Cloning and nucleotide sequencing of a 15 kb region of the *Bacillus subtilis* genome containing the *iol* operon

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Within the framework of an international project on the sequencing of the whole *Bacillus subtilis* genome, a 15 kb chromosome segment, which contains the *iol* operon involved in inositol utilization, has been cloned and sequenced. This region (14974 bp) contains 12 complete open reading frames (ORFs; genes) and two partial ones; the seventh gene (*E83G*) is the *idh* gene encoding inositol dehydrogenase. All the genes identified are transcribed in the same direction as that of the movement of the replication fork. A homology search for their products deduced from the 12 complete ORFs revealed that eight of them exhibit significant homology to known proteins such as fructokinase, acetolactate synthase, fructose-1,6-bisphosphate aldolase (*B. subtilis*), and PhoB and FtsE proteins (*Escherichia coli*). It also implied that two genes (*B65D* and *B65E*) might encode a set of two-component regulatory proteins and that the *B65F* gene might encode a protein belonging to the ATP-binding cassette (ABC) family. Based on the features of the nucleotide sequence determined and the results of the homology search, the primary structure of the *iol* operon is predicted.

Keywords: *Bacillus subtilis*, genomic sequencing, inositol operon, OmpR subfamily, ABC family

INTRODUCTION

Recent developments of new techniques for DNA sequencing and analysis, including improvement of DNA sequencers and microcomputer software, allows us to sequence the whole genome of an organism. But even now, a vast amount of work is required to determine the entire nucleotide sequence of a relatively small bacterial genome, so it is important to develop a productive and systematic strategy. The systematic sequencing of a small genome will provide much productive information and allow the technical developments required to determine the entire sequence of the large genomes of higher eukaryotes.

Recently, several sequencing projects on small genomes have been started. Among them, the *Bacillus subtilis*

sequencing project is an international cooperative effort between Japan and Europe, the aim of which is to complete the entire sequence within the next five years. We are participating in this project, and are responsible for the sequencing of an approximately 180 kb chromosomal region between *sacS* (333°) and *gnt* (344°), in which the *iol* operon involved in *myo*-inositol utilization is located (Fujita & Fujita, 1983).

In this first communication, we describe our sequencing strategy for this project and report the determination of the nucleotide sequence of a 14974 bp region containing the *idh* gene (Fujita *et al.*, 1991) which belongs to the *iol* operon.

METHODS

Bacterial strains, phages, plasmids and growth media. *B. subtilis* strain 1A1 (Marburg 168; *trpC2*) was chosen as our target strain. *Escherichia coli* strains P2392, a P2 lysogen of strain LE392 [*hsdR514*($r_k^- m_k^+$) *supE44 supF58 lacY* or $\Delta(lacIZY)6 galT22 galK2 metB1 trpR55 mcrA^- mcrB^+$], and XL1-Blue [*endA1 hsdR17*($r_k^- m_k^+$) *supE44 thi-1 recA1 gyrA96 relA1 \Delta(lac) F'[proAB^+ lacI^q lacZ\Delta M15 Tn10Tet^r]*] were used as cloning hosts for λ DASH II and M13mp19 phage clones, respectively.

Abbreviations: aa, amino acid; ABC, ATP-binding cassette; GP, translated protein sequence from NCBI-GenBank Nucleotide Sequence Database; SD, Shine-Dalgarno; SP, Swiss Prot Protein Sequence Database.

The EMBL/GenBank/DDJB accession number for the nucleotide sequence (14974 bp) reported in this paper is D14399.

Fig. 1. Cloning, physical mapping and sequencing of the *iol* region. λ DASH II clones (H2.65-1D and EM11 and 14), the inserts of which are indicated by thick solid lines, were obtained using DNA fragments as probes (solid boxes; H2.65 and EM). A physical map of the cloned 24 kb region with respect to *EcoRI* (E), *BamHI* (B), *HindIII* (H), *SalI* (S) and *NotI* (N) is shown, in which the *idh* gene and the insert of plasmid pNEXT40 (Itaya & Tanaka, 1991) are located. This map was constructed by connecting the physical maps of the inserts of the three λ DASH II clones, which were obtained as described in Methods. The direction of the location of *gnt* and *sacS* is indicated at the top of the figure. The sequenced regions are indicated as stippled boxes [M76431, accession no. in the EMBL/GenBank/DBJ Nucleotide Sequence Database (Fujita *et al.*, 1991); E83 and B65]. In the lower part of the figure, the deduced 14 ORFs in the connected sequenced region (14974 bp) are shown; the partial ORFs are shown in parentheses. The direction of movement of the replication fork is indicated at the bottom of the figure.

Strain 1A1, strains P2392, XL1-Blue and λ DASH II, and M13mp19 were obtained from the *Bacillus* Genetic Stock Center (Ohio) via M. Itaya (Mitsubishi Kasei Institute of Life Sciences, Tokyo), Stratagene (La Jolla), and Takara Shuzo (Kyoto), respectively.

Plasmids pIOL02 and pNEXT40 were used for preparation of DNA probes for hybridization. pIOL02 carries the *idh* gene (Fujita *et al.*, 1991). pNEXT40 is one of the *NotI* linking clones located at 4082 kb on the *B. subtilis* chromosome (Itaya & Tanaka, 1991); it was supplied by M. Itaya.

NZY broth [5 g NaCl l⁻¹, 2 g MgSO₄·H₂O l⁻¹, 5 g yeast extract l⁻¹ and 10 g NZ amine (Wako, Osaka) l⁻¹; pH 7.5] and L-broth (10 g tryptone l⁻¹, 5 g yeast extract l⁻¹ and 5 g NaCl l⁻¹) were used for growth of the *E. coli* strains to propagate λ DASH II and M13mp19 phage clones, respectively.

Preparation of a *B. subtilis* genomic library. A *B. subtilis* genomic library using λ DASH II was prepared according to the instruction manual from Stratagene. The chromosomal DNA of *B. subtilis* strain 1A1 was isolated and partially digested with *Sau3AI* to yield DNA fragments of approximately 15 kb. Fragments ranging from 10 to 20 kb were fractionated by agarose gel electrophoresis, dephosphorylated with calf intestinal alkaline phosphatase (Boehringer Mannheim), and ligated to the arms of λ DASH II cleaved with *BamHI*. The

ligation mixture was incubated with an *in vitro* packaging mixture (GIGAPACK II XL, Stratagene), and the resulting phage particles were propagated on strain P2392 for Spi/P2 selection. Nineteen thousand independent phage clones, from several packaging experiments, were amplified by plaque formation to yield a *B. subtilis* genomic library (2.5×10^7 p.f.u. ml⁻¹).

Isolation of λ DASH II clones containing a DNA insert carrying the *iol* locus and construction of a physical map of the DNA inserts. Using an aliquot of the *B. subtilis* genomic library, approximately 12000 plaques were formed in total and subjected to plaque hybridization (Benton & Davis, 1977) with an appropriate DNA fragment as probe. *EcoRI* digests of phage DNA prepared from positive recombinant clones were subjected to agarose gel electrophoresis and Southern hybridization (Southern, 1975) to confirm that the DNA inserts actually carried the *iol* locus. A physical map of the region cloned in λ DASH II was constructed according to the method described by Kohara *et al.* (1987), as follows. Recombinant phage DNA was partially digested with *EcoRI*, *HindIII*, *SalI*, *BamHI* or *NotI*, and subjected to gel electrophoresis using 0.4% (w/v) agarose (0.7 V cm⁻¹, 40 h), and Southern hybridization using the 9 kb arm of λ DASH II as probe.

Shotgun cloning for random sequencing. λ DASH II recombinant phage DNA carrying the *iol* locus was isolated,

digested with an appropriate restriction enzyme, and approximately 5 µg of the DNA fragment to be sequenced (5–10 kb in length) was purified. This DNA fragment was self-ligated and partially digested with DNaseI (Takara Shuzo) in the presence of Mn²⁺ to generate random fragments 400–1000 bp in length. The resulting fragments were blunt-ended by stepwise treatment with T4 DNA polymerase and the Klenow fragment of DNA polymerase I. The blunt-ended fragments were ligated with M13mp19 replicative-form DNA, which had been digested with *Sma*I, extensively dephosphorylated with calf intestine alkaline phosphatase, and transferred to competent cells of strain X1.1-Blue for the screening of white plaques on plates [H-broth (10 g tryptone l⁻¹ and 5 g NaCl l⁻¹)] containing 5-bromo-4-chloro-3-indolyl β-D-galactoside and isopropyl β-D-thiogalactopyranoside. Hundreds of white plaques were randomly selected, picked up with toothpicks, and phage particles were suspended in 50 µl L-broth and incubated at 37 °C for 5 h in 96-well microtitre plates to propagate the recombinant phage clones. The insert DNA of each recombinant M13 phage was amplified by 30 cycles of PCR (20 s at 95 °C, 20 s at 50 °C and 1 min at 72 °C) with Vent DNA polymerase (New England Biolabs), using a pair of primers, M4 and RV (1 µM each; Takara Shuzo), and the propagated-phage suspensions directly as template solutions with a Perkin Elmer GeneAmp PCR System 9600 (Takara Shuzo). The amplified products were analysed by agarose gel electrophoresis, and the recombinant phages carrying insert DNA ranging from 400 to 1000 bp were selected for preparation of templates in subsequent DNA sequencing reactions.

