CodY Regulates Expression of the *Bacillus subtilis* Extracellular Proteases Vpr and Mpr

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ABSTRACT

CodY is a global transcriptional regulator in low-G+C Gram-positive bacteria that is responsive to GTP and branched-chain amino acids. By interacting with its two cofactors, it is able to sense the nutritional and energetic status of the cell and respond by regulating expression of adaptive genetic programs. *In B. subtilis*, more than 200 genes, including those for peptide transporters, intracellular proteolytic enzymes, and amino acid degradative pathways, are controlled by CodY. In this study, we demonstrated that expression of two extracellular proteases, Vpr and Mpr, is negatively controlled by CodY. By gel mobility shift and DNase I footprinting assays, we showed that CodY binds to the regulatory regions of both genes, in the vicinity of their transcription start points. The *mpr* gene is also characterized by the presence of a second, higher-affinity CodY-binding site located at the beginning of its coding sequence. Using strains carrying vpr- or mpr-lacZ transcriptional fusions in which CodY-binding sites were mutated, we demonstrated that repression of both protease genes is due to the direct effect by CodY and that the *mpr* internal site is required for regulation. The vpr promoter is a rare example of a sigma H-dependent promoter that is regulated by CodY. In a *codY* null mutant, Vpr became one of the more abundant proteins of the *B. subtilis* exoproteome.

IMPORTANCE

CodY is a global transcriptional regulator of metabolism and virulence in low-G+C Gram-positive bacteria. *In B. subtilis*, more than 200 genes, including those for peptide transporters, intracellular proteolytic enzymes, and amino acid degradative pathways, are controlled by CodY. However, no role for *B. subtilis* CodY in regulating expression of extracellular proteases has been established to date. In this work, we demonstrate that by binding to the regulatory regions of the corresponding genes, *B. subtilis* CodY negatively controls expression of Vpr and Mpr, two extracellular proteases. Thus, in *B. subtilis*, CodY can now be seen to regulate the entire protein utilization pathway.

First identified in *B. subtilis* (1), CodY is a global transcriptional regulator whose homologues are found almost ubiquitously in low-G+C Gram-positive bacteria (2). DNA microarray and chromatin immunoprecipitation with microarray (ChIP-chip) experiments and, most recently, *in vitro* DNA binding assays coupled with massively parallel sequencing (the IDAP-Seq method) and genome-wide profiling of transcription by transcriptome sequencing (RNA-Seq) (3–5) have shown that in *B. subtilis* CodY regulates over 200 genes, many of which encode components of metabolic pathways, are repressed during growth in the presence of excess nutrients, and are involved in adaptation to poor growth conditions (6). Although CodY acts mainly as a repressor, some *subtilis* genes are under positive CodY regulation (3, 5, 7).

In all other CodY-expressing species examined to date, CodY also controls multiple metabolic pathways (2, 8–12). In pathogenic species, key virulence genes are also under CodY control (10–23). CodY can control transcription by binding in the vicinity of the promoter region of the target genes, by competing with a positive regulator for binding, or by serving as a roadblock to RNA polymerase (24).

Binding of CodY to DNA requires in most cases at least a moderately conserved version of a 15-nucleotide (nt) consensus motif (AATTTCWGAAAT) (4, 9, 25, 26) and is enhanced by its interaction with two classes of effector molecules that act as signals of the nutritional status of the cell: the branched-chain amino acids isoleucine, leucine, and valine (ILV) (27, 28) and GTP (29–31). Varying the concentration of activated CodY results in a hierarchical, programmed regulation of gene expression that presumably allows the cell to adapt in different ways to various levels of nutritional availability (5).

