The 52°–55° segment of the *Bacillus subtilis* chromosome: a region devoted to purine uptake and metabolism, and containing the genes *cotA*, *gabP* and *guaA* and the *pur* gene cluster within a 34 960 bp nucleotide sequence

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Within the framework of the international project for sequencing the entire *Bacillus subtilis* genome, we have determined the complete sequence of the segment flanking the *purE-D* gene cluster (55°) as far as *cotA* (52°). This segment (34 960 bp) contains, as well as 12 genes already identified as part of the *pur* operon, 17 putative ORFs and one partial one. Two of them (*gabP* and *guaA*) are known *B. subtilis* genes. The gene product of *cotA* (formerly *pig*) shows significant similarity to oxidoreductases (phenoxazine synthase and bilirubin oxidase). The putative products of ORFs *yeaB* (Czd protein), *yeaC* (MoxR), *yebA* (CNG-channel and cGMP-channel proteins from eukaryotes), *yebB* (hypothetical 32.9 kDa protein of *Escherichia coli*), *yecA* (amino acid permease) and *yecB* (adenine deaminase) were similar to proteins in data banks.

Keywords: *Bacillus subtilis*, genome sequencing, *cotA*, *guaA*, *purF*

INTRODUCTION

The chromosomal region of *Bacillus subtilis* 168 extending from 52° to 55°, between *cotA* and the *pur* gene cluster (Anagnostopoulos *et al.*, 1993), has been shown to contain many genes involved in purine uptake and metabolism. Most of these have been found clustered within a polycistronic operon (Ebbole & Zalkin, 1987; Saxild & Nygaard, 1987, 1988; Mäntsälä & Zalkin, 1992). The region lies about 700 kb downstream from the zero position of the *B. subtilis* physical map and approximately 670 kb downstream from the replication origin *oriC* (Itaya & Tanaka, 1991). As part of the cooperative *B. subtilis* genome project between European and Japanese laboratories to determine the entire nucleotide sequence of the *B. subtilis* genome, we have sequenced and analysed 34 960 bp of the region flanking the *pur* gene cluster. The sequence and the genetic organization of the 13 160 bp region containing the *pur* gene cluster have been reported by Ebbole & Zalkin (1987).

In this contribution, we summarize the main features of 18 ORFs identified 5' and 3' from the *pur* operon. In

addition, we present an updated physical map of the 60 kb region which has been assigned to our laboratory by the EU genome sequencing project.

METHODS

Bacterial strains, plasmids and phages. *B. subtilis* strain 1 A1 (Marburg 168, *trpC2*) was chosen as our target strain. *Escherichia coli* strains DH5 α (*supE44* Δ *lacU169* ϕ 80d *lacZ* Δ M15 *hsdR17* *recA1* *gyrA96* *thi-1* *relA1* F⁻), TP611 (*cya* *pcn*, Glaser *et al.*, 1993) and JM101 (*recA1* *supE44* *endA1* *hsdR17* *gyrA96* *relA1* *thi-1* Δ (*lac-proAB*) F' [*traD36* *proAB* *lacI*^q *lacZ* Δ M15]) were used as cloning hosts for plasmids rescued from *B. subtilis* and M13mp18 phage clones, respectively.

Plasmids pDE64 and pDE81, containing fragments of the *pur* operon, were obtained from H. Zalkin (Ebbole & Zalkin, 1987); pNEXT38 containing the *NotI*-763 flanking region was obtained from M. Itaya (Itaya & Tanaka, 1991) and plasmids pGA13 and pGA14, containing parts of the *guaA* gene region, were kindly given by P. Mäntsälä (Mäntsälä & Zalkin, 1992). Plasmid pDIA5304, used as an integration vector, was constructed by P. Glaser (Glaser *et al.*, 1993).

Cloning and DNA techniques. DNA from phages and plasmids was isolated by standard procedures according to Sambrook *et al.* (1989). Minimal salts medium used for DNA transformation of *B. subtilis* was that of Anagnostopoulos & Spizizen (1961). Plasmid rescue was done according to Glaser *et al.* (1993). Long Range PCR (LR PCR) was carried out using the Expand Long

Abbreviation: LR PCR, Long Range PCR.

The GenBank accession numbers for the sequences reported in this paper are U51115 (HUB5), J02732 (HUB1) and J02732 (HUB2A).

Template PCR System (Boehringer Mannheim), according to the supplier's instructions.

Sequencing and data handling. LR PCR fragments and plasmid inserts were digested with DNase I to obtain fragments of 0.5–2 kb. The fragments were blunt-end repaired with T4 DNA polymerase (30 min) and Klenow polymerase (15 min) and ligated into dephosphorylated M13mp18 or pUC18 vectors. The clones were sequenced using an automatic sequencing system (Autoread Sequencing Kit, Pharmacia). Sequencing data were evaluated using the software packages GENE SKIPPER V1.2 (EMBL) and PCGENE V6.85 (IntelliGenetics). Homology searches were performed using programs BLAST and FASTA. Program BLASTX was especially valuable for detecting frame shifts in ORFs. Remaining gaps between the contigs were filled by primer walking.