DNA sequencing. Sequencing reactions were performed using a Dye-Primer Cycle Sequencing Kit with –21M13 Dye Primer (Applied Biosystems). Template DNAs were insert DNAs of the recombinant M13 phages selected, which were amplified by 30 cycles of PCR (30 s at 95 °C, 30 s at 50 °C and 2 min at 72 °C), with AmpliTaq DNA polymerase (Perkin-Elmer Cetus), using M4 and RV (0.05 µM each) as primers with a Program Temp Control System PC-700 (Astec, Fukuoka), and purified by precipitation with polyethylene glycol. The reaction products were applied to and sequenced with a DNA sequencer (373A, Applied Biosystems). Sequences determined randomly were compiled and automatically connected using a personal computer with the GENETYX-MAC 6.0.1 and GENETYX-MAC/ATSQ programs (Software Development Co., Tokyo), respectively; to sequence a 10 kb fragment as a target, for example, 150 independent sequences on average were needed to form several islands of connected sequences. The insert DNAs of M13 recombinant clones located at the extremities of each of these islands were sequenced with an anti-sense primer (M13 Reverse Dye Primer, Applied Biosystems). Sequences newly and previously determined were all automatically connected to shorten the gaps between the islands. The islands were physically aligned on a constructed physical map of the target DNA fragment by Southern hybridization using the DNA inserts belonging to different islands as probes. The gap regions neighbouring islands were amplified by PCR, using *B. subtilis* chromosomal DNA as a template, and synthetic primer pairs each consisting of an approximately 20 base sequence complementary to one of the extremities of a neighbouring island and the same nucleotide sequence as one of each set of different dye-primers (18–20 bases). New sequences were obtained using the PCR products as templates, which were automatically connected with all those previously determined. The last process was repeated until sequencing of the target fragment was completed.

Computer analysis. The DNA sequences determined were

analysed with the GENETYX-MAC 6.0.1 program. A homology search for putative gene products was performed with the FASTA program (Pearson & Lipman, 1988) using an NEC PC-9801 personal computer with GENETYX-CD Version 22.0.0 (Software Development Co.) and a GenomeNet FASTA server (Supercomputer Laboratory, Institute for Chemical Research, Kyoto University) (e-mail: fasta@genome.ad.jp).

RESULTS AND DISCUSSION

Cloning, physical mapping and sequencing of the *iol* region

Screening of the *B. subtilis* genomic library constructed using λ DASH II was started with a *Hind*III 2.65 kb fragment (H2.65) derived from plasmid pIOL02 (Fujita *et al.*, 1991) as a probe (Fig. 1), which contained the *idb* gene, the nucleotide sequence of which was reported previously (Fujita *et al.*, 1991). With this probe, a λ DASH II recombinant clone, H2.65-1D, carrying the *Hind*III 2.65 kb fragment was isolated. Using a 3 kb *Eco*RI–*Mlu*I fragment (EM) derived from DNA of recombinant clone H2.65-1D as another probe (Fig. 1), λ DASH II clones EM11 and EM14 were obtained. Fig. 1 also shows the physical map of the 24 kb region containing the *idb* gene with respect to *Eco*RI, *Bam*HI, *Hind*III, *Sal*I and *Not*I. This map was constructed by connecting those of the insert DNAs of the three recombinant clones, produced by the method of Kohara *et al.* (1987). The *Hind*III insert in plasmid pNEXT40, which is one of the *Not*I linking clones located at 4082 kb on the *B. subtilis* chromosome (Itaya & Tanaka, 1991), hybridized with the insert of the EM11 clone (data not shown), suggesting that the *Not*I location of the EM11 insert DNA is at 4082 kb. The *idb*

Table 1. General features of ORFs found in the 14974 bp *iol* region sequenced

ORF	Endpoints (bp)	Size (aa)	Translation start*
<i>E83A</i> †	1	1253	
<i>E83B</i>	1331	2143	271 gAGGAGGgcttctctatg
<i>E83C</i>	2170	3144	325 gAGGAGtgGctgtttacgatg
<i>E83D</i>	3351	5090	580 cAGGAtGcaggccatttg
<i>E83E</i>	5110	6000	297 ggGGAGGcaccatagatg
<i>E83F</i>	6018	7334	439 tgGGgaaTGaatatg
<i>E83G (idb)</i>	7360	8391	344 AAGGAGtgGctgtcaatg
<i>B65A</i>	8414	9280	289 gAGGAGtgaaatgatg
<i>B65B</i>	9369	10202	278 AtGGGGGaGcttgccatg
<i>B65C</i>	10226	11095	290 AAGGAGGgGtgaaatgatg
<i>B65D</i>	11207	11893	229 cgGGAGtgGttcagtttg
<i>B65E</i>	11893	12867	325 gctGAGGgcgcagtcgatg
<i>B65F</i>	13014	13784	257 AAGGAGGattttcatg
<i>B65G</i> †	13762	14974	ggGGAAacGcaaatg

* Putative initiation codons are shown in bold face. The same nucleotides as those of a consensus SD sequence of AAGGAGGTG are shown in upper case.