Extracellular proteases are thought to be involved in nutrient acquisition. *B. subtilis* produces at least eight characterized extracellular or cell wall-associated proteases. The alkaline serine protease subtilisin (AprE) and the neutral metallopeptase NprE, commonly referred to as the major extracellular proteases, account for more than 95% of the total extracellular protease activity of *B. subtilis* (32). The remaining protease activity is due to minor extracellular proteases, which include the serine proteases Epr...
Bacterial strains and culture media. The B. subtilis strains constructed and used in this study were all derivatives of strain SMY (47) and are listed in Table 1. They were grown at 37°C in DSM nutrient broth or in TSS minimal medium with 0.5% glucose as a carbon source and 0.2% NH₄Cl as a nitrogen source (48). The same media with the addition of agar were used for growth of bacteria on plates. TSS medium was supplemented as indicated below with a mixture of 16 amino acids (aa) (49), which contained all amino acids commonly found in proteins except for glutamine, asparagine, histidine, and tyrosine; the branched-chain amino acids ILV were added at a final concentration of 200 μg/ml each. In TSS medium plus 13 aa, ILV were omitted. For proteomics experiments, TSS medium was supplemented with 2 mM CaCl₂·2H₂O and 10 μM MnSO₄·4H₂O. Escherichia coli strains JM107 (50) and DH5α (51) were used for isolation of plasmids and were grown in LB medium (52). The following antibiotics were used when appropriate: tetracycline (15 μg/ml), spectinomycin (50 μg/ml), or chloramphenicol (5 μg/ml) or the combination of erythromycin (0.5 μg/ml) and lincomycin (12.5 μg/ml) for B. subtilis strains and ampicillin (50 μg/ml) for E. coli strains.

DNA manipulations. Methods for common DNA manipulations and bacterial transformation were previously described (26, 53). Chromosomal DNA of B. subtilis strain SMY or plasmids constructed in this work were used as templates for PCR. Plasmids isolated from E. coli strain DH5α were subjected to rolling-circle amplification using the Illustra TempliPhi 100 amplification kit (GE Healthcare) before being used to transform B. subtilis competent cells. The oligonucleotides used in this work are described in Table 2. All cloned PCR-generated fragments were verified by sequencing.

Gel mobility shift experiments using ≤1 fmoI of end-labeled DNA fragments and DNase I footprinting experiments using 20 to 40 fmoI of labeled DNA were performed following procedures described in detail previously (26, 54).

Construction of transcriptional lacZ fusions. To construct plasmid pGB3, containing a vpr-lacZ transcriptional fusion, a 680-bp vpr product, corresponding to positions −656 to +24 with respect to the vpr start codon, was synthesized by PCR using oligonucleotides oGB9 and oGB10 and cloned between the Xbal and HindIII sites of an integrative plasmid pHK23 (erm) (26). Plasmid pGB4 (mpr-lacZ) was created as described above for pGB3 by cloning the 302-bp mpr PCR product, obtained with oGB13 and oGB14 and corresponding to positions −260 to +42 with respect to the start codon.

B. subtilis strains carrying the vpr-lacZ or mpr-lacZ fusion at the amyE locus (Table 1) were isolated after transforming strain BB2511 (amyE:: lacA) with the appropriate plasmids, selecting for resistance to erythromycin conferred by the plasmids, and screening for loss of the spectinomycin resistance marker, which indicated a double-crossover, homologous recombination event. Strain BB2511 and all of its derivatives have
very low endogenous β-galactosidase activity due to a null mutation in the lacZ gene (55).

**Mutations in the CodY-binding sites.** Mutations in the vpr regulatory region were introduced by two-step overlapping PCR. In the first step, 0.6-kb products containing the 5′ part of the vpr regulatory region were synthesized by using oligonucleotide oGB9 as the forward primer and mutagenic oligonucleotide oGB11 (vpr
\(_{p1}\)) as the reverse primer. In a similar manner, 0.1-kb products containing the 3′ part of the regulatory region and the first 24 bp of the vpr coding sequence were synthesized by using mutagenic oligonucleotide oGB12 (vpr
\(_{p3}\)) or oGB16 (vpr
\(_{p5}\)) or oGB18 (vpr
\(_{p6}\)) as the reverse primer.

