3, 1.5 pmol; lanes 4 and 7, 2.25 pmol).
the yxdL transcriptional start site. This fragment encompasses fragment B and a part of fragment A (Fig. 2). As shown in Fig. 3, a 38 bp region of the minus-strand, extending from base -41 to -78, was efficiently protected by YxdJ from DNase I digestion. Analysis of the protected region sequence revealed a 9 nucleotide direct repeat, TTAMRAAAA. Spacing of 21 nucleotides between the repeats indicates that they lie on the same side of the DNA helix, a feature that is expected from regulatory regions controlled by the OmpR-subfamily members acting as multimers (Makino et al., 1988; Rampersaud et al., 1989; Tsung et al., 1989). Thus, the YxdJ binding site extends between nucleotides -41 to -78 in the regulatory region of the yxdLMyxeA operon, and contains two direct repeats.

Whole-genome transcription analysis of the YxdJ targets

To assess the extent of the YxdJ regulon, microarray hybridizations using RNA isolated from cells overproducing or not overproducing YxdJ were compared. Overproduction of YxdJ in strain BSmsr112 was used to mimic the unknown physiological conditions that normally lead to YxdJ activation. Three repetitive microarray analyses were carried out using RNA isolated from independent cell cultures of strains BSmsr139 and BSmsr112 grown in Luria–Bertani medium. IPTG was added at mid-exponential growth and cells were harvested after 30 min further incubation and used for RNA preparation. The growth rates of the two strains were indistinguishable (not shown). After 30 min induction, only seven genes were shown to be induced (stimulation index above 3) and none was repressed (stimulation index below 0.33) (Table 3).

As expected, the stimulation indices of the yxdJ, yxdL and yxdM genes were found to be very high. This indicated that (i) the response regulator overproducing system is working efficiently; and (ii) the yxdL and yxdM genes are positively controlled by the YxdJ response regulator as we previously described (Joseph et al., 2002). However, a large difference in expression was observed between the three genes within the operon yxdLMyxeA: the stimulation index was 64 for yxdL, 6 for yxdM and below the threshold of detection for yxeA, which might reflect rapid degradation of this polycistronic mRNA. Similar results were obtained when real-time RT-PCR experiments were performed on the same RNA preparations (data not shown).
Table 3. Transcriptome analysis

RNA was extracted from cells of BSmsrs112 and BSmsrs139 30 min after induction with 2 mM IPTG. Induction index values are ratios of BSmsrs112 signal over BSmsrs139 signal. Results are expressed as means ± SD based on results of three independent experiments. Only genes with induction ratios >3 are listed (no genes were repressed, i.e. none showed a stimulation index <0.33). Genes are grouped when they belong to the same operon.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Known or putative function</th>
<th>Stimulation index</th>
</tr>
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<tbody>
<tr>
<td>yxdJ</td>
<td>Putative two-component response regulator</td>
<td>122 ± 20</td>
</tr>
<tr>
<td>yxdk</td>
<td>Putative two-component sensor histidine kinase</td>
<td>12 ± 4</td>
</tr>
<tr>
<td>yxdl</td>
<td>Putative ABC transporter (ATP-binding protein)</td>
<td>64 ± 11</td>
</tr>
<tr>
<td>yxdM</td>
<td>Putative ABC transporter (permease)</td>
<td>6 ± 1.4</td>
</tr>
<tr>
<td>yvcr</td>
<td>Putative ABC transporter (ATP-binding protein)</td>
<td>4.2 ± 0.9</td>
</tr>
<tr>
<td>dltA</td>
<td>D-Alanyl-D-alanine carrier protein ligase</td>
<td>3 ± 0.3</td>
</tr>
<tr>
<td>dltD</td>
<td>D-Alanine transfer from Dcp to undecaprenol phosphate</td>
<td>3 ± 0.4</td>
</tr>
</tbody>
</table>