† Partial ORF.

Table 2. Results of comparison of eight ORFs with protein databases

ORF*	Homologous protein	Database: entry†	FASTA optimized score
E83C	Fructokinase		
	(<i>Solanum tuberosum</i>)	GP:STFRUCM_1	312
	(<i>Vibrio alginolyticus</i>)	PIR:jQ0782	243
	(<i>Klebsiella pneumoniae</i>)	PIR:s18523	237
	(<i>Salmonella typhimurium</i>)	PIR:s18524	235
E83D	Acetolactate synthase		
	(<i>Saccharomyces cerevisiae</i>)	PIR:a23808	334
	(<i>Spirulina platensis</i>)	PIR:b44857	302
	(<i>Lactococcus lactis</i>)	SP:ILVB_LACLA	298
	(<i>Bacillus subtilis</i>)	GP:BACILNB_1	295
	Acetohydroxy acid synthase		
	(<i>Corynebacterium glutamicum</i>)	GP:CORAIA_2	282
	Pyruvate dehydrogenase		
	(<i>Escherichia coli</i>)	PIR:a23648	198
E83F	Quinolone resistant protein NorA		
	(<i>Staphylococcus aureus</i>)	PIR:a37838	235
	Methylenomycin A resistant protein MMR		
	(<i>Bacillus subtilis</i>)	PIR:s22742	175
	D1 transporter		
	(<i>Leishmania donovani</i>)	GP:LEID1TRA_1	161
	Arabinose transporter		
	(<i>Escherichia coli</i>)	PIR:a28075	158
E83G (<i>idh</i>)	Streptomycin biosynthesis protein StrI		
	(<i>Streptomyces griseus</i>)	PIR:s17779	285
B65C	Tsr protein		
	(<i>Bacillus subtilis</i>)	PIR:c32354	599
	Fructose biphosphate aldolase I		
	(<i>Rhodobacter sphaeroides</i>)	PIR:b40767	221
	Fructose biphosphate aldolase II		
	(<i>Rhodobacter sphaeroides</i>)	PIR:d41080	195
B65D	Phosphate regulon regulatory protein PhoB		
	(<i>Shigella dysenteriae</i>)	PIR:a44753	383
	(<i>Escherichia coli</i>)	PIR:rgcfb	382
	Transcriptional regulatory protein OmpR		
	(<i>Escherichia coli</i>)	PIR:a25024	351
B65E	Sensor protein BvgC		
	(<i>Bordetella pertussis</i>)	SP:BVGCBORPE	178
	ChvG protein		
	(<i>Agrobacterium tumefaciens</i>)	GP:ATUCHVG_1	169
B65F	Cell division protein FtsE		
	(<i>Escherichia coli</i>)	PIR:ceecfe	375
	ABC protein		
	(<i>Escherichia coli</i>)	SP:ABC_ECOLI	365
	Sulfate permease CysA		
	(<i>Synechococcus</i> sp.)	PIR:grycs7	364

* ORFs which possess known proteins exhibiting significant homology (FASTA optimized score > 175) are listed.

† Database entries of GP are followed by an underlined space and an ordinal number for each of the coding sequences.

11910 11920 11930 11940 11950 11960 11970 11980 11990 12000
 GTTCTCCGGTCTCATGTCAGTGTGATTCTGCTTTTCTCCTTCAAGGGCTGTTTGTCTTCTTTTATTACTGGTTTGCGGGTCTTCATTCTTTTCTCAC
 F L R S H A V L I L L F L L Q G L F V F F Y Y W F A G L H S F S H

 12010 12020 12030 12040 12050 12060 12070 12080 12090 12100
 TTGTTTACATATTGGGCGTGCAGCTTCTTATTTTGGCGGGCTATCTTGCTATCGCTGGTACAAAGACCGCGCGTGTATCATTGGCTCAGCTCGGGGC
 L F Y I L G V Q L L I L A G Y L A Y R W Y K D R G V Y H W L S S G Q

 12110 12120 12130 12140 12150 12160 12170 12180 12190 12200
 AGGAAGGGACGGATATCCCGTATCTCGGTCTTCAGTGTCTGTTTCAAGCTGTATGAAAAGCAGATGGAGCTGATCCGGCTGCAGCACCAGAAGCTCCA
 E G T D I P Y L G S S V F C S E L Y E K Q M E L I R L Q H Q K L H

 12210 12220 12230 12240 12250 12260 12270 12280 12290 12300
 TGAGACCGAAGCCAAGCTTGACGCGCGGGTGACGTATATGAATCAATGGGTGCACCAAGTAAAGACGCCGCTGTCGGTCATTAATTTAATCATTCAAGAA
 E T E A K L D A R V T Y M N Q W V **H** Q V K T P L S V I N L I I Q E