The appropriate pairs of PCR products were used in a second step of PCR as overlapping templates to generate modified fragments containing the entire vpr regulatory region; oligonucleotides oGB9 and oGB10 served as forward and reverse primers, respectively. A 391- or 434-bp PCR product containing the regulatory region of the lacA gene (55).

**lacZ**

<table>
<thead>
<tr>
<th>Oligonucleotide type and name</th>
<th>Sequence (5′–3′)</th>
<th>Specificity</th>
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<tr>
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<td></td>
</tr>
<tr>
<td>Forward</td>
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<tr>
<td>oGB9</td>
<td>CAGGACTCTGAGCACGGCCTTTCTTGGTATG</td>
<td>vpr</td>
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<td>oGB13</td>
<td>CAGGACTCTGAGGGAAAGCCGATAAAGAAAGCC</td>
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<td>GCTTCTAAGTTTTTTC</td>
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<tr>
<td>Reverse</td>
<td></td>
<td></td>
</tr>
<tr>
<td>oGB10</td>
<td>CCGAAGATGCTTAAAGGGAATGATCCTCTTTTTC</td>
<td>vpr</td>
</tr>
<tr>
<td>oGB14</td>
<td>CGCACAGCCTGATAAGGCAACATTGTTTCTGATTC</td>
<td>mpr</td>
</tr>
</tbody>
</table>
| oGB15                        | CAGAACGCTTGAAAGCCGAAACATTGTTTGGATTC | mpr
\(_{p4}\) |
| oGB24                        | CGAACGCTGTTAAGGCAACATTGTTTCTATCCTATGGAAC | mpr
\(_{p5}\) |
| oGB25                        | CACACGCTGTTAAGGCAACATTGTTTCTATCCTATGGAAC | mpr
\(_{p5}\) |
| oBB102                       | CACCTTCTCCATATAAAAAAGC | spoVG-lacZb |
| oBB253                       | GGTTTTCCGGTTCGCAC | lacZb |

<table>
<thead>
<tr>
<th>Internal mutagenic primers</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward</td>
<td></td>
</tr>
</tbody>
</table>
| oGB12                         | GACCAAAGGATTTTTTTTAATCTTTCAAGAAATATATAC | vpr
\(_{p3}\) |
| oGB16                         | GACACAGAGTTTTTTTTGTTGAAAGATATATAC | vpr
\(_{p2}\) |
| oGB18                         | GACACAGAGTTTTGTTGAAATGGTTC | vpr
\(_{p1}\) |
| Reverse                       |                 |
| oGB11                         | GTATATATTCCTTTGAAATATCAAGAAATATATAC | vpr
\(_{p2}\) |
| oGB17                         | GTATATATGCTTTGCAAATTTGAACAAATTTGAAC | vpr
\(_{p1}\) |
| oGB19                         | CTTGAAATTTGCAAATTTGAACAAATTTGAAC | vpr
\(_{p1}\) |

\(\ast\) The altered nucleotides in the CodY-binding motifs are in lowercase. Restriction sites are underlined.

\(\ast\ast\) This target is located on plasmid pHK23.

**TABLE 2** Oligonucleotides used in this work

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**Protein spot quantification and protein identification.** Gels were scanned and the images were analyzed with Delta2D software version 4.3 (Decodon). Protein spot quantification was conducted according to the method of Wolf et al. (59). Briefly, gel images for proteins from wild-type and mutant strains were overlaid and a fusion gel image, which was generated in *silico* using the image fusion function of the Delta2D software, was used for spot detection. After spot editing, protein spots were transferred to the single-gel images. Spot quantities were calculated with Delta2D software as a fraction (percentage) of the total intensity of all protein spots present on the gel attributable to the intensity of an individual protein spot.

For identification, spots were excised from gels with the Ettan Spot Picker (GE Healthcare) using pick lists generated with Delta2D software. Digestion with trypsin and spotting of the resulting peptide solutions onto matrix-assisted laser desorption ionization (MALDI) targets were performed in the Ettan Spot Handling Workstation (GE Healthcare). Mass spectrometry (MS) analysis was done by MALDI–time-of-flight (TOF)
Binding of CodY to the vpr regulatory region. (A) Sequence of the vpr insert used to construct the vpr-lacZ fusions. Coordinates are reported with respect to the putative transcription start point (60), indicated by the bent arrow. The core CodY-binding site identified by IDAP-Seq (4) is in boldface. Two overlapping CodY-binding motifs with 4 and 3 mismatches to the consensus are underlined; two more motifs with 5 mismatches to the consensus can be found in the same region and are not shown. The CodY-protected region, detected in DNase I footprinting experiments, is underlined with the dashed line. The direction of translation of vpr and ywcI are indicated by the long arrows. The initiation codons of the two genes are in boldface. (B) Gel mobility shift assay of CodY binding to a radioactively labeled vpr PCR fragment, obtained with oligonucleotides oBB67 and oBB102, in the presence of 10 mM ILV. CodY monomer concentrations are reported below each lane. (C) DNase I footprinting analysis of CodY binding to the vpr fragment. The labeled vpr PCR fragment, obtained with oligonucleotides oGB9 and oBB253, was incubated with increasing amounts of purified CodY in the presence of 10 mM ILV and 2 mM GTP and then with DNase I. Concentrations of CodY monomers are reported above each well. The corresponding A+G sequencing ladder of the bottom DNA strand is on the left. The protected area is shown by the vertical line and the corresponding sequence is reported; the bent arrow indicates the transcription start point and the direction of transcription. The core CodY-binding site identified by IDAP-Seq is in boldface. (D) Gel mobility shift assay of CodY binding to a radioactively labeled vpr$p2$ fragment, obtained with oligonucleotides oBB67 and oBB102, in the presence of 10 mM ILV. CodY monomer concentrations are reported below each lane.
tandem MS (MS/MS) using a Proteome Analyzer 4800 (Applied Biosystems) (59). Peak lists were searched against a B. subtilis database with the MASCOT search engine version 2.1.0.4 (Matrix Science) using search parameters as described previously (59).

RESULTS

CodY-mediated regulation of the vpr gene. To assess the ability of CodY to regulate vpr expression, a vpr-lacZ transcriptional fusion containing a 680-bp fragment including the entire intergenic region upstream of vpr and the first 24 nucleotides of the coding sequence was constructed (Fig. 1A). Under conditions of maximal CodY activity, in a glucose-ammonium minimal medium containing ILV and a mixture of 13 other amino acids (TSS medium plus 16 aa), expression of the vpr fusion under steady-state growth conditions was about 10-fold higher in the codY null mutant strain GB1011 than in the wild-type strain GB1003 (Table 3). In the wild-type strain, the activity of the fusion increased 3-fold when ILV was omitted (TSS medium plus 13 aa) and was completely derepressed when TSS medium was unsupplemented with amino acids (Table 3). Though this pattern of amino acid-dependent expression is common to CodY-regulated genes, the inability of partly active CodY, present in TSS medium-grown cells, to repress vpr at all is rather unusual (26).

Binding of CodY to the vpr regulatory region. The ability of CodY to bind to the vpr promoter was established by gel mobility shift assay (Fig. 1B) and DNase I footprinting (Fig. 1C). In a gel mobility shift assay, CodY bound to the vpr regulatory region with an apparent Kₐ (equilibrium dissociation constant, defined as the protein concentration needed to shift 50% of the DNA fragments under conditions of CodY excess over DNA) of ~10 nM (Fig. 1B). A DNase I footprinting experiment revealed that CodY protects a 30-nt site, corresponding to positions −20 to +10 with respect to the putative vpr transcription start point determined in a tiling array transcriptome analysis of B. subtilis (60) (Fig. 1A and C). This binding site overlaps the transcription start point and fully encompasses a core CodY-binding site, at positions −11 to +5, previously identified by IDAP-Seq as a sequence in which each base pair is essential for CodY binding (4).

CodY binding has been associated with the presence of a 15-bp consensus sequence (AAAGAATTCAAGAAATA) (4, 9, 25, 26) here referred as the CodY-binding motif. A bioinformatics search revealed that the vpr CodY-binding site includes two partially overlapping 15-nt CodY-binding motifs, I and II, with four and three mismatches, respectively, with respect to the consensus sequence (positions −15 to −1 and −6 to +9 with respect to the vpr transcription start point) (Fig. 1A).

Mutagenesis of the vpr CodY-binding site. Three different double-nucleotide substitution mutations were introduced upstream of the transcription start point and within the two overlapping CodY-binding motifs of the vpr gene (Table 3). All mutations were aimed at decreasing the similarity of one or both of the motifs to the CodY-binding consensus sequence. As shown in Table 3, all three mutations significantly reduced the ability of CodY to repress expression from the vpr promoter. Moreover, the vprp1 and vprp3 mutations strongly decreased the affinity of CodY for the vpr regulatory region (Fig. 1D; the p1 and p3 mutations have not been tested).

The decreased activities of the vprp1- and vprp3-lacZ fusions in the absence of CodY suggest that the p1 and p3 mutations, being located in the proximity of the −10 region of the promoter, affect its intrinsic activity (Table 3, compare strains GB1011, GB1015, and GB1030).

CodY-mediated regulation of the mpr gene. The ability of CodY to regulate mpr expression was tested using an mpr-lacZ transcriptional fusion comprising the entire 260-bp intergenic region upstream of the gene and the first 14 codons of the coding sequence (Fig. 2A). Expression of the fusion in TSS medium plus 16 aa was almost 13-fold higher in the codY null mutant strain GB1012 than in the wild-type strain GB1004. In the wild-type strain, the activity of the fusion was derepressed 4-fold when ILV were omitted and was further increased 2.5-fold in TSS medium without added amino acids (Table 4).

TABLE 3 Expression of vpr-lacZ fusions

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>Fusion version</th>
<th>vpr CodY-binding motif</th>
<th>Addition to the medium</th>
<th>β-Galactosidase activity</th>
</tr>
</thead>
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<td>Wild type</td>
<td>vpr-lacZ</td>
<td>ATTTTTTGAAATTCAAGAAATA</td>
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<td>7.9 ± 1.3, 103%</td>
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<td></td>
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<td>13 aa</td>
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<tr>
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<td></td>
<td>16 aa</td>
<td>0.7 ± 0.1, 9%</td>
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<td>GB1101</td>
<td>codY</td>
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<td>7.0 ± 1.2, 91%</td>
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<td></td>
<td>16 aa</td>
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<td>16 aa</td>
<td>0.3 ± 0.1, 58%</td>
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<td>vpr-lacZ</td>
<td>ATTTTTTGAAATTCAAGAAATA</td>
<td>16 aa</td>
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<td>GB1030</td>
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<td>vpr-lacZ</td>
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<td>16 aa</td>
<td>2.1 ± 0.4, 100%</td>
</tr>
</tbody>
</table>

a Cells were grown in TSS glucose-ammonium medium, unsupplemented or containing a mixture of 13 aa or the same mixture with ILV added (16 aa). β-Galactosidase activity was assayed and is expressed as means ± standard deviations (SDs). All values are averages from at least two experiments.

b All strains contained a lacA null mutation.

c Sequences of positions −15 to +9 with respect to the putative transcription start point (double underline) of the vpr-lacZ fusions. CodY-binding motifs I and II are italicized and in boldface, respectively. The core CodY-binding site is underlined. The mutated nucleotides are in lowercase.

d β-Galactosidase activity of each fusion in TSS medium plus 16 aa in a strain containing a codY null mutation was normalized to 100%.

Additions and deletions to the vpr promoter were constructed by mutagenesis of an 850-bp fragment including the entire intergenic region upstream of vpr. The consensus sequence (AATTTTCWGAAAATT) (4, 9, 25, 26) here referred as the CodY-binding motif. A bioinformatics search revealed that the vpr CodY-binding site includes two partially overlapping 15-nt CodY-binding motifs, I and II, with four and three mismatches, respectively, with respect to the consensus sequence (positions −15 to −1 and −6 to +9 with respect to the vpr transcription start point).
FIG 2  Binding of CodY to the mpr regulatory region. (A) Sequence of the mpr insert used to construct the mpr-lacZ fusions. Coordinates are reported with respect to the putative transcription start point (60), indicated by the bent arrow. The core CodY-binding sites identified by IDAP-Seq (4) are in boldface. The CodY-binding motifs of sites I and II are underlined. The CodY-protected regions, detected in DNase I footprinting experiments, are underlined with the dashed lines. The directions of translation of purT and mpr are indicated by the long arrows. The mpr initiation codon is in boldface. (B) Gel mobility shift assay of CodY binding to a radioactively labeled mpr PCR fragment, obtained with oligonucleotides oBB67 and oBB102, in the presence of 10 mM ILV. CodY monomer concentrations are reported below each well. (C) DNase I footprinting analysis of CodY binding to a radioactively labeled mpr PCR fragment obtained with primers oGB13 and oBB253. Increasing amounts of purified CodY were incubated with the mpr fragment in the presence of 10 mM ILV and 2 mM GTP before treatment with DNase I. The corresponding A + H11001/G sequencing ladder of the bottom DNA strand is shown on the left. The protected areas corresponding to sites I and II are shown by the continuous and dashed vertical lines, respectively, and their sequences are reported in the corresponding boxes. The core CodY-binding sites identified by IDAP-Seq are in boldface. Concentrations of CodY monomers are reported above each well. (D) Gel mobility shift assay of CodY binding to a radioactively labeled mprp1 fragment, obtained with oligonucleotides oBB67 and oBB102, in the presence of 10 mM ILV. CodY monomer concentrations are reported below each lane.
The expression of mpr-lacZ (and vpr-lacZ) in the codY null mutant was unaffected by the amino acid composition of the medium, indicating that CodY is the major relevant regulator of the mpr and vpr genes under the conditions tested.

**Binding of CodY to the mpr regulatory region.** CodY binding to the mpr region was demonstrated by gel mobility shift (Fig. 2B) and DNase I footprinting (Fig. 2C) experiments. Purified CodY bound a labeled mpr fragment with an apparent K_D of ~30 nM (Fig. 2B). As revealed by a DNase I footprinting experiment, CodY binding occurred at two sites. The upstream, lower-affinity site I mapped to positions −24 to +23 with respect to the putative transcription start point of the gene (60); the best possible CodY-binding motif in this region (positions −9 to +6) contains 5 mismatches to the consensus sequence. The higher-affinity site II extended from positions +63 to +99 with respect to the transcription start point of mpr (positions −7 to +30 with respect to the translation start point of the gene) and included a 15-bp CodY motif with four mismatches to the consensus (positions +15 to +29 with respect to the translation start site) (Fig. 2A and C).

**Mutagenesis of the internal mpr CodY-binding site.** Three different double-nucleotide substitution mutations aimed at decreasing the similarity of the CodY-binding motif associated with the higher-affinity mpr CodY-binding site II to the consensus motif were introduced separately 84 to 93 bp downstream of the transcription start point and corresponding to positions 23 and 24, 20 and 21, or 15 and 16 with respect to the mpr translation start site (Table 4). While the introduction of the p1 mutation reduced 6-fold the ability of CodY to repress mpr-lacZ transcription, both p2 and p3 abolished CodY-dependent regulation of mpr (Table 4). Even the least effective mutation, p1, strongly decreased the affinity of CodY for the mpr regulatory region (Fig. 2D; the p2 and p3 mutations have not been tested). CodY-mediated repression of mpr expression appears therefore to be mediated mainly by binding at the high-affinity site II.

Somewhat unexpectedly, two of the mutations affected expression of the mpr-lacZ fusion in a negative (p1) or positive (p3) way, even in a codY null strain, indicating that they altered either the intrinsic activity of the promoter or stability of mpr-lacZ mRNA or efficiency of lacZ translation (Table 4).

**Role of the sigma H factor in vpr expression.** Based on DNA microarray experiments, the vpr gene was reported to be expressed from a σ^H-dependent promoter (61). σ^H, a product of the sigH (spo0H) gene, is an alternative sigma factor of RNA polymerase, which controls induction of many genes during the transition from exponential to stationary phase and is required for sporulation (62). In TSS medium plus 16 aa, expression of the vpr-lacZ fusion was almost completely abolished in a sigH null mutant,

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### TABLE 4 Expression of mpr-lacZ fusions

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>Fusion version</th>
<th>mpr CodY-binding motif</th>
<th>Addition to the medium</th>
<th>β-Galactosidase activity</th>
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<td>mpr-lacZ</td>
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<td>16 aa</td>
<td>2.9 ± 0.5 (52)</td>
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<td>BB3950</td>
<td>codY sigH</td>
<td>mpr-lacZ</td>
<td>ATGAAATTAGTCCAAGATggGAAACAAC</td>
<td>16 aa</td>
<td>19.1 ± 0.6 (93)</td>
</tr>
</tbody>
</table>

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a Cells were grown and β-galactosidase activity (expressed as means ± SDs) was assayed as described for Table 3.

b All strains contained a lacZ null mutation.

c Sequences of positions +70 to +98 with respect to the transcription start point of the mpr-lacZ fusions. The leftmost three nucleotides correspond to the mpr initiation codon.

d The CodY-binding motif is in boldface, and the core CodY-binding site is underlined. The mutated nucleotides are in lowercase.

e β-Galactosidase activity of each fusion in TSS medium plus 16 aa in a strain containing a codY null mutation was normalized to 100%.

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**FIG 3** Expression of extracellular Vpr in *B. subtilis* during exponential (A), transition (B), and stationary (C) phases of growth. A section of a 2D-PAGE containing protein spots corresponding to Vpr is shown. The presence of multiple Vpr spots and spot groups may be caused by carbamidomethylation or other unknown modifications of proteins. Additional minor Vpr spots were detected on the gel but are not shown. The quantification of Vpr spots is reported in Table 5.
indicating that the vpr promoter is indeed dependent on $\sigma^H$ even during steady-state growth (Table 3). In contrast, expression of the mpr-lacZ fusion, which is not known to require $\sigma^H$, was not affected by a sigH mutation (Table 4). Thus, the vpr promoter is the second example of a $\sigma^H$-dependent promoter that is repressed by CodY; previously, the ureAp2 promoter was found to be under CodY control (63).

**Overproduction of the Vpr protein in codY mutant cells.** The ability of CodY to affect expression of the vpr gene at the protein level in TSS medium plus 16 aa, and the extracellular proteins were isolated at different stages of growth and analyzed as described in Materials and Methods. All values are averages $\pm$ SDs from three independent experiments. Several protein spots containing Vpr, but of different intensities, were identified in the exoproteome. Protein spot abundance for the main spot group, labeled as Vpr (Fig. 3), and for all Vpr spots was calculated as a fraction of total protein in all spots on the gel.

### Table 5: Abundance of Vpr in the *B. subtilis* exoproteome

<table>
<thead>
<tr>
<th>Growth stage and protein spot group</th>
<th>Value for:</th>
<th>codY/ wild-type ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wild type</td>
<td>codY mutant</td>
</tr>
<tr>
<td>Exponential growth</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Main Vpr spot group</td>
<td>0.019 ± 0.008</td>
<td>0.611 ± 0.591</td>
</tr>
<tr>
<td>All Vpr spots</td>
<td>0.051 ± 0.018</td>
<td>0.748 ± 0.615</td>
</tr>
<tr>
<td>Transition phase</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Main Vpr spot group</td>
<td>0.020 ± 0.012</td>
<td>0.823 ± 0.620</td>
</tr>
<tr>
<td>All Vpr spots</td>
<td>0.040 ± 0.024</td>
<td>0.929 ± 0.705</td>
</tr>
<tr>
<td>Stationary phase</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Main Vpr spot group</td>
<td>0.018 ± 0.005</td>
<td>0.889 ± 0.321</td>
</tr>
<tr>
<td>All Vpr spots</td>
<td>0.045 ± 0.018</td>
<td>1.121 ± 0.327</td>
</tr>
</tbody>
</table>

* Cells were grown in TSS medium plus 16 aa, and the extracellular proteins were isolated at different stages of growth and analyzed as described in Materials and Methods. All values are averages $\pm$ SDs from three independent experiments. Several protein spots containing Vpr, but of different intensities, were identified in the exoproteome. Protein spot abundance for the main spot group, labeled as Vpr (Fig. 3), and for all Vpr spots was calculated as a fraction of total protein in all spots on the gel.

### DISCUSSION

In response to the intracellular levels of ILV, *L. lactis* CodY is able to regulate the expression of genes encoding products involved in protein degradation and peptide assimilation for nitrogen supply (9). In nitrogen-rich media, *L. lactis* CodY represses expression of the PrtP protease, Opp transporter, and PepN, PepC, and PepO1 peptidases (25). In *B. subtilis*, the genes encoding peptide transporters Dpp and App, intracellular peptidase DppA, and intracellular protease IspA are known to be negatively regulated by CodY (1, 3–5, 64).

In this work, we demonstrated that the genes coding for two *B. subtilis* minor extracellular proteases, Vpr and Mpr (36, 39, 40, 65, 66), are also repressed by CodY. CodY repression at the vpr locus is exerted by binding at the single CodY-binding site located in the vicinity of the transcription start point of the gene, implying that the mechanism of repression is by competition with RNA polymerase. The repression of mpr expression appears to be mediated mainly by binding of the repressor at the high-affinity site II, internal to the coding sequence of the gene. CodY binding at sites located within the coding regions of target genes was previously reported to cause efficient repression of gene expression by transcriptional roadblocking (4, 54, 67, 68). It seems likely that CodY binding to the mpr site II also creates a transcriptional roadblock for elongating RNA polymerase. In this case, however, the proximity of mpr site II to the translation initiation codon may also cause the stalled RNA polymerase to interfere with translation initiation. The location and relative strengths of the vpr and mpr CodY-binding sites, determined in this study by DNase I footprinting and gel mobility shift experiments, were found to correlate very well with the results of IDAP-Seq (4).

Mpr was shown to be a broad-range (glutamate-specific) endopeptidase (69). This is in accord with the assumption that the principal function of extracellular proteases is to supply amino acids for growth via degradation of extracellular proteins, although *B. subtilis* exoproteases have also been assigned additional physiological roles. Extracellular proteases, including Vpr, may affect multicellular behavior, such as swarming motility and biofilm formation (70, 71). Interestingly, Vpr was identified as one of the most abundant proteins in biofilms produced by some *B. subtilis* cells (71). Moreover, this minor serine protease was demonstrated to play a role in the processing of the peptide antibiotic subtilin (72) and production of two quorum sensing signaling peptides, PhrA and CSF, by cleavage of their precursors (73).

Interestingly, CodY negatively regulates the rapA-phrA operon (3–5, 74). RapA is a Spo0F phosphatase involved in the regulation of sporulation; PhrA is processed extracellularly to produce a pentapeptide that inhibits RapA activity (75). Therefore, CodY regulates the activity of RapA both at the level of rapA-phrA expression and at the level of Vpr-dependent proteolytic processing of the full-length PhrA protein.

In ongoing work, we have found that two other *B. subtilis* genes coding for extracellular proteases, aprE and nprE, for which no CodY-mediated regulation was previously detected (3, 5), are, in fact, direct targets of CodY but also of a second regulator whose expression is repressed by CodY (G. Barbieri, A. M. Albertini, E. Ferrari, A. L. Sonenshein, and B. R. Belitsky, unpublished data). Thus, CodY is a direct repressor of at least four *B. subtilis* extracellular proteases.

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