Previous experiments done by real-time PCR (Joseph et al., 2002) showed that neither the yxdk gene, encoding the histidine kinase partner of Yxdl, nor the yvcr gene, encoding an ABC transporter NBD, was induced upon Yxdl overexpression. These results are totally different from those obtained using the microarray approach and we believe that the latter are artefactual. First, to construct the recombinant plasmid for Yxdl overproduction we used a DNA fragment containing 29 bp that overlap with the 5' end of the yxdk gene. Thus, a large amount of an mRNA containing this overlap is produced when overproducing Yxdl. After reverse transcription and labelling, hybridization of the 29 bp fragment to the yxdk probe might easily occur on the microarray and produce the artefactual signal. Second, among the NBD-encoding genes, yvcr is by far the best yxdl homologue in B. subtilis (62.1% identity at the DNA sequence level). In addition, the yvcr and yxdl DNA sequences contain long stretches of identical nucleotides. Thus, in the conditions of high-level yxdl mRNA production, cross-hybridization between the yxdl labelled target and the yvcr probe might occur.

The dltA and dltD genes, involved in alamination of lipoteichoic and teichoic acids, are also induced. Using longer IPTG induction times, we have observed that dltB, dltC and dltE were also induced (data not shown) as well as the ywaA gene, which is predicted to encode a putative branched-chain amino acid aminotransferase. All these genes (dltABCDE and ywaA) are predicted to constitute an operon.

**DISCUSSION**

The yxdk and yxdM genes, encoding the TCS and ABC transporter systems, respectively, and belonging to separate operons (Joseph et al., 2002; Yoshida et al., 2000) were shown to be functionally related (Joseph et al., 2002). As reported previously (Yoshida et al., 2000) and in the present study, both operons are constitutively expressed at a very low level during the growth phase of B. subtilis. This complicates any studies of their promoter sequence and regulation. In conditions of overproduction of the Yxdl response regulator that mimics Yxdk activation, the level of yxdlMyxeA operon expression is dramatically increased (Joseph et al., 2002), allowing its transcriptional start to be precisely located. Further analyses presented here show that the Yxdl response regulator positively controls the yxdlMyxeA operon transcription by direct interaction with its promoter region. In retardation experiments a complete shift occurred with 15 pmol Yxdl, representing a 50-fold molar excess of the regulator to DNA. This ratio is of the same order of magnitude or even smaller than that needed to gain the complete shift of promoter region by response regulators of the OmpR family, for instance YycF and its fisZA promoter in B. subtilis (Fukuchi et al., 2000). The purified Yxdl protein protects from DNase I degradation a 38 nucleotide region located between positions −41 and −78 relative to the yxdl transcription start. Two TTAMRAAAA repeats were found in this protected region. Deletion of one base at position −70 in one repeat together with the flanking protected region (−71 to −78) (Fig. 3) results in a complete loss of Yxdl binding to the DNA fragment in a gel shift assay. These results suggest that the two repeats (TTAMRAAAA) are the Yxdl binding site. Thus, this pattern was used to screen the whole B. subtilis genome using bioinformatics tools. Even with weak constraints on the sequence of the repeats and variable distance between them (1, 2 or 3 helix–turns), we were unable to identify any region containing this motif apart from that in the yxdlMyxeA promoter region. This result is in concordance with the experimental search for these targets using transcriptome analysis.

Data collected from microarray experiments indicate a very restricted regulon of Yxdl: only four genes show significant change in their transcription level upon conditions mimicking TCS induction. Indeed, after 30 min of IPTG-mediated Yxdl overproduction, we detected only the expression of the cognate ABC transporter genes yxdM,
confirming our previous observation (Joseph et al., 2002), and the induction of the\( dltA \) and\( dltD \) genes.

The experimental conditions we used were slightly different from those of Kobayashi et al. (2001) in a similar approach. We used a wild-type strain rather than a\( yxdK \)-deleted mutant and a more efficient expression system (Joseph et al., 2001) giving a\( yxdJ \) overexpression ratio that reaches 122 (Table 3) whereas Kobayashi et al. (2001) used a 30-fold increase in\( yxdJ \) expression (data available at ftp://ftp.genome.ad.jp/pub/kegg/expression/ex0000286.dat). A very restricted gene expression pattern change was obtained in both cases with a common characteristic, a strong positive control of the\( yxdLM \) cognate ABC transporter gene expression by the\( YxdJ \) response regulator.

Using longer IPTG induction time, we have seen that all the genes of the\( dlt \) operon were induced, including the\( ywaA \) gene encoding a putative branched-chain amino acid aminotransferase (data not shown). The five\( dlt \) gene products are involved in teichoic acidpolyalanylation (Perego et al., 1995). The promoter region of the\( dlt \) operons did not show detectable DNA binding of His-\( YxdJ \) in the gel mobility shift assays (data not shown). This probably indicates indirect regulation of expression of these genes by\( YxdJ \) and presumably explains the lack of detection of any\( YxdJ \) binding site sequence using bioinformatic approaches. Interestingly, expression of the\( dlt \) operon from\( Streptococcus agalactiae \) was also controlled by a TCS belonging to the OmpR family, the\( DltS/DltR \) system (Poyart et al., 2001). The exact physiological role of the\( yxd \) gene cluster remains to be elucidated. From our results it appears that the\( YxdJ \) regulon is limited to a set of genes encoding systems responsible for compound efflux, such as the membrane pump of the\( ABC \) family, and cell wall biosynthesis/modification.\( YxdL \) shows strong similarity to several\( NBD \)\( ABC \) transporters, such as SalX, involved in salicarvin resistance in\( Streptococcus salivarius \) and\( Streptococcus pyogenes \) (Upton et al., 2001), VraD and VraF from\( Staphylococcus aureus \) (Kuroda et al., 2000), and MbrA, responsible for bacitracin resistance in\( Streptococcus mutans \) (Tsuda et al., 2002). It was also shown recently that the\( bce \) system (formerly\( yds \)), which is paralogous to the\( yxd \) system, is involved in bacitracin resistance (Bernard et al., 2003; Mascher et al., 2003; Ohki et al., 2003). The increase in\( D \)-alanyl esterification of teichoic acids caused by activation of\( dlt \) transcription should result in a neutralization of the negative charge of adjacent phosphoryl residues of this anionic polymer, eventually leading to an increased resistance of the cells to some antibiotics. In fact, mutants lacking\( D \)-alanine on teichoic acids displayed an increased sensitivity; of\( B. subtilis \) to methicillin (Wecke et al., 1997), of\( S. agalactiae \) to several cationic antimicrobial peptides (Poyart et al., 2003) and of\( S. aureus \) to gallidermin or nisin (Peschel et al., 1999).

We therefore suggest that the\( yxdLMYxaA \) operon gene products might be involved in resistance to an as yet unknown group of antibiotics, and that\( yxdJK \) encodes the corresponding signal detector/transducer system.

Related to\( yxdLM \), the gene\( yxaA \) encodes a long peptide which is conserved in several Gram-positive bacteria:\( Bacillus anthracis \),\( Enterococcus faecalis \),\( Lactococcus lactis \),\( Listeria innocua \),\( Listeria monocytogenes \) and\( Staphylococcus aureus \). This peptide might participate in the proposed antibiotic resistance mechanism as an immunity peptide interacting with and neutralizing the antibiotic. It is predicted to be processed (Nielsen et al., 1997) and exported via the general secretion pathway. In that case the\( YxdLM \)\( ABC \) transporter might work in conjunction with\( YxeA \) as an antibiotic efflux pump. However, one cannot exclude that\( YxeA \) might be exported by the\( ABC \) transporter\( YxdLM \) to protect the cell as indicated above. We are currently studying these different possibilities.

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