 12310 12320 12330 12340 12350 12360 12370 12380 12390 12400
 GAGGATGAGCCCGTTTGAACAAATCAAAAAAGAAGTCCGGCAGATTGAATTCGGGCTGGAGACGCTTCTTTATTCTTCAAGGCTCGACCTGTTTGAAC
 E D E P V F E Q I K K E V R Q I E F G L E T L L Y S S R L D L F E R

 12410 12420 12430 12440 12450 12460 12470 12480 12490 12500
 GGGATTTAAATCGAAGCGGTTCCCTGTCCGAGCTTCTCCAATCAGTTATTCAAAGCTATAAACGATTTTTATTCAATATCGCGTTTATCCGAAAAT
 D F K I E A V S L S E L L Q S V I Q S Y K R F F I Q Y R V Y P K M

 12510 12520 12530 12540 12550 12560 12570 12580 12590 12600
 GAACGTTTGCGATGATCATCAGATTTACACCGATGCCAAATGGCTCAAAATTGCGATTGGCCAAGTGGTCACAAATGCGGTCAAGTATTACGCGGGCAAG
 N V C D D **H** Q I Y T D A K W L K F A I G Q V V T **N** A V K Y S A G K

 12610 12620 12630 12640 12650 12660 12670 12680 12690 12700
 AGTGACAGACTTGAGCTGAATGTCTTCTGCGATGAAGACAGGACGGTGTCTGAAGTCAAAGATTACGGTGTGCGGATTCCCTCACAAGACATCAAGCGGG
 S D R L E L N V F C D E D R T V L E V K **D** Y **G** V **G** I P S Q D I K R V

 12710 12720 12730 12740 12750 12760 12770 12780 12790 12800
 TGTTTGACCCTTATTACACGGGTGAAAAACGGGCGGCGTTTCCAAGAATCTACGGGAATCGGCCTCCATCTCGTCAAAGAAATTACAGATAAGCTCAATCA
 F D P Y Y T G E N G R R F Q E S T G I G L H L V K E I T D K L N H

 12810 12820 12830 12840 12850 12860 12870 12880 12890 12900
 TACGGTGGACATCAGCTCGTCTCCCGGTGAAGGACATCAGTTGATTTTCAATTTCTTACAAAAATGTAAGGCTGCGTTAAGAAAACAACATAGCCCCGT
 T V D I S S S P **G** E **G** T S V R F S F L T K M *

 12910 12920 12930 12940 12950 12960 12970 12980 12990 13000
 TTGTTTCCCTTGATAACATGGATTTATGTCAGGGGATTTTATGTTGAACGTCCCGGCACTACATGAAAGCAGCCAGATCAACAAGAATAAAACCAT
 Terminator-like sequence

B65F

13010 13020 13030 13040 13050 13060 13070 13080 13090 13100
AAGGAGGATTTTCATGCGGAATATGCTTGAAGTCAAACATATAAATAAAACCTATAAAGGACAAGTGTCTATCAGGCCTTAAAGCAAATTTCA
 SD M A N M L E V K H I N K T Y K G Q V S Y Q A L K Q I S F S

 13110 13120 13130 13140 13150 13160 13170 13180 13190 13200
 ATAGAAGAAGCGAGTTCACGGCGGTGATGGGCGCGTCCGGGTCCGGAACAAACGCTTTTGAATATCATTTCACGATTGACCGTCCAGATTACAGCG
 I E E G E F T A **V** M **G** P S **G** S **G** K T T L L N I **I** S T I D R P D S G D
 ATP-binding cassette

Fig. 2. For legend see facing page.

13210	13220	13230	13240	13250	13260	13270	13280	13290	13300
ATATCCTGATTAAACGGAGAAAACCCGCATCGCCTGAAGCGGACAAAGCTCGCTCATTTCGCCGCGCAAGCAGCTCGGATTGTGTTTCAGGATTTC AACCT									
I L I N G E N P H R L K R T K L A H F R R K Q L G F V F Q D F N L									
13310	13320	13330	13340	13350	13360	13370	13380	13390	13400
TCTGGATACGCTGACGATCGGTGAAAATATCATGCTGCCGTTAACGCTTGA AAAAAGAACGCCGCTCTGTAATGGAAGAAAACTGCACGGCATCGCGGCA									
L D T L T I G E N I M L P L T L E K E A P S V M E E K L H G I A A									
13410	13420	13430	13440	13450	13460	13470	13480	13490	13500
AAGCTCGGTATCGAAAACCTGCTTAAACAAGCGGACGTTTGAAGTATCCGGAGGCCAGCGCCAGCGGGCCGCATCGCAAGAGCGGTCATTTCATAAGCCGT									
K L G I E N L L N K R T F E V S G G Q R Q R A A I A R A V I H K P S									
Transmitter									
13510	13520	13530	13540	13550	13560	13570	13580	13590	13600
CACTCATCTGGCCGATGAACCAACTGGAACCTTGATTCCAAAGCGACAAAAGACGTCATGGAACGTTGCAGAGCCTGAATCGGGATGATCATGTCTAC									
L I L A D E P T G N L D S K A T K D V M E T L Q S L N R D D H V T									
13610	13620	13630	13640	13650	13660	13670	13680	13690	13700
GGCTTTGATGGTGACCCACGATCCGGTTTCGGCAAGCTACTGCCGCCGCGTCATCTTTATAAAAGACGTCGTACTGTTTAAATGAGATTTCACGCGGGGAA									
A L M V T H D P V S A S Y C R R V I F I K D V V L F N E I Y R G E									
B65G									
13710	13720	13730	13740	13750	13760	13770	13780	13790	13800
AACCGCCAAGTATTTTATGAACAAATTCGATGTGCTGTCTATGCTGGGGGGAACGCAAAATGACCTTCTTCAGTTTCGCTTATAAAAACGTCACGAGA									
N R Q V F Y E Q I L D V L S M L G G N A N D L S S V R L *									
SD					M T F L Q F A Y K N V T R				

Fig. 2. Specific features found in the nucleotide sequence of the *iol* region. Two sequence segments derived from the sequence (1 to 14974 nt) obtained by sequencing both strands independently are presented; (a) is that of the region upstream of the *E83D* gene (3181 to 3380 nt) and (b) is that containing the *B65C* (partial ORF, 11001 to 11095 nt), *B65D* (11207 to 11893 nt), *B65E* (11893 to 12867 nt), *B65F* (13014 to 13784 nt), and *B65G* (partial ORF, 13762 to 13800 nt) genes. The SD sequences for these ORFs are underlined and labelled SD. The aa sequences of their products are shown as single letters with the respective termination codons indicated by asterisks. The '–35' (3198 to 3203 nt) and '–10' (3221 to 3226 nt) regions of the putative promoter-like sequence for the *iol* operon are located upstream of the *E83D* gene (doubly underlined). Two palindromic sequences within the respective *p*-independent terminator-like sequences (11108 to 11129 and 12906 to 12938 nt) are indicated by underlining; one might be a transcription terminator for the putative *iol* operon, which is located downstream of the *B65C* gene, and the other might be that for the *B65D* and *B65E* genes comprising the two-component regulatory system, which is located between the *B65E* and *B65F* genes. The two aspartate (D) and one lysine (K) residues boxed in the aa sequence of the *B65D* protein are those highly conserved in most response regulators of the two-component regulatory systems of bacteria (Stock *et al.*, 1989). The boxed residues in the aa sequence of the *B65E* protein are the highly-conserved residues among histidine protein kinases of the two-component regulatory systems (Stock *et al.*, 1989). In the aa sequence of the *B65F* protein, the residues in the ATP-binding domain are boxed and labelled as the 'ATP-binding cassette' (Higgins *et al.*, 1990); those of another conserved region are boxed and labelled as the 'Transmitter', proposed to be involved in the transmission of an ATP-dependent conformational change to the membrane-associated sites (Gill *et al.*, 1986).

gene is located within the 30N fragment between 4029 and 4082 kb (M. Itaya, personal communication). The locations of the 2.65 kb *Hind*III fragment and the *Hind*III insert in plasmid pNEXT40 were determined by analysing their Southern hybridization patterns using restriction enzyme fragments of the inserts of these recombinant clones (data not shown). With reference to the physical map of the *B. subtilis* chromosome (Itaya & Tanaka, 1991), these results clearly indicate that the direction of *oriC* is from the *idh* gene to this *Not*I site, as shown for the direction of *gnt*, *sacS* and the movement of the replication fork (Fig. 1).

The nucleotide sequence of the 1.6 kb region containing

the *idh* gene has been reported previously (Fujita *et al.*, 1991). To extend the sequences of the adjacent regions to the *idh* gene, an 8.3 kb *Eco*RI fragment (E83) of the EM11 clone and a 6.5 kb *Bam*HI fragment (B65) of the EM14 clone (Fig. 1), which are located upstream and downstream of the *idh* gene, respectively, were isolated and sequenced systematically. Connection of the nucleotide sequences of the 8.3 kb and 6.5 kb fragments newly determined and the 1.6 kb region resulted in a 14974 bp nucleotide sequence for the region containing the *idh* gene. Analysis of the 14974 bp sequence revealed 12 complete open reading frames (ORFs; genes) [*E83B*, *E83C*, *E83D*, *E83E*, *E83F*, *E83G* (*idh*), *B65A*, *B65B*, *B65C*, *B65D*, *B65E* and *B65F*] and two partial ones

(*E83A* and *B65G*) (Fig. 1). The endpoints of these genes in the sequence and their features, such as the molecular size and the putative SD sequence and initiation codon, are shown in Table 1. (The *B65F* gene overlaps the *B65G* gene by 23 bp.)

Eleven out of the 13 genes, except the first partial ORF-*E83A*, were predicted to use ATG as initiation codon, though the *E83D* and *B65D* genes might use TTG, suggesting that the genes in the *iol* region might utilize ATG more preferentially than those in other regions (personal communications from other Japanese groups participating in the *B. subtilis* genomic sequencing project; Glaser *et al.*, 1993). More interesting is that the direction of the transcription of all these genes is the same as that of the movement of the replication fork. This is often observed in regions located relatively close to the chromosome origin like the *iol* region (Zeigler & Dean, 1990; Glaser *et al.*, 1993).

Functions of the newly identified genes

Among the putative gene products encoded by the 12 complete ORFs, only the product of the *E83G* gene was previously identified as the *idh* gene encoding inositol dehydrogenase (Fujita *et al.*, 1991). As a prerequisite for determining the functions of these putative gene products, we searched for homology with known protein sequences present in sequence libraries, using the FASTA program to obtain the FASTA optimized score (Pearson & Lipman, 1988). (The last homology search was performed in November 1993.) Eight out of the 12 gene products exhibited significant homology to known proteins in the PIR, SP and GP Protein Databases (FASTA optimized score > 175) (Table 2). However, four products (*E83B*, *E83E*, *B65A* and *B65B*) seemed to be unique, exhibiting no significant homology to known proteins (FASTA optimized score < 100).

We discuss below the functions of the putative gene products exhibiting significant homology to known proteins. The *E83C* protein showed homology to various fructokinases including the *Solanum tuberosum* L. enzyme (Smith *et al.*, 1993), implying that this protein might be a fructokinase. The *E83D* protein showed homology to many acetolactate synthases, including a yeast enzyme (Falco *et al.*, 1985), acetohydroxy acid synthase, and the pyruvate dehydrogenase of *E. coli*, implying that this protein might have a functional relationship with these enzymes. The *E83F* protein exhibited homology to the methylenomycin A resistant protein and the NorA protein of *Staphylococcus aureus* participating in the active efflux pump for hydrophilic quinolones (Yoshida *et al.*, 1990), and to D1 and arabinose transporter proteins. This suggests that it might be involved in a membrane-associated transport system. The gene product of *E83G* (*idh*), which was identified as inositol dehydrogenase of *B. subtilis* (Fujita *et al.*, 1991), showed high homology to the StrI protein of *Streptomyces griseus*, which was predicted to catalyse one of the numerous steps dependent on dehydrogenases or epimerases in the biosynthesis of hexose-

derived moieties of streptomycin (Mansouri & Piepersberg, 1991). The *B65C* protein exhibited homology to many fructose-bisphosphate aldolases including the *Rhodobacter sphaeroides* enzyme (Gibson *et al.*, 1991), implying that this protein might have a similar function to this enzyme. Moreover, this protein exhibited very high homology to the Tsr protein of *B. subtilis*, which is required for RNA synthesis (Trach *et al.*, 1988). Recently, Mitchell *et al.* (1992) found that this *tsr* gene was a portion of the *fla1* gene encoding the fructose-1,6-bisphosphate aldolase. Moreover, Sukhodolets *et al.* (1983) reported that the *B. subtilis dra* gene encoding deoxyriboaldolase was located at 339°, which is very close to the *B65C* gene. It remains to be examined whether or not the *dra* gene is the *B65C* gene.

The *B65D* protein showed high homology to many proteins belonging to the OmpR subfamily of response regulator proteins of the two-component regulatory systems of bacteria (Stock *et al.*, 1989), such as PhoB proteins including that of *E. coli* (Makino *et al.*, 1986), and the OmpR protein of *E. coli* (Comeau *et al.*, 1985). When the sequence of this protein was aligned with those of these proteins, two aspartate residues and a lysine residue highly conserved in most response regulators were found in it, as shown in Fig. 2. These two aspartate residues are considered to be phosphorylated by the phosphohistidine of the corresponding histidine protein kinase (Stock *et al.*, 1989). Moreover, the *B65E* protein exhibited homology to histidine protein kinases of the two-component regulatory systems (Stock *et al.*, 1989) including the sensor protein BvgC of *Bordetella pertussis* (Arico *et al.*, 1989). Alignment of the sequence of this protein with those of these histidine protein kinases revealed histidine residues in *B65E* which might be phosphorylated and aa residues conserved in these histidine kinases (Fig. 2). Interestingly, the *B65D* gene is adjacent to *B65E*, so their gene products very likely constitute a set of the two-component regulatory system (a response regulator protein and its histidine protein kinase). The function of this set remains to be determined.

The *B65F* protein showed very high homology to many proteins belonging to the ABC family (Higgins *et al.*, 1990), such as the ABC protein of *E. coli*, the sulfate permease of *Synechococcus* sp. and the FtsE protein of *E. coli* (Gill *et al.*, 1986), suggesting that it might function as an ATP-dependent transporter for an unidentified substrate. A domain of the *B65F* protein for the ATP-binding cassette was predicted by alignment of the sequence of this protein with those of transporter proteins belonging to the ABC family, as shown in Fig. 2 (Higgins *et al.*, 1990). Fig. 2 also shows another conserved domain of this family of proteins, which was recently proposed to be involved in transmission of an ATP-dependent conformational change to membrane-associated sites (Gibbs *et al.*, 1992). The *B65F* gene is located immediately downstream of the *B65D* and *B65E* genes comprising the two-component regulatory system, although a stem-loop structure which might possibly function as transcription terminator exists between the *B65E* and *B65F* genes (Fig.

2). It would be very interesting to elucidate the actual functions of these three genes and their mutual relation.

Structure of the *iol* operon

The 15 kb region, the nucleotide sequence of which was determined, is believed to contain the *iol* operon, involved in *myo*-inositol catabolism in *B. subtilis*, because not only the *idb* gene encoding inositol dehydrogenase but also many *iol* mutations rendering the cells unable to grow on *myo*-inositol are located in this region (Y. Fujita, unpublished observation). Although the pathway for *myo*-inositol degradation is not well studied in *B. subtilis*, an outline of this pathway in *Aerobacter aerogenes* (*Klebsiella pneumoniae*) has been elucidated (Berman & Magasanik, 1966; Anderson & Magasanik, 1971). *myo*-Inositol transported into cells is dehydrogenated by inositol dehydrogenase. The resulting 2-keto-*myo*-inositol is dehydrated to D-2,3-diketo-4-deoxy-*epi*-inositol, which is then hydrated to 2-deoxy-5-keto-D-gluconic acid, the first open-chain intermediate. After this intermediate has been phosphorylated by a kinase, an aldolase catalyses the cleavage of the kinase product (2-deoxy-5-keto-D-gluconic acid 6-phosphate) to yield dihydroxyacetone phosphate and malonic semialdehyde. The malonic semialdehyde is metabolized by malonic semialdehyde oxidative decarboxylase, which catalyses the conversion of malonic semialdehyde, co-enzyme A and NAD⁺ to acetyl-CoA, CO₂ and NADH + H⁺.

To identify the genes of the *iol* operon, we first searched for promoter-like sequences recognized by the major σ^A factor of *B. subtilis* using a personal computer, and found in the 14974 bp sequence a plausible promoter-like sequence only immediately upstream of the *E83D* gene, the '−35' and '−10' regions of which are shown in Fig. 2. We also looked for a *p*-independent terminator which might be located downstream of the *E83G* (*idb*) gene encoding inositol dehydrogenase, and found a terminator-like palindromic sequence followed by a stretch of T residues downstream of the *B65C* gene, the location of which is shown in Fig. 2. These results also imply that the *B65D* and *B65E* genes might be transcribed by RNA polymerase associated with another σ factor. Thus, it was considered, on the basis of the location of *idb*, and the above transcriptional promoter- and terminator-like sequences, that the *iol* operon might consist of seven genes (*E83D*, *E83E*, *E83F*, *idb*, *B65A*, *B65B* and *B65C*). Moreover, assuming that the pathway for *myo*-inositol degradation in *B. subtilis* may be similar to that in *Aerobacter aerogenes*, we could hypothesize as to the functions of some of these genes from the results of the homology search for their products. The third protein, *E83F*, showed homology to various membrane-associated transporter proteins, as described above, which suggests that it might be involved in the transport of *myo*-inositol. The transported *myo*-inositol could be dehydrogenated by the inositol dehydrogenase encoded by *idb*. The resulting open-chain phosphorylated compound, 2-deoxy-5-keto-D-gluconic acid 6-phosphate, might be cleaved by the *B65C* protein, which showed homology to various

aldolases. The resulting malonic semialdehyde might be finally metabolized to acetyl-CoA, CO₂ and NADH + H⁺ by the *E83D* protein, because this protein showed homology not only to various acetolactate synthases but also to pyruvate dehydrogenase, an enzyme similar to malonic semialdehyde oxidative decarboxylase.

The expression of the *idb* gene is induced by the addition of *myo*-inositol to the medium (Fujita & Freese, 1981), suggesting that expression of the *iol* operon may be induced by *myo*-inositol. Thus, there should be a transcriptional regulator for the *iol* operon. However, no gene encoding a protein showing homology to DNA-binding regulatory proteins was found within the putative *iol* operon, suggesting that the gene for the transcriptional regulator should be located elsewhere. Experimental analysis of the organization and transcription of the *iol* operon is in progress.

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