RESULTS AND DISCUSSION

Cloning and mapping of the sequence flanking the *pur* operon

A 517 bp DNA fragment from the *purE* gene was amplified, cloned into pDIA5304 and integrated into *B. subtilis* 168. Chromosomal DNA isolated from four transformants was restricted with *EcoRI*. The religated DNA was transformed into *E. coli* TP611. Only one chloramphenicol-resistant clone containing plasmid pSP303 was obtained. Sequencing of the 5.5 kb insert of pSP303 revealed two ORFs (*yebB* and *yebC*) encoding putative membrane-associated proteins. ORF *yebC* was found to be interrupted at amino acid 213 by an IS1A element. The corresponding DNA sequence obtained by LR PCR amplification of *B. subtilis* chromosomal DNA does not contain the IS element, suggesting that the insertion was due to cloning in *E. coli*. Further cloning of the region 5' from *pur* was carried out by plasmid rescue using a 1 kb *HincII* fragment derived from plasmid pGA14 (Mäntsälä & Zalkin, 1992). The resulting plasmid, pSP312, contained the *gua* sequence and regions 5' to *guaA*. The LR PCR fragments containing *cotA*, *gabP* and

guaA were also used for sequencing of a 15 kb region flanking the 5' end of the *pur* operon. The distances determined between the known genetic markers *cotA* (52°), *guaA* (54°) and *purE* (55°) were shorter than expected: *cotA*-9 kb-*guaA*-6 kb-*purE* (Figs 1 and 2).

To clone the *B. subtilis* DNA downstream from the *pur* gene cluster, the 1.6 kb *HindIII*-*NotI* fragment obtained from plasmid pNEXT38 was used for the plasmid rescue strategy described above. A DNA fragment covering 15.5 kb of the region between *NotI*-763 and the 3' end of the *pur* gene cluster was obtained (pRS205). The gap between the *pur* sequence and pRS205 was closed by applying LR PCR using oligonucleotide primers *pur*Rout and 205P1. The amplified 4.6 kb fragment (PCR209) was found to cover the remaining gap between *pur* and pRS205, suggesting that the distance between the *purE*-*D* gene cluster and *NotI*-763 is 20 kb.

According to the physical map, the *NotI*-763 restriction site lies 68 kb downstream of *cotA* which has been located 695 kb from the zero position of the *B. subtilis* chromosome (Itaya & Tanaka, 1991). Our data indicate that the distance between *cotA* and *NotI*-763 is only 49.5 kb. We also cloned a LR PCR fragment with the 3' flanking region of *NotI*-763 containing the *opuE* gene. *opuE* stands for osmoprotectant uptake and it encodes the OpuE protein (493 aa, *M_r* 53400) which complements *E. coli* strains with defects in the proline transport system (von Blohm *et al.*, 1996). The whole region cloned in this study has the gene order *cotA*-*gabP*-*guaA*-*purE*-*D*-*opuE* and consists of 60.5 kb (Fig. 1).

Identification of ORFs and gene organization in the 34960 bp region

Evaluation of putative coding regions revealed 18 ORFs, besides the 12 previously reported genes of the *pur* cluster. One of them, *yeaA*, is partial. Each ORF is preceded by a

Fig. 1. Genetic map of the *cotA*-*opuE* region located at 705–765 kb according to the physical map of Itaya & Tanaka (1991). The cloned region, 60 kb, is indicated by a thin line. The sequenced area is hatched. The sequence covering the *purE*-*D* gene cluster (Ebböle & Zalkin, 1987) is indicated by a box. Plasmids and LR PCR fragments obtained in this study are also indicated.

Fig. 2. ORFs identified within the *cotA*–*purE-D* region covering 34960 bp. Their positions according to the genetic map (Anagnostopoulos *et al.*, 1993), the physical map (Itaya & Tanaka, 1991) and the direction of transcription are also indicated. Factor-dependent termination signals are shown by vertical arrows. Genes previously identified are in bold.

Table 1. Putative ORFs in the 52°–55° region of the *B. subtilis* chromosome

ORF*	Endpoints (nt)	G + C (%)	Codon usage (GC-bias)	SD consensus sequence (upper case) and initiation codon (bold)
<i>yeaA</i>	1 < 798	39.1	41.7	AcAAAGGAGcTtttt ATG
<i>cotA</i>	899 < 2431	47.6	48.1	AgAtaAGGacag ATG
<i>gabP</i>	2586 < 3992	47.0	53.4	AAAGGAGGTaActt ATG
<i>yeaB</i>	4390 > 5259	45.4	45.7	AGGAGGTtttgt ATG
<i>yeaC</i>	5415 > 6734	48.1	48.6	AGGAGGaaaata ATG
<i>yeaD</i>	6761 > 7570	48.9	45.9	AAAcGAGcaTGAcg ATG
<i>yebA</i>	7770 > 9734	45.4	47.6	AGGaTGAg GTG
<i>guaA</i>	9970 > 11509	45.8	46.9	gtGAGGTGAