Coordinated patterns of cytochrome *bd* and lactate dehydrogenase expression in *Bacillus subtilis*

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A variety of pathways for electron and carbon flow in the soil bacterium *Bacillus subtilis* are differentially expressed depending on whether oxygen is present in the cell environment. This study characterizes the regulation of the respiratory oxidase cytochrome *bd* and the NADH-linked fermentative lactate dehydrogenase (LDH). Transcription of the *cydABCD* operon, encoding cytochrome *bd*, is highly regulated and only becomes activated at low oxygen availability. This induction is not dependent on the gene encoding the redox regulator Fnr or the genes encoding the ResDE two-component regulatory system. The DNA-binding protein YdiH was found to be a principal regulator that controls *cydABCD* expression. Transcription from the *cyd* promoter is stimulated 15-fold by a region located upstream of the core promoter. The upstream region may constitute a binding site for an unidentified transcription activator that is likely to influence the level of transcription but not its timing, which is negatively controlled by YdiH. This report provides evidence that YdiH also functions as a repressor of the *ldh* gene encoding LDH and of a gene, *ywcJ*, which encodes a putative formate-nitrite transporter. Based on the similarity between YdiH and the Rex protein of *Streptomyces coelicolor*, it is proposed that YdiH serves as a redox sensor, the activity of which is regulated by cellular differences in the free levels of NAD⁺ and NADH. It is suggested that *ydiH* be renamed as *rex*.

Received 15 April 2005

Revised 14 July 2005

Accepted 15 July 2005

INTRODUCTION

Aerobic and facultative aerobic bacteria can respond to changes in the environment by synthesizing different respiratory pathways (Poole & Cook, 2000; von Wachenfeldt & Hederstedt, 2002). During aerobic growth the final step in respiration, the four-electron reduction of dioxygen to two water molecules, is catalysed by a group of membrane-bound enzymes called terminal oxidase. The free energy released in this process is used to establish and maintain a charge and pH gradient across the cytoplasmic membrane. This electrochemical gradient drives the synthesis of ATP and other energy-requiring reactions in the cell. Most prokaryotes have the capacity to produce multiple terminal oxidases: they have branched pathways for electron transfer from different low-redox-potential electron donors to oxygen. The spore-forming, low-G+C Gram-positive bacterium *Bacillus subtilis* has genes that encode three

distinct terminal oxidases: cytochrome *caa*₃, cytochrome *aa*₃ and cytochrome *bd* (von Wachenfeldt & Hederstedt, 2002). In addition, the presence of a fourth terminal oxidase of *bd* type, YthAB, can be predicted from the genome sequence (Winstedt *et al.*, 1998; von Wachenfeldt & Hederstedt, 2002). No single terminal oxidase is essential, but one of the quinol oxidases, cytochrome *aa*₃ or cytochrome *bd*, is required for aerobic growth of *B. subtilis* (Winstedt & von Wachenfeldt, 2000). By changing the levels of the terminal oxidases and other respiratory enzymes, bacteria can conserve energy under widely varying environmental conditions. *B. subtilis* is also able to use nitrate (NO₃[−]) or nitrite (NO₂[−]) as electron acceptors for respiration or to grow fermentatively (Nakano & Zuber, 2002). In *B. subtilis*, the respiratory nitrate reductase is encoded by the *narGHI* genes of the *narGHIJ* gene cluster (Cruz Ramos *et al.*, 1995). Nitrate reductase is a membrane-bound complex, which probably contributes to the proton-motive force by a redox loop in which oxidation of menaquinol and release of two protons takes place at the positive side (outer side) of the membrane. Nitrite reduction is catalysed by a soluble NADH-dependent nitrite reductase that is encoded by *nasD* and *nasE* (Nakano & Zuber, 2002). Under fermentative growth conditions, sugars are converted to reduced organic

Abbreviations: CcpA, carbon catabolite protein A; LDH, lactate dehydrogenase; MUG, 4-methylumbelliferyl-β-D-galactoside.

Text files of transcriptome data, light absorption difference spectra of membranes from strains 1A1 and LUW210 (Supplementary Fig. S1) and oligonucleotide sequences (Supplementary Table S1) are available with the online version of this paper.

compounds, such as ethanol, lactate, acetoin and 2,3-butanediol. Lactate dehydrogenase (LDH), encoded by *ldh*, is a cytoplasmic NADH-linked enzyme that converts pyruvate to lactate. During fermentation, LDH is the key enzyme involved in reoxidation of the NADH formed by glycolysis (Cruz Ramos *et al.*, 2000).

In *Escherichia coli*, oxygen represses the anaerobic respiratory pathways and fermentation, whereas nitrate is the preferred electron acceptor under anaerobic conditions. Nitrate acts to induce nitrate reductase expression and to repress synthesis of other anaerobic pathways (Gennis & Stewart, 1996). The transcriptional regulation of the respiratory enzymes parallels the redox potentials of the corresponding electron-acceptor couples (Gennis & Stewart, 1996). A similar hierarchical transcriptional regulation may be present in *B. subtilis*. The two-component regulatory system ResDE and the redox regulator Fnr are important components of the regulatory system for anaerobic adaptation. Several enzymes necessary for anaerobic growth are under the control of the ResDE two-component system (Nakano & Zuber, 2002). ResD encodes a putative oxygen sensor kinase and ResE encodes a transcription regulator (Geng *et al.*, 2004). Full activation of *fnr* expression requires ResE (Nakano & Zuber, 2002). Fnr is in turn an activator of the *narGHJI* operon, *narK* encoding a putative nitrite extrusion protein, and *arfM* encoding an anaerobic respiration and fermentation modulator (Cruz Ramos *et al.*, 1995; Marino *et al.*, 2000).

Transcription of the *cydABCD* operon, encoding cytochrome *bd*, is highly regulated and only becomes activated when oxygen is limiting (Winstedt *et al.*, 1998). Cytochrome *bd* is, in analogy to the orthologous *E. coli* enzyme, likely to be a high-oxygen-affinity terminal oxidase. Here, we show that regulation of the *cydABCD* operon is independent of the *resDE* or *fnr* gene products. It has recently been shown that in the high-G+C Gram-positive bacterium *Streptomyces coelicolor* A3(2), expression of the cytochrome *bd*-encoding genes is negatively regulated by the DNA-binding Rex protein (Brekasis & Paget, 2003). In *B. subtilis*, the orthologous protein is encoded by the *ydiH* gene. Here, we provide evidence that *B. subtilis* YdiH functions as a repressor not only of the *cydABCD* operon but also of the *ldh* *lctP* operon and of a putative formate-nitrite transporter gene, *ywcJ*. During the course of this work, Schau *et al.* (2004) reported that YdiH is a negative regulator of the *cydABCD* operon.

A regulatory model is proposed in which the NADH/NAD⁺ ratio is key to a control YdiH activity. During the transition to oxygen-limited growth, an increased level of NADH is expected, since it is less efficiently reoxidized to NAD⁺ as a result of reduced respiration. This leads to the production of cytochrome *bd*, LDH and YwcJ.

METHODS

Bacterial strains, plasmids, media and growth conditions. The bacterial strains and plasmids used in this study are listed in

Table 1. *B. subtilis* strains were grown at 37 °C in nutrient sporulation medium with phosphate (NSMP) and in NSMP supplemented with 0.5% (w/v) glucose (NSMPG) (Winstedt *et al.*, 1998). Tryptose blood agar base medium (TBAB) was used for growth of bacteria on plates. L broth or L agar was used for the growth of *E. coli* strains (Sambrook & Russel, 2001). The following antibiotics were used when required: chloramphenicol (5 mg ml⁻¹), tetracycline (15 µg ml⁻¹), spectinomycin (150 µg ml⁻¹), and a combination of erythromycin (0.5 µg ml⁻¹) and lincomycin (12.5 µg ml⁻¹) for *B. subtilis* strains, and ampicillin (100 µg ml⁻¹) for *E. coli* strains.

DNA manipulations and transformation. Methods for plasmid isolation, agarose gel electrophoresis, use of restriction and DNA-modification enzymes, DNA ligation, PCR and electroporation of *E. coli* cells were performed according to standard protocols (Sambrook & Russel, 2001). Isolation of chromosomal DNA and transformation of *B. subtilis* cells with chromosomal or plasmid DNA were done as described by Hoch (1991).

RNA isolation. To isolate RNA, a 12.5 ml culture sample was rapidly added to a 50 ml centrifuge tube filled with 5 g crushed ice. The sample was centrifuged at 8000 g for 10 min at 4 °C, and the pellet was suspended in 1 ml ice-cold TES buffer (50 mM Tris/HCl, pH 7.5, 5 mM EDTA, 50 mM NaCl) and transferred to a 2 ml test tube containing 0.6 ml acidic phenol, 0.12 ml chloroform and 0.75 ml zirconium-silica beads (0.1 mm diameter). The test tube was placed in a Mini-Beadbeater (Biospec Products, USA) and shaken at full speed for 80 s. The tube was then centrifuged at 5000 g for 5 min. The aqueous phase was recovered and extracted twice with 0.6 ml acidic phenol and 0.12 ml chloroform, and once with 0.7 ml chloroform. Total RNA was precipitated from the aqueous phase by adding a 1/10 volume of 3 M sodium acetate (pH 4.8) and two volumes of ice-cold 95% (v/v) ethanol. After centrifugation and washing with ice-cold 70% (v/v) ethanol, the pellet was suspended in 0.2 ml water pre-treated with diethyl pyrocarbonate. The RNA was then treated with 3 µl (3 U) DNase I (Invitrogen) for 30 min at 37 °C, extracted once more with 0.6 ml acidic phenol and 0.12 ml chloroform, and finally with 0.7 ml chloroform. RNA was recovered by precipitation, as described above, and suspended in 60 µl water pre-treated with diethyl pyrocarbonate. The concentration and quality of the RNA was checked by electrophoresis in a 0.8% (w/v) agarose gel containing ethidium bromide.

cDNA labelling and microarray hybridization conditions. For cDNA synthesis, 20 µl of total RNA (1 µg ml⁻¹) was mixed with 1 µl (0.1 pmol) of a mixture of coding-sequence-specific primers (Eurogentec), 8 µl of Superscript II buffer (Invitrogen), 3 µl of a 20 mM mixture of dATP, dGTP and dTTP (containing equimolar amounts of the nucleotides), 1 µl of 2.5 mM dCTP, 1 µl of 1 mM Cy3- or Cy5-labelled dCTP analogues (Amersham Biosciences) and 4 µl of 0.1 M DTT. The mixture was incubated at 65 °C for 5 min and subsequently at 42 °C for 5 min. Then 1 µl (40 U) of RNasin (Promega) and 2 µl (400 U) of SuperScript II reverse transcriptase (Invitrogen) were added and the mixture was incubated at 42 °C for 1 h. To stop the reaction and hydrolyse RNA, 5 µl of 50 mM Na/EDTA (pH 8.0) and 2 µl of 10 M NaOH were added, and the sample was incubated at 65 °C for 20 min. Unincorporated dye-labelled dCTP was removed and cDNA was concentrated to 1–5 µl by using a microconcentrator (Microcon YM-30, Millipore). DNA microarrays consisting of duplicated spots of PCR products representing 3925 of the ~4100 predicted ORFs of the *B. subtilis* 168 genome (Eurogentec) were prehybridized using 47.5 µl DIG Easy Hyb solution (Roche) and 2.5 µl yeast RNA (10 mg ml⁻¹ in DIG Easy Hyb solution) at 42 °C for 2 h. Solutions were applied to the microarrays by capillary action under a coverslip. The coverslip was removed by dipping the slides into 0.1 × SSC. The slides were dried by centrifugation for 30 s. For hybridization with cDNA, 42.5 µl

Table 1. Bacterial strains and plasmids used in this study

Strains* and plasmids	Relevant characteristics	Source or reference†
<i>E. coli</i>		
XL-1 Blue	<i>endA1 gyrA96 thi hsdR17</i> ($r_k^- m_k^+$) <i>supE44 relA1 lac/F' proAB lacI^q lacZΔM15 Tn10</i>	Stratagene
TOP10	<i>F⁻ mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZDM15 ΔlacX74 deoR recA1 araD139 Δ(ara-leu)7697 galU galK rpsL endA1 nupG</i>	Invitrogen
<i>B. subtilis</i>		
BSIP1185	<i>trpC2 ldh-lacZ1 cat</i>	Cruz Ramos <i>et al.</i> (2000)
BGW2	<i>trpC2 lys-3 ccpA::Tn917</i>	Kruger <i>et al.</i> (1993)
BSM21	<i>trpC2 rsbU::kan</i>	Brigulla <i>et al.</i> (2003)
BSM30	<i>trpC2 rsbP::spc</i>	Brigulla <i>et al.</i> (2003)
MH5081	<i>pheA1 trpC2 resDE::tet</i>	Sun <i>et al.</i> (1996)
QB5407	<i>trpC2 ccpA::spc</i>	Presecan-Siedel <i>et al.</i> (1999)
1A1	<i>trpC2</i>	BGSC‡
LUW38	<i>amyE::(pDG1661 cat)</i>	This study
LUW49	<i>fnr::spc</i>	pDIA5350→1A1
LUW95	<i>amyE::(cyd(−270 to +199)-lacZ cat)</i>	This study
LUW96	<i>amyE::(cyd(−116 to +199)-lacZ cat)</i>	This study
LUW107	<i>amyE::(cyd(−37 to +199)-lacZ cat)</i>	This study
LUW108	<i>amyE::(cyd(−7 to +199)-lacZ cat)</i>	This study
LUW210	<i>ccpA::Tn917</i>	BGW2→1A1
LUW214	<i>amyE::(cyd(−270 to +199)-lacZ cat) ccpA::spc</i>	QB5407→LUW95
LUW217	<i>amyE::(PglpD-cyd(+1 to +199)-lacZ cat)</i>	This study
LUW219	<i>yjbIH::spc</i>	pΔyjbIH1→1A1
LUW221	<i>amyE::(cyd(−270 to +199)-lacZ cat) yjbIH::spc</i>	LUW219→LUW95
LUW241	<i>amyE::(cyd(−270 to +10)-lacZ cat)</i>	This study
LUW243	<i>amyE::(cyd(−92 to +199)-lacZ cat)</i>	This study
LUW244	<i>amyE::(cyd(−74 to +199)-lacZ cat)</i>	This study
LUW245	<i>amyE::(cyd(−60 to +199)-lacZ cat)</i>	This study
LUW264	<i>resDE::tet</i>	MH5081→1A1
LUW269	<i>resDE::tet amyE::(cyd(−270 to +199)-lacZ cat)</i>	LUW95→LUW264
LUW270	<i>amyE::(cyd(−89 to +199)-lacZ cat)</i>	This study
LUW271	<i>amyE::(cyd(−79 to +199)-lacZ cat)</i>	This study
LUW273	<i>ydiHΩpYDIH1 Sp^r</i>	pYDIH1→1A1
LUW274	<i>amyE::(cyd(−270 to +199)-lacZ cat) ydiHΩ pYDIH1 Sp^r</i>	LUW273→LUW95
LUW275	<i>amyE::(ywcJ-lacZ cat)</i>	This study
LUW276	<i>amyE::(ldh-lacZ cat)</i>	BSIP1185→1A1
LUW277	<i>amyE::(PglpD-cyd(+1 to +199)-lacZ cat) ccpA::spc</i>	QB5407→LUW217
LUW279	<i>amyE::(cyd(−270 to +11)-lacZ cat) ydiHΩpYDIH1 Sp^r</i>	LUW273→LUW241
LUW280	<i>amyE::(ywcJ-lacZ cat) ydiHΩpYDIH1 Sp^r</i>	LUW273→LUW275
LUW281	<i>amyE::(ldh-lacZ cat) ydiHΩpYDIH1 Sp^r</i>	LUW273→LUW276
LUW287	<i>amyE::(cyd(−270 to +11)-lacZ cat) fnr::spc</i>	LUW49→LUW95
LUW289	<i>rsbU::kan</i>	BSM21→1A1
LUW290	<i>rsbP::spc</i>	BSM30→1A1
LUW291	<i>rsbU::kan rsbP::spc</i>	BSM30→LUW289
LUW292	<i>ydiHΩEm^r</i>	YDIHd→1A1
LUW293	<i>ydiHΩEm^r rsbU::kan rsbP::spc</i>	YDIHd→LUW291
LUW294	<i>ydiHΩEm^r rsbU::kan</i>	YDIHd→LUW289
LUW295	<i>ydiHΩEm^r rsbP::spc</i>	YDIHd→LUW290
Plasmids		
pDG1661	Insertional Sp ^r plasmid carrying promoterless <i>lacZ</i>	Guerout-Fleury <i>et al.</i> (1996)
pDG1726	Ap ^r Sp ^r	Guerout-Fleury <i>et al.</i> (1996)
pDG1727	Ap ^r Sp ^r	Guerout-Fleury <i>et al.</i> (1996)
pDIA5350	Ap ^r Sp ^r	Cruz Ramos <i>et al.</i> (1995)

Table 1. cont.

Strains* and plasmids	Relevant characteristics	Source or reference†
pYDIH1	Insertional Sp ^r plasmid carrying an internal part of <i>ydiH</i>	This study
pYjbI1	Ap ^r Sp ^r	This study
pΔyjbIH1	Ap ^r Sp ^r	This study

*All LUW strains are derivatives of 1A1 and thus carry the *trpC2* auxotrophic marker.

†Arrows indicate transformation and point from donor DNA to recipient strain.

‡BGSC, Bacillus Genetic Stock Center, Department of Biochemistry, Ohio State University, Columbus, OH. The *ydiH* pMUTIN2 insertion mutant YDIHd is described in the BSORF database (<http://bacillus.genome.jp/>).

DIG Easy Hyb solution was mixed with 2.5 µl yeast RNA and 5 µl dye-labelled cDNA. The slides were incubated with this mixture for 16 h at 42 °C. Microarray slides were washed twice for 5 min each at room temperature with 0.1 × SSC, 0.1 % (w/v) SDS and again for 1 min in 0.1 × SSC. Slides were dried by centrifugation and were immediately scanned with a confocal laser scanner with excitation at 635 and 532 nm (Axon GenePix 4000B; Axon Instruments, Inc.). Synthesis of cDNA from RNA isolated from untreated (aerobic) and argon-exposed cells was made in the presence of Cy5 and Cy3, respectively. Each experiment, except with LUW219, was performed at least two times, with RNA isolated from independent bacterial cultures.

Microarray data analysis. Images were analysed using GenePix Pro 4.1 (Axon Instruments). The images were inspected for artifacts. Spots affected by, for example, smears were excluded from further analysis. The results from the image analysis, including background and median spot intensities, were transferred into the software BASE 1.2 (BioArray Software Environment) (Saal *et al.*, 2002). The data were normalized with a LOWESS (locally weighted scatterplot smoothing) algorithm [0.33 window size (fraction of points), 0.1 minimum log (intensity) step, 4 iterations]. For detection of differentially expressed genes, a standard deviation analysis obtaining a Z score representing the change in expression for each gene was performed (Saal *et al.*, 2002; Yang *et al.*, 2003). Briefly, the Z scores were calculated as follows. From within the assay, *n* spots were selected using a sliding window across, where *Ch1* and *Ch2* were the background-corrected intensities from the scanning at 635 and 532 nm, respectively. The sliding window was set to 400 spots.

For each spot *i*, the Z score was calculated as:

$$Z(i) = \frac{M(i) - M(w)}{SD(w)}$$

where *M(w)* is the mean log₂ ratio of the spots in the window surrounding *i*:

$$M(w) = \frac{\sum_{i \in w} M(i)}{n}$$

SD(*w*) is the corresponding standard deviation.

An arithmetic mean of the Z scores was calculated from all replicates in each assay. Genes present in less than $n_{\text{(total replicates)}} - 1$ were excluded from the results. The Z score cut-off was chosen as ±3SD. This corresponds to a confidence level of 99.73 %, or a *P* value of 7.3×10^{-6} for a gene to be differentially expressed for two slides (outside the Z score cut-off for both slides). With 3925 data points, this results in 0.03 errors in this experiment.

β-Galactosidase activity measurements. β-Galactosidase activity was assayed using MUG (4-methylumbelliferyl-β-D-galactoside) as

substrate. Liquid culture measurements were done essentially as described by Youngman (1990). The bacteria were grown in 25 ml cultures in 250 ml Erlenmeyer flasks with indentations. The cultures were first inoculated to an OD₆₀₀ of 0.1, grown to an OD₆₀₀ of 0.8–1.0 and then diluted to an OD₆₀₀ of 0.1. At various times during growth, 1 ml samples were removed and frozen in liquid nitrogen. Samples were stored at –80 °C until assayed for β-galactosidase activity. One unit of activity is defined as 1 nmole of MUG hydrolysed per millilitre of culture sample per minute, normalized for culture cell density (OD₆₀₀).

Construction of *B. subtilis* strains carrying *cyd-lacZ* or *ywcJ-lacZ* fusions. Transcriptional *lacZ* fusion plasmids were constructed in vector pDG1661. Strains LUW95, LUW96, LUW107, LUW108, LUW217, LUW243, LUW244, LUW245, LUW270, LUW271, LUW241 and LUW275 were constructed by using pDG1661 and primer pairs (see Supplementary Table S1): CYDA6/CYDA10, CYDA6/CYDA9, CYDA6/CYDA11, CYDA6/CYDA12, glpDcyd/CYDA6, CYDA6/CYDA22, CYDA6/CYDA20, CYDA6/CYDA21, CYDA6/CYDA24, CYDA6/CYDA25, CYDA6/CYDA30, CYDA6/CYDA31 and YWCJ1/YWCJ2, respectively. The various DNA fragments were digested with *EcoRI* and *BamHI* and ligated into pDG1661 digested with the same enzymes, and then transformed into *E. coli* XL-1 Blue selecting for ampicillin resistance. Plasmids extracted from *E. coli* were used to transform *B. subtilis* 1A1 to chloramphenicol resistance. In the resulting transformants, the promoter–*lacZ* fusion had integrated at the *amyE* locus by double-crossover recombination. All constructs generated from PCR-amplified fragments were subjected to sequence analysis to verify the fidelity of amplification.

Construction of a *yjbIH* null mutant. The upstream and downstream regions flanking the *yjbIH* genes were amplified by PCR from *B. subtilis* 1A1 chromosomal DNA using primer pairs YJB16/YJB17 and YJB18/YJB19, respectively (see Supplementary Table S1). The YJB16/YJB17 PCR fragment was cut with *NdeI* and *EcoRI* and ligated into pDG1726 digested with the same enzymes and transformed into *E. coli* TOP10 selecting for ampicillin resistance. The resulting plasmid was named pYjbI1. The YJB18/YJB19 PCR fragment was cut with *SphI* and ligated into pYjbI1 cut with *SphI* to give plasmid pΔyjbIH1. This plasmid was used to transform strain 1A1 to spectinomycin resistance. The deletion–insertion within the chromosomal *yjbIH* locus arising from a double crossover recombination event was confirmed by sizing the PCR fragments generated from this chromosomal region (data not shown).

Inactivation of *ydiH*. Plasmid pYdiH1, carrying an internal fragment of *ydiH*, was integrated into the chromosome of *B. subtilis* strain 1A1 via a single-crossover recombination event. Integration of pYdiH1 in the *ydiH* gene was confirmed by sizing of PCR fragments generated from this chromosomal region (data not shown). The integration disrupts the ORF of *ydiH*. To obtain plasmid pYdiH1, a

663 bp DNA fragment was amplified using PCR with *B. subtilis* 1A1 chromosomal DNA as template and the primers YDIH1 and YDIH2. The DNA product was cut with *DraI* and *EcoRI* and ligated into *HindIII/EcoRI*-cut pDG1727.

Miscellaneous methods. For membrane preparations, liquid cultures of *B. subtilis* were grown in 1.5 l batches in 5 l Erlenmeyer flasks with indentations. The cultures were incubated at 37 °C on a rotary shaker (200 r.p.m.). Membranes were isolated as described by Hederstedt (1986) and suspended in 20 mM MOPS buffer (pH 7.4). Absorption spectra were recorded as described previously (Schiött *et al.*, 1997). The absorption difference at 650–626 nm was used to evaluate the relative concentration of cytochrome *bd* in the membranes. Protein concentrations were estimated using the bicinchoninic acid (BCA) method (Pierce) with BSA as standard. Dissolved oxygen measurements were done with a Mettler Toledo InPro 6000 series oxygen sensor. The RSAT tools were used to search genomic sequences for putative YdiH binding sites (van Helden *et al.*, 2000). The WebLogo was prepared according to Crooks *et al.* (2004).

RESULTS

Role of the *cyd* leader

The expression of cytochrome *bd* in *B. subtilis* is induced in cells grown in broth media containing glucose and reaches its maximum when the cells are entering the early stationary growth phase (Winstedt *et al.*, 1998). *B. subtilis* LUW95 that carries a transcriptional *cyd-lacZ* fusion was grown in a broth medium with (NSMPG) or without glucose (NSMP), and β -galactosidase activities were recorded. High expression of the *lacZ* reporter gene was only detected in NSMPG-grown cells harvested in the stationary phase (Fig. 1A). To study if the leader alone is responsible for the observed expression pattern, the LUW241 strain was constructed. This strain carries a *cyd-lacZ* fusion lacking most of the DNA corresponding to the untranslated *cyd* leader region. Removing the leader region drastically increased expression of *cyd-lacZ* in cells grown in NSMP (Fig. 1B). In NSMPG, an increased expression in exponentially growing cells was observed (Fig. 1A). When the *cyd* untranslated leader region followed by *lacZ* was placed downstream of the constitutive σ^A -type *glpD* promoter (Holmberg & Rutberg, 1992) (strain LUW217), a similar expression pattern as seen for LUW95 was observed (Fig. 1A, B).

Role of the upstream region of the *cyd* promoter

To define the minimal upstream sequences required for transcription of the *cydABCD* operon, a series of 5' deletion variants of the *cyd* promoter were used to drive expression of the *lacZ* reporter gene (Fig. 2). Upstream of the *cydA* transcription start site, a potential housekeeping σ^A -type –10 (TAAAGT) sequence is present. It is preceded by a poor –35 signal (TTTATT), suggesting the involvement of a transcription activator. The deletion analysis shows that transcription from the *cyd* promoter is stimulated approximately 15-fold by a *cis*-acting DNA sequence located between –79 and –74 upstream of the transcription start point (Fig. 2). Deletion of the upstream region affected the

maximum expression level but not the timing of expression of the *cyd-lacZ* fusions (data not shown).

Carbon catabolite protein A (CcpA) indirectly affects expression of the *cydABCD* operon

The expression pattern of the *cydABCD* genes (Fig. 1A, B) suggests that they are subject to glucose activation. Data from transcriptome analysis indicates that the glucose activation is mediated by the transcription factor CcpA (Moreno *et al.*, 2001). A mutation in the *ccpA* gene was found to suppress the maximal transcription of the *cyd-lacZ* fusion approximately 2.5-fold without affecting the timing of the transcription (Fig. 1C). The reduced level of expression was confirmed by an approximately three-fold reduction of cytochrome *bd* enzyme produced (see Supplementary Fig. S1). The *cyd* untranslated leader region was next placed downstream of the constitutive *glpD* promoter. In this construct also, the *ccpA* mutation reduced transcription (approx. 3.5-fold), indicating that CcpA activation does not involve the *cydABCD* core promoter region or upstream regions (Fig. 1D).

Role of Fnr, ResDE and YjbIH in regulation of *cydABCD*

Cytochrome *bd* shows an apparent oxygen-dependent expression pattern and is maximally expressed at low oxygen tension (Winstedt *et al.*, 1998). Several of the O₂-responsive gene regulators of bacteria are members of the Fnr protein family of transcriptional regulators (Korner *et al.*, 2003). However, mutations in *fnr* had no apparent effect on *cydABCD* expression in NSMP, but may influence the maximum level of expression in NSMPG (Fig. 1E). ResD of the ResD–ResE two-component signal transduction system has been suggested to directly sense a decreased oxygen tension leading to activation of the ResDE regulon (Geng *et al.*, 2004). Deletion of *resDE* had little effect on the expression of a *cyd-lacZ* fusion (Fig. 1F).

The *yjbI* gene of the putative *yjbIH* operon encodes a truncated haemoglobin (Giangiacomo *et al.*, 2005). The physiological roles of truncated haemoglobins are largely unknown. However, experimental data suggest a variety of roles for these proteins related to the binding of oxygen for storage, transfer or sensing (Wittenberg *et al.*, 2002). A *yjbIH* mutation drastically decreased *cyd-lacZ* expression (Fig. 3A). It was noted that the *yjbIH* mutation also affected bacterial growth and oxygen consumption in NSMPG (Fig. 3B, C). To investigate if YjbIH is a direct oxygen sensor, we used transcriptome analysis to see which genes are differentially expressed when an aerobic culture is rapidly challenged with oxygen limitation. Wild-type and *yjbIH* mutant cells grown aerobically to exponential phase in NSMPG were left untreated or exposed to argon to rapidly deplete oxygen from the culture medium. After 5 min, total RNA was isolated from the untreated and argon-exposed cultures. The *cydAB* (encoding cytochrome

Fig. 1. Time-courses of β -galactosidase activity of various *cyd-lacZ* fusions. Cells were grown at 37 °C on a rotary shaker (100 r.p.m.) in NSMPG (A, C–F) or NSMP (B). Samples were collected and assayed as described in Methods. t_0 marks the end of exponential growth. Data were averaged from at least three independent trials. Error bars represent the standard deviation of the means. One unit of activity is defined as 1 nmole of MUG hydrolysed per millilitre of culture sample per minute, normalized for culture cell density (OD_{600}). Strains: (A, B, C, E, F) LUW95 (*cyd* region –270 to +199) (●), (A, B) LUW241 (*cyd* region –270 to +10) (○), (A, B) LUW217 (*cyd* region +1 to +199) (▼), (C) *ccpA::spc* mutant strain LUW214 (○), (D) *PglpD-cyd-lacZ* strain LUW217 (●), *ccpA::spc* mutant strain LUW277 (○), (E) *fmr::spc* mutant strain LUW287 (○), (F) *resDE::tet* mutant strain LUW269 (○).

bd), *ldh* *lctP* (encoding LDH and lactate permease, respectively) and *ywcJ* (encoding a putative formate-nitrite transporter) transcripts showed a significant accumulation in the wild-type samples exposed to argon (Fig. 4A). Transcriptome analysis of the *yjbIH* mutant showed significant induction of *cydA* and *ldh* expression (Fig. 4B). The results indicate that YjbIH is not directly involved in the regulation of these genes.

YdiH regulates *cyd*, *ldh* and *ywcJ*

DNase I footprinting experiments indicate that YdiH *in vitro* binds to three regions located in the 5'-untranslated region preceding *cydA*. The reported YdiH DNA-binding consensus sequence is TTTGTGAADTABTGAKCAAWDT (Schau *et al.*, 2004). By searching the *B. subtilis* genome with the consensus sequence, highly related sequences are

Fig. 2. Deletion analysis of the *cyd* promoter. *B. subtilis* strains carrying *cyd* promoter variants were grown at 37 °C on a rotary shaker (100 r.p.m.) in NSMPG, and the promoter activity was detected at t_{+1} . The promoter activity of LUW95 was used for the calculation of the relative promoter activity. The 100% activity corresponds to 26.8 ± 2.2 MUG units according to the mean and standard deviation from three independent experiments. The other promoter activities are calculated as the mean percentage expression of the full-length promoter (–270) from three independent experiments. The promoter activity for each construct is plotted versus the 5'-deletion end-point relative to the *cydA* transcriptional start point. The strains used were: LUW95 (–270), LUW96 (–116), LUW243 (–92), LUW270 (–89), LUW271 (–79), LUW244 (–74), LUW245 (–60), LUW107 (–37) and LUW108 (–7).

found in the *ldh* and *ywcJ* promoter regions (Fig. 5), indicating that YdiH also regulates these genes. In addition, a related but reversed sequence is present upstream of *yjlC* that encodes a protein of unknown function. A related sequence is also found to overlap the –10 region in the *alsSD* promoter. As described above, *ldh* and *ywcJ*, together with the *cyd* genes, are induced when an exponentially

growing aerobic culture is rapidly challenged with oxygen limitation (Fig. 4A). When the same experiment was done with a YdiH mutant, no induction of *cydAB*, *ldh*, *lctP* or *ywcJ* transcription could be detected (Fig. 4C). The relatively high signal intensities observed for *cydAB*, *ldh*, *lctP* and *ywcJ* indicate that these genes were constitutively expressed at high levels in the mutant (Fig. 4C). In the YdiH mutant, oxygen limitation induced transcription of at least 103 genes, of which 54 are known to be under the control of the general stress sigma factor σ^B (Petersohn *et al.*, 2001; Price *et al.*, 2001) (Fig. 4C and supplementary data text files 3, 4 and 5).

Activation of σ^B is achieved through a complex phosphorylation/dephosphorylation cascade in response to various

Fig. 3. (A) Time-courses of β -galactosidase activity of a *cyd-lacZ* fusion in wild-type or in a *yjbIH* mutant. Cells were grown at 37 °C on a rotary shaker (100 r.p.m.) in NSMPG. Sampling was done as described in the legend to Fig. 1. Strains: parental strain LUW95 (●), *yjbIH::spc* mutant strain LUW219 (○). (B) Growth and (C) dissolved oxygen concentrations in cultures of LUW95 (●) and LUW219 (○) in NSMPG. In (C), the horizontal line (10 mbar of O₂) indicates the approximate limit of microaerobic growth conditions (Arras *et al.*, 1998).

cellular stimuli. Two independent signalling pathways regulate σ^B activity. The energy pathway requires the RsbP phosphatase, and the environmental pathway depends on the RsbU phosphatase (Hecker & Völker, 2001). To identify the pathway by which σ^B is activated in the YdiH mutant in response to oxygen starvation, an RsbP/YdiH and an RsbU/YdiH mutant strain were tested under these conditions (Fig. 6). In the RsbU/YdiH mutant, the σ^B regulon was activated. In contrast, in the RsbP/YdiH mutant or in an RsbP/RsbU/YdiH mutant strain, no activation of the σ^B regulon could be detected, indicating that the activation is RsbP dependent.

To further investigate experimentally if YdiH is a regulator of *ldh* and *ywcJ*, *ldh-lacZ* and *ywcJ-lacZ* expression in

Fig. 4. Changes in gene expression as determined by microarray analysis of exponentially growing cells and cells challenged with 5 min of oxygen limitation. *B. subtilis* strains were grown aerobically in NSMPG until reaching OD₆₀₀ 0.6. At this point, the cells were left untreated or exposed to thorough bubbling with argon. This treatment rapidly removes oxygen from the culture medium. (A) wild-type, (B) LUW219 ($\Delta yjbIH$), (C) LUW273 (*ydiH* insertion mutant). The horizontal line marks the Z score threshold value that is considered significant. The intensity-dependent Z score measures the number of standard deviations of a particular data point from the mean (for details see Methods). Data points above this line represent genes which are significantly upregulated in oxygen-depleted cells. In (C), circled dots represent genes that are transcribed from σ^B -dependent promoters, and the boxed dots show genes that were found to be upregulated in wild-type cells (A). It should be noted that the microarrays used do not contain functional probes for *cydC* and *cydD*. The transcriptome datasets can be found in supplementary data text files 1, 2, 3, 4 and 5.

wild-type and YdiH mutant cells was compared. Expression of the *ldh-lacZ* and *ywcJ-lacZ* constructs was induced in cells grown in media containing glucose (NSMPG). The expression reached its maximum levels when the cells entered the early stationary growth phase, similar to that seen for the *cyd-lacZ* fusion (Fig. 7). When the cultures were grown in NSMP or NSMPG at high aeration, expression of *cyd-lacZ*, *ldh-lacZ* and *ywcJ-lacZ* was essentially undetectable. In contrast, in a YdiH mutant, expression of *cyd-lacZ*, *ldh-lacZ* and *ywcJ-lacZ* was relatively high, both in NSMP and in the highly aerated NSMPG-grown cells (Fig. 7). Taken together, these data suggest that YdiH is a direct regulator not only of the *cydABCD* operon but also of *ywcJ* and the *ldh lctP* operon.

Refining the YdiH recognition sequence by a bioinformatics approach

B. subtilis YdiH is highly similar to the corresponding proteins predicted to be present in several low-G+C-content Gram-positive bacteria. We selected YdiH homologues that have identical amino acid sequences in the presumed DNA-binding region. It is likely that these proteins bind to a common DNA sequence. Thus we scanned the selected genome sequences with a YdiH DNA-binding consensus sequence derived from the five predicted binding sites in *B. subtilis* (TGTGAADTRBTKMDCAA) and found related sequences in the promoter regions of genes homologous to *cydABCD*, *ldh lctP* and *ywcJ*. The YdiH DNA-binding regions were used to create a weight matrix graphically represented as a sequence logo in Fig. 5(B).

DISCUSSION

For many bacteria, changes in oxygen tension serve as an important environmental cue to trigger adaptive changes in metabolism (Green & Paget, 2004; Poole & Cook, 2000). Bacteria have evolved different mechanisms to directly and

Fig. 5. (A) Putative YdiH-binding sites and location in different *B. subtilis* promoter regions. Grey rectangles show potential -10 and -35 σ^A -type promoter recognition sites and black rectangles indicate the position of YdiH-binding sites. The arrows indicate transcription start sites. The transcription start site of the *cydABCD* operon (Winstedt *et al.*, 1998) and of the *ldh* *lctP* operon was obtained from primer extension analysis (Cruz Ramos *et al.*, 2000). The location of the promoter and of the transcription start site of *ywcJ* was predicted (Jarmer *et al.*, 2001). The predicted *ywcJ* promoter sequences are: -10 (TATACT) and -35 (GTGAAA). The upstream region of *ywcJ* was sequenced and found to be identical to the published genomic sequence (Glaser *et al.*, 1993), with the exception of a single change of C to T corresponding to the most-5' nucleotide of the -10 sequence. This change has been confirmed by directly sequencing a PCR product originating from this region of the chromosome. (B) Sequence logo of the YdiH-binding sites identified in the genome sequences of: *Bacillus anthracis* str. Ames, *Bacillus cereus* ATCC 10987, *B. cereus* ATCC 1457, *B. cereus* E33, *Bacillus halodurans* C-125, *Bacillus licheniformis* ATCC 14580, *B. subtilis* 168, *Bacillus thuringiensis* serovar konkukian str. 97-27, *Bacillus clausii* KSM-K16, *Geobacillus kaustophilus* HTA426, *Listeria innocua* Clip11262, *Listeria monocytogenes* str. 4b F2365, *L. monocytogenes* EGD-e and *Oceanobacillus iheyensis* HTE831. The Shannon information content is shown on the y axis; Shannon's unit of non-randomness is the bit (short for 'binary digit') (Crooks *et al.*, 2004). The proposed 18 bp YdiH-binding consensus sequence is shown above the sequence logo. It is composed of a tandem inverted repeat of two weakly conserved 7 bp half-sites, as indicated by the arrows. *B. subtilis* Fnr- (Makita *et al.*, 2004) and ResD- (Nakano *et al.*, 2000) binding consensus sequences are also shown.

indirectly sense oxygen levels. Here we show that in *B. subtilis*, three transcriptional units, *cydABCD*, *ldh* *lctP* and *ywcJ*, previously known to be induced under microaerobic or anaerobic growth conditions, are directly regulated by YdiH. In a recent study, YdiH has also been shown to function as a negative regulator of cytochrome *bd* (*cydABCD*) gene expression in *B. subtilis* by binding to the DNA corresponding to the 5'-untranslated region of the *cydABCD* transcript (Schau *et al.*, 2004). The predicted *ydiH* translation product shares significant similarity with Rex from *S. coelicolor* A3(2), a regulator suggested to modulate transcription in response to changes in the cellular NADH/NAD⁺ ratio. Rex binds both the oxidized and reduced form of NAD(H). Binding of NADH results in the disruption of a Rex–DNA complex (Brekasis & Paget, 2003). Database searches indicate that Rex-related proteins are present in many Gram-positive bacteria, particularly in the low-G+C group. The structure of a Rex family repressor from *Thermus aquaticus* in complex with NADH has been determined (Sickmier *et al.*, 2005). The protein has an N-terminal DNA-binding domain with a winged helix–turn–helix fold and a C-terminal NAD(H)-binding domain. A local sequence alignment between *S. coelicolor* Rex and *B. subtilis* YdiH shows that about 40% of the amino acid residues are invariant. Comparative structural modelling (data not shown) suggests that the overall structure of YdiH is similar to that of *T. aquaticus* Rex. Based on analogy with the *T. aquaticus* and *S. coelicolor* Rex proteins, YdiH is

likely to serve as a redox sensor that is regulated by cellular differences in the free levels of NAD⁺ and NADH.

The ResDE two-component system and the redox regulator Fnr play important roles in *B. subtilis* during transition from aerobic to anaerobic growth (Nakano & Zuber, 2002). Our work demonstrates that ResDE does not appear to influence transcription of the *cydABCD* genes encoding the cytochrome *bd* terminal oxidase. In an Fnr mutant, the maximum level of β -galactosidase activity from the *cyd-lacZ* reporter was slightly reduced, but the timing of expression was not influenced. The recently described regulator YdiH seems to be a principal regulator of *cydABCD* expression in *B. subtilis*. Three putative binding sites for YdiH are located downstream from the transcription start site (Schau *et al.*, 2004), suggesting that repression of the *cyd* operon occurs via a transcriptional roadblock mechanism that inhibits transcript elongation. However, it is possible that additional factors control expression of the *cyd* genes. Our observation that the sequence upstream (up to position -79) of the -35 hexamer of the core *cyd* promoter significantly stimulates promoter activity suggests the involvement of an as-yet-unidentified transcription activator. Transcription activation by the putative regulator is likely to influence the level of transcription but not its timing, which is negatively controlled by YdiH.

The *ldh* *lctP* genes, which encode LDH and lactate permease, respectively, and the *ywcJ* gene, which encodes a predicted

Fig. 6. An RsbP-dependent signal transduction cascade controls induction of σ^B -dependent promoters in a YdiH mutant under oxygen starvation. The isogenic set of *B. subtilis* strains (A) LUW292, YdiH⁻; (B) LUW289, YdiH⁻ RsbU⁻; (C) LUW295, YdiH⁻ RsbP⁻; (D) LUW293, YdiH⁻ RsbU⁻ RsbP⁻; was grown and analysed as described in the legend to Fig. 7. Black dots represent genes that are transcribed from σ^B -dependent promoters. Circles and arrows indicate the positions of signals corresponding to *sigB* and *gsiB* transcripts, respectively. The *gsiB* gene is often used as a reporter for activation of the σ^B regulon as it is transcribed exclusively from a σ^B -dependent promoter (Brigulla *et al.*, 2003). The presented data are from a single experiment. The horizontal line marks Z score=3, corresponding to a confidence level of 95%. The intensity-dependent Z score measures the number of standard deviations of a particular data point from the mean. Data points above this line represent genes which are significantly upregulated in oxygen-depleted cells. The transcriptome datasets are available in supplementary data text file 6.

formate-nitrite transporter, show a pattern of expression that is very similar to that of the *cyd* genes. The present work demonstrates that YdiH acts as a negative regulator that coordinates the expression of these genes during the transition from aerobic to microaerophilic and finally to anaerobic growth. When aerobically and exponentially growing bacteria encounter depletion of oxygen in the medium, NADH reoxidation to NAD⁺ by the respiratory chain becomes less efficient. The oxygen limitation leads to induction of the *cyd* genes and production of cytochrome *bd*, an enzyme that is likely to have a higher affinity for O₂ but a lower energetic efficiency with respect to proton translocation compared to the cytochrome *aa₃* haem-copper type oxidase (Jünemann, 1997). To further increase NADH reoxidation, expression of the genes encoding

the fermentative NADH-linked LDH is induced. By the action of cytochrome *bd* and LDH, the NADH/NAD⁺ ratio is decreased, leading to repression of the expression of the corresponding genes by YdiH. From the available data and the sequence similarity between Rex and YdiH, we suggest that *ydiH* be renamed *rex*.

Our results show that a rapid shift to oxygen-starved conditions activates the σ^B -dependent general stress response in a strain lacking *ydiH*. Moreover, activation of σ^B under these circumstances is primarily dependent on the energy-stress sensor RsbP. Induction of σ^B by energy stress correlates with a drop in the intracellular levels of ATP. It is not known if ATP itself is the signal that communicates energy stress to RsbP (Hecker & Völker,

Fig. 7. Time-courses of β -galactosidase activity of *cyd-lacZ*, *ldh-lacZ* and *ywcJ-lacZ* fusions. Cells were grown at 37 °C at different aeration levels by setting the rotary shaker to 100 (A–C, J–L, G–I, P–R) or 400 r.p.m. (D–F, M–O). The growth medium was NSMPG (A–F, J–O) or NSMP (G–I, P–R). Sampling was as described in the legend to Fig. 1. Strains: LUW95 (A, D, G), LUW276 (B, E, H), LUW275 (C, F, I), LUW274 (J, M, P), LUW281 (K, N, Q) and LUW280 (L, O, R).

2001). However, sequence analysis suggests that the protein is composed of two domains, a PAS domain and a catalytic protein phosphatase 2C (PP2C)-like serine phosphatase domain. PAS domains are involved in several signalling

proteins, where they are used as cytosolic signalling modules. It has been suggested that the PAS domain of RsbP could directly sense a change in the redox potential of the cytoplasm (Vijay *et al.*, 2000). Further studies are needed

to understand why RsbP is activated in the YdiH (Rex) mutant strain.

Many factors and growth conditions contribute to changes in the ratio of NADH to NAD⁺. For instance, the observed reduction of *cyd* expression in the mutant lacking the truncated haemoglobin (YjbIH mutant) is most likely an indirect effect due to altered growth properties of the YjbIH mutant. During growth of the YjbIH mutant, the levels of dissolved oxygen in the medium did not decrease below 10 mbar of O₂. In contrast, growth of the wild-type resulted in very low O₂ tensions in the stationary growth phase. In a similar way, the involvement of the transcription regulator CcpA in *cyd* expression is most likely mediated via YdiH (Rex). Glucose repression of the *ctaCDEF* genes encoding the cytochrome *caa*₃ terminal oxidase is reported to be dependent on CcpA (Yoshida *et al.*, 2001). Analysis of membranes from a *ccpA* mutant (see Supplementary Fig. S1) indicates the presence of cytochrome *caa*₃. Increased levels of cytochrome *caa*₃ may contribute to NADH reoxidation, leading to YdiH (Rex)-mediated repression of the *cyd* genes.

Nitrate can be used as an electron acceptor for anaerobic growth of *B. subtilis*. It has previously been shown that nitrate represses transcription of *ldh* and *lctP* (Cruz Ramos *et al.*, 2000). The mechanism for this regulation has not been reported. However, it has been shown that expression of the respiratory nitrate reductase is needed to allow nitrate regulation (Cruz Ramos *et al.*, 2000). Nitrate respiration contributes to the oxidation of NADH. This strongly indicates that the observed nitrate repression of *ldh* *lctP* is mediated via YdiH (Rex) due to the nitrate-respiration-dependent NADH oxidation.

The role of Fnr in the regulation of *ldh* *lctP* expression is less clear. It is interesting to note that the potential binding site for Fnr, and also to some extent ResD, overlaps with the proposed YdiH (Rex)-binding sequence (Fig. 5B). Putative Fnr-binding sites are found in the *ycwJ* and the *ldh* *lctP* promoter regions, and a ResD-binding site overlaps with the middle YdiH (Rex) box in the *cyd* promoter (Fig. 5A). It remains to be investigated if Fnr and ResD can function as transcriptional repressors. The interplay between multiple regulatory systems could help to ensure the appropriate expression of cytochrome *bd* and LDH in response to changes in the oxygen tension and redox state of the cells.

ACKNOWLEDGEMENTS

We thank Lars Rutberg for valuable comments on the manuscript, Isabelle Martin-Verstraete, Dieter Jahn, Jörg Stülke, Kazuo Kobayashi, Yoshito Sadaie, Philippe Glaser and Marion Hulett for the gift of *B. subtilis* strains, and Markus Ringnér for advice on statistical analysis of microarray data. This work was supported by grants from Carl Tryggers Stiftelse and Emil och Wera Cornells Stiftelse.

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