A 23 911 bp region of the *Bacillus subtilis* genome comprising genes located upstream and downstream of the *lev* operon

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Within the framework of the European project to sequence the whole *Bacillus subtilis* 168 genome, a 23 911 bp long chromosomal DNA fragment located around 233° on the *B. subtilis* genetic map was cloned and sequenced. From the generated sequencing data and the results of the homology search, the primary structure of this region was determined. In addition to the whole *lev* operon, the region contains putative genes for an amino acid permease, two different alcohol dehydrogenases, a chitosanase, a protein belonging to the LysR family of transcriptional regulators, a protein related to the MerR transcriptional regulator, up to four proteins related to the product of the *spoF* gene, and genes coding for nine more inferred proteins of unknown function.

Keywords: *Bacillus subtilis*, genomic sequencing, *adh*, *cns*, *lysR* family

INTRODUCTION

The structural gene for the enzyme levanase of *Bacillus subtilis* (*sacC*) is the distal gene of a fructose-inducible operon containing five genes, *levD*, *levE*, *levF*, *levG* and *sacC* (Martín-Vestraete *et al.*, 1990); this operon is regulated at the transcriptional level by the product of the gene *levR*, which precedes the operon in the *B. subtilis* genome (Débarbouillé *et al.*, 1991). The region of the genome assigned to us as part of the European *B. subtilis* genome sequencing project contains *sacC*, the last gene of the *lev* operon, located at 233° on the *B. subtilis* chromosome (Martín *et al.*, 1987). We have chosen *sacC* and *levR* whose sequences are known (Martín *et al.*, 1987; Débarbouillé *et al.*, 1991) as the points to start sequencing in both directions, continuing downstream from *sacC* and upstream from *levR*. By walking in this way along the *B. subtilis* chromosome we were able to determine the sequence of a contiguous region of 23 911 bp that contains the entire *lev* operon and apparently possesses coding capacity for an additional 19 proteins.

METHODS

Bacterial strains and plasmids. *B. subtilis* 168 (*trpC2*; Anagnostopoulos *et al.*, 1993) was the source of genomic DNA and was provided by F. Kunst. *Escherichia coli* NM514 was used for the propagation of plasmid pRM1 (R. P. Mellado, unpublished), which contains the pUC18 polylinker (Norrander *et al.*, 1983) and is a derivative of pRM1*cat* (Parro *et al.*, 1991) from which the *cat* gene has been removed. *E. coli* XL1-Blue (Stratagene) was used to propagate pUC19. Plasmid pRL2 (a gift from G. Rapoport; Martín *et al.*, 1987) was used as a source of *B. subtilis* DNA.

Standard media and DNA manipulations. Procedures for the growth and manipulation of *E. coli* and *B. subtilis*, and general recombinant DNA manipulations were as described elsewhere (Sambrook *et al.*, 1989; Harwood & Cutting, 1990). DNA was isolated from *B. subtilis* or *E. coli* cultures grown in LB at 37 °C. When necessary, ampicillin (100 µg ml⁻¹) was added to the cultures.

Genome libraries. Genome libraries on plasmid pRM1 were made by cloning gel-purified 4–8 kb long chromosomal DNA fragments produced by digestion with *Hind*III. A *Sau*3A genome library made in bacteriophage lambda GEM11 (a gift from C. Harwood), containing clones harbouring 10–15 kb long inserts of *B. subtilis* chromosomal DNA, was also screened for recombinant phages containing sequences around the *lev* operon. When chromosome walking by cloning of the DNA fragments was not possible, large pieces of chromosomal

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DNA were generated by inverse long-range PCR (Barnes, 1994; Cheng *et al.*, 1994). Up to 10 µg purified chromosomal DNA was digested with *Kpn*I, ligated and then subjected to several cycles of PCR amplification (GeneAmpXL kit, Perkin Elmer) using the oligonucleotide primers K1 (AAGAACGGAGTCAGAAATTATGCG) and LR1 (CGATACTAGTTCAAACATGTACGG) from positions 13412 to 13389 and 13848 to 13871 of the sequenced DNA, respectively. The amplified DNA fragment was purified and its sequence (ACTAGTGAGTGCTACAATAACGGG) determined by extension of the K1 primer. This fragment was designated K3. Ten micrograms of chromosomal DNA was then subjected to PCR amplification using oligonucleotides K3 and LR1 as primers in 10 separate identical reactions. The resulting amplified material was pooled and used as substrate for direct sequencing or cloning. Pooling together the separated reactions minimized the probability of sequencing putative single point mutations arising during the amplification procedure. The pooled DNA was digested with DNase and resulting 0.5–0.8 kb long DNA fragments were purified and blunt-end inserted into plasmid pUC18 (Ready-To-Go, Pharmacia) through its unique *Sma*I site. The ligation mixture was used to transform *E. coli* XL1-Blue and the recombinant clones randomly sequenced.

DNA sequencing and computer analysis of sequences.

Sequencing of DNA was by the dideoxy chain-termination method (Sanger *et al.*, 1977), using the 7-deaza-dGTP reagent kit from US Biochemical as recommended by the manufacturer. Direct and reverse pUC19 primers (*f*mol kit, Promega) were used to randomly sequence the recombinant plasmids generated by the DNase treatment. Purified recombinant lambda or plasmid DNA was sequenced extending synthetic oligonucleotide primers (Isogen) using PCR (*f*mol kit, Promega). Alternatively, recombinant plasmid DNA was extracted from freshly grown colonies, in which case, cells were dispersed in 12 µl lysis solution (10 mM Tris/HCl pH 7.5, 1 mM EDTA, 50 µg proteinase K ml⁻¹) by vortexing, incubated for 15 min at 55 °C and the proteinase K inactivated by further incubation at 80 °C for an additional 15 min; the mixture was cooled by addition of 20 µl sterile water and incubated on ice for 1 min before centrifugation at 12000 g for 3 min at room temperature. Ten microlitres of the resulting supernatant was used for the cycle sequencing following the supplier's recommendations. In all three cases, the sequencing mixtures were heated at 94 °C for 2 min, then subjected to 30 cycles of incubation at 94 °C (1 min), 50 °C (1 min), and 72 °C (2 min) before allowing the reaction to cool down at 4 °C.

Sequence alignments were done using the program FRAGMENT ASSEMBLY from the UWGCG package and XDAP (Dear & Staden, 1991). The DNA sequence was analysed for ORFs using CODON PREFERENCE (UWGCG) and XNIP (Dear & Staden, 1991). Amino acid sequences were analysed using programs from the UWGCG package (version 8.0, September 1994). Protein sequences were compared with the PIR and SWISS-PROT protein databases using the FASTA algorithm (Pearson & Lipman, 1988).

RESULTS AND DISCUSSION

Cloning, physical mapping and sequencing of genomic DNA

Two synthetic oligonucleotides, GACGGGAGCGA-GTCAGGC and GCGATCGCCCTCGGCCTC, were used to synthesize the DNA between them by PCR

amplification, and the amplified region, encoding part of the carboxyl end of the *B. subtilis* levanase (*SacC*), was gel-purified to be used as a probe. Genomic DNA was digested with the restriction endonuclease *Hind*III and subjected to Southern blotting hybridization. DNA, 6–8 kb long, hybridizing to the probe, was purified and inserted into the plasmid pRM1 through its unique *Hind*III site and the recombinant plasmids propagated in *E. coli*. The pre-sized genome library was then screened with the same probe. The selected recombinant plasmid pSU1000 contained the whole *sacC* gene as expected, the approximately 6.5 kb long insert spanning over 3.8 kb downstream of *sacC* (Fig. 1). This 3.8 kb long region was entirely sequenced, extending 23–24 nt long oligonucleotide primers generated as the sequencing proceeded (Fig. 1). The rest of *B. subtilis* DNA contained in plasmid pRL2, including the *lev* operon, was sequenced in the same manner.

The 1.5 kb long *Pst*I–*Eco*RI fragment upstream of the *lev* operon (P–E, Fig. 1) was used as a probe to screen the lambda GEM11 library. Among the five selected recombinant phages only lambda L1003 contained DNA not previously cloned; the 888 bp long *Sau*3A–*Pst*I fragment (S–P, Fig. 1) situated within the 12 kb long insert of lambda L1003 was sequenced directly from the phage DNA.

Up to 90 different clones obtained from the inverse-long-range-PCR-amplified chromosomal DNA (*Kpn*I sites K1–K2, Fig. 1) were randomly sequenced; sequence alignment of the generated sequences allowed us to specifically derive 23–24-mer oligonucleotides that were used as primers to fill up the gaps and to link the different aligned contigs. This region was also sequenced using the BA4 × AA3 fragment generated by PCR as described by Bolotin *et al.* (1996). On average, both strands of the resulting DNA were sequenced between two and three times. The base composition of the sequenced DNA was 41.2 mol% G + C, close to the value of 42 mol% G + C of the *B. subtilis* genome as a whole (Sharp *et al.*, 1990).

Identification of ORFs

Putative ORFs were searched for in all possible reading frames. Twenty-five putative ORFs were identified based on the length of the ORF and the presence of a preceding RBS (Table 1). The initiation codons for two of them were TTG, the other 23 started with ATG. Suitable RBS sequences were found in all cases except *yraC*. ORFs *yraB*, *yraH*–*J*, *csn* and *yraN*–*O* are transcribed in the same sense as the *lev* operon, the rest of the ORFs are transcribed in the opposite orientation.

Table 2 shows putative *p*-independent transcription terminator sites identified behind ORFs other than those included in the *lev* operon. Putative promoter-like regions containing *B. subtilis* major RNA polymerase holoenzyme –10 consensus sequences were identified in front of *aapA* (TATAAT), *csn* (TATACT) and *yraN* (TATAAT), but the corresponding –35 consensus

Fig. 1. Physical map and ORF organization of the sequenced region. The upper part shows the ORFs contained in the 23911 bp long contig. Regions upstream and downstream of the *lev* operon are shown enlarged in the lower part. The arrows indicate the direction of transcription. S, *Sau3A*; P, *PstI*; E, *EcoRI*; H, *HindIII*; K, *KpnI*.

Table 1. ORFs in the 23911 bp sequence around the 233° region of the *B. subtilis* chromosome

ORF	Endpoints (bp)	Size (aa)	Translation start*
AapA	379–1755	459	tactGGAGGaaaataaaaTTG
LevR	2472–5285	938	atctaataAGGACaaaggATG
LevD	5478–5915	146	aggaAAGGAGcaatagatATG
LevE	5918–6403	162	aagagAGGAtGaattatgATG
LevF	6423–7229	269	aatcAAGGgGaTGagaatATG
LevG	7253–8077	275	gatgAGGgGGaagaaaaATG
SacC	8237–10267	677	agAGGAGcgaaggaacaaATG
YraA	10361–10822	154	ttattAGGAGGgttttttATG
AdhA	10956–12002	349	aagaAAGGAtagGttcgtATG
YraB	12510–12929	140	gatAgaGAGGTGaagatcATG
YraC	12915–13187	91	ccAacccttTaacatacaATG
YraD	13197–13493	99	tgAGGAGaTGaataaatcATG
YraE	13512–13706	65	aaaAcGGAGGTtacagaaATG
AdhB	13728–14861	378	cactGGAGGTtttacagcATG
YraF	14883–15248	122	aaAgGGAGGTaagcctctTTG
YraG	15269–15511	81	aAAGGAGGTtacatcaatATG
YraH	15760–16143	128	ttatAGGAGGcactcgatATG
YraI	16587–17018	144	tatgGGAGGgGagggggcATG
YraJ	17068–17427	120	ggaattgAAGaAaGaGggATG
YraK	17966–18652	229	gaAAGGAaGgtcctacaaATG
YraL	18792–19052	87	aatAAGGAGcTGgcagaaATG
Csn	19495–20325	277	ttagaAAGGgGaaGtgaaATG
YraM	20604–21704	367	aagAtGAGGatgatgacgATG
YraN	21849–22715	289	ctagaAAGGAaGatgaaaATG
YraO†	22833–23911	359	cattcttGGAGGTGtaatATG

* Putative initiation codons are shown in upper case bold. Nucleotides conserved from the consensus RBS sequence AAGGAGGTG are shown in upper case.

† Partial ORF.

sequences did not seem to be present. *yraH* and *yraI* have -35 consensus sequences (TTGACA and TTGAAA, respectively) in front of them, but they lack the corresponding -10 ones. Putative -35 regions likely to be recognized by other sigma factors appeared

in front of *yraL* and *yraM* (CTAAA, sigma D) or *adhA* (AGGTTT, sigma B), but again the corresponding -10 regions seemed to be missing. Transcription initiation and termination mapping experiments are needed to ascertain these possibilities.

Table 2. Putative ρ -independent transcription terminators

Inverted repeats are indicated in upper case.

ORF	Putative ρ -independent transcription terminator
AapA	cgaaaATAaaAAAAAGACAAGGGAactcacTCCCTTGTCTTTTTaTATgtgtc
YraA	ataacAAGAAGGCACAGACTgttcgGGTCTGTGCCTTTTTtaaaa
AdhA	cggtcAATCTTCCTGttATTCTatgaatagAGAGTtatTAGGAGGGTTtttta
YraB	tgctGTGaAGAGGGCAGGTCAaAAACGAaggattTTGTTTcTGGCCTGTTTTCTcCATcaaga
YraD	gatttGCTGAGGAACAGTatTGattggcCAaACTGTTTCCTCAGCttcaa
AdhB	cgcctTAcACGAAGTGCCTTGAGgttCAGGaaatgaCTGcaTTTAAGaCACTTTGTttgac
YraH	aggttGCGGACACTGaACttgCAGCatttacGCTGatttGTaTGGTGTCCGTttttg
YraJ	gacgaACCATAACCAACaacaataGTTGGTATGGTttttg
YraK	gggagCTtTTTCcttATcGTTgCGCgaacgcctaGCGgAACaATtGGAAAcAGaatggg
YraL	aacggAAGCACTGATaaaaataATCAGTGCTTtttat
Csn	gatgaACTCAAtctAAAAGATACCAAtgatgTTGGTATCTTTTtatTTGcAGTaaaaa
YraM	atgtaGATGGACTGTGCAaAAGCcTCCCCctTCCATCgttaataGATGGAttGAatgcGCcTTcTTGCACAGaTCCA- TTgtaat
YraN	atttaAAGTTAtgATAGAatTTTTTTTgaacacagTAAAAAAaTTTATtTAACTTattac

Function of the newly identified ORFs

The amino acid sequence of the putative product of each ORF was compared with the non-redundant protein databases and the results are summarized in Table 3. Apart from the ORFs included in the *lev* operon and its regulator *levR* (Martín *et al.*, 1987; Débarbouillé *et al.*, 1991), four more ORFs, *aapA*, *adhA*, *adhB* and *csn*, were found to have putative products similar to known enzymes and were named after them. Four other ORFs, *yraD*–*G*, showed homology to the two proteins encoded by the *spoF* gene (Cutting *et al.*, 1991) and another one, *yraB*, to the regulatory protein product of *merR* (Fleischmann *et al.*, 1995). The putative polypeptide YraN seemed to be a member of the LysR family of transcriptional regulators and the product of *yraO* showed homology to the hypothetical one reported for the *B. subtilis* gene *yxiQ* (Yoshida *et al.*, 1995; Wolf *et al.*, 1995). YraA showed homology to a hypothetical 20.3 kDa protein product of the *yhbO* gene from *E. coli*, as registered in the databases. No homologous polypeptides were found for the remaining seven putative ORF products.

aapA encodes a polypeptide of 459 aa and its sequence showed homology with many amino acid permeases from *E. coli*, *B. subtilis* and *Salmonella typhimurium* with identity percentages ranging from 31.4 to 51.8; it showed the highest identity with the D-serine/D-alanine/glycine transporter from *E. coli* (Burland *et al.*, 1995). The 349 aa long putative polypeptide encoded by *adhA* showed a high degree of homology (61.8% identity) with a *Mycobacterium bovis* NADP-dependent alcohol dehydrogenase, belonging to the family of zinc-containing alcohol dehydrogenases, which is widely distributed among the eukaryotic and prokaryotic kingdoms; all the residues considered to be needed for enzyme activity (Stélandre *et al.*, 1992) are also conserved. The putative protein encoded by *adhB* is 378 aa

long and showed homology to another group of alcohol dehydrogenases (identity percentages between 27.2 and 30 in most cases), the NAD-dependent alcohol dehydrogenases, which are also widely distributed in nature. It showed the greatest homology with the enzyme from baltic cod *Gadus callarias* (Danielsson *et al.*, 1992). The *csn* putative product, 277 aa long, showed homology (36.4% identity) to the chitosanase product of the *Streptomyces* sp. *csn* gene (Masson *et al.*, 1994).

spoF encodes a 160 aa long polypeptide that is cleaved to generate two spore coat proteins: one, with a molecular size of 5 kDa, containing residues 5–76 and another one of 8 kDa, containing the rest of the original molecule (Cutting *et al.*, 1991). Two sets of two ORFs, *yraG*–*yraF* and *yraE*–*yraD*, flank *adhB* (see Fig. 1); *yraG* and *yraE* encode polypeptides homologous to the 5 kDa protein (30.7% and 40% identity, respectively), and *yraF* and *yraD* encode polypeptides homologous to the 8 kDa protein (39.9% and 40% identity, respectively). *yraG* and *yraE* are homologous but not identical proteins, as is also the case for *yraD* and *yraF*. The chromosomal arrangement is such that the ORF for the 5 kDa-protein-like product always precedes the ORF for the 8 kDa-protein-like product, as if this particular order has to be preserved. Perhaps the 5 kDa and 8 kDa proteins head two families of tightly linked proteins that need to be coordinately synthesized. The existence of an operon comprising *yraG*–*yraF*–*adhB*–*yraE*–*yraD*–*yraC* cannot be ruled out from the sequencing data, although *yraC* does not seem to have a consensus RBS preceding the translation start codon (Table 1).

The ORF *yraN* precedes *yraO*, these being the two last ORFs at one of the ends of the 23911 bp long contig. The putative protein encoded by *yraN* is 289 aa long and showed homology to proteins included in the prokaryotic LysR family of transcriptional regulators, particularly with the *B. subtilis* hypothetical Ywbl

Table 3. Comparison of predicted ORF products with protein database entries

ORF*	Homologous protein	Database entry	FASTA-optimized score†
AapA	Amino acid permease	P39312	1427
LevR	Transcriptional regulatory protein	P23914	4446
LevD	Fructose-specific IIA component	P26379	684
LevE	Fructose-specific IIB component	P26380	729
LevF	Fructose-specific IIC component	P26381	1254
LevG	Fructose-specific IID component	P26382	1427
SacC	Levanase precursor	P05656	3418
YraA	Hypothetical 20·3 kDa protein	P45470	255
AdhA	NADP-dependent alcohol dehydrogenase	P31975	1116
YraB	Mercury resistance operon regulatory protein	P22896	182
YraC	Unknown		
YraD	Spore coat protein F	P23261	172
YraE	Spore coat protein F	P23261	105
AdhB	NAD-dependent alcohol dehydrogenase	P26325	313
YraF	Spore coat protein F	P23261	137
YraG	Spore coat protein F	P23261	111
YraH	Unknown		
YraI	Unknown		
YraJ	Unknown		
YraK	Unknown		
YraL	Unknown		
Csn	Chitosanase precursor	P33665	379
YraM	Unknown		
YraN	LysR family of transcriptional regulators	P39592	232
YraO	YxiQ, hypothetical 45·5 kDa protein	P42308	1053

*ORFs with a FASTA-optimized score below 150 were considered not to show homology with proteins contained in databases and therefore as previously unknown, with the exception of YraE, YraF and YraG (see text for details)

†Only the entry having the highest score is given.

protein (25·1% identity, Glaser *et al.*, 1993); YraO is an incomplete, 359 aa long polypeptide, showing homology (52·6% identity) with the product of the *B. subtilis* *yxiQ* gene, a hypothetical integral membrane protein with a molecular size of 45·5 kDa (Yoshida *et al.*, 1995; Wolf *et al.*, 1995); the potential transmembrane domains are conserved along the 359 aa overlap between the two proteins.

The putative product of *yraB* is a 140 aa long polypeptide that showed homology with the mercury resistance operon regulatory protein MerR from *Haemophilus influenzae*, *Thiobacillus ferrooxidans*, *Bacillus* sp. and *Staphylococcus aureus* (29·0%, 29·6%, 26·5% and 29·7% identity, respectively) and others; it showed the greatest homology with the hypothetical MerR (YhdM) from *H. influenzae* (Fleischmann *et al.*, 1995). The YraB potential helix–turn–helix motif (residues 3–22) seemed to be conserved among all the proteins showing homology to MerR; although all the essential residues for the metal-binding properties of the regulatory protein seemed to be missing in both YraB and YhdM, and *yraB* does not precede the *mer* operon as reported in another *Bacillus* sp. (Wang *et al.*, 1989).

The rest of the putative products encoded by ORFs in the sequenced region showed little homology with the proteins contained in the databases and were considered to be unknown proteins.

The average codon usage for all the ORFs does not differ much from the codon usage in any individual ORF (data not shown). However, the relative synonymous codon usage values, both average and individual, are very different from those of the highly expressed genes of *B. subtilis* (Sharp *et al.*, 1990). This could suggest that the genes included in the sequenced region of the genome belong either to the class of *B. subtilis* genes that are constitutively expressed at a low level, or to a class which, if ever expressed, are tightly regulated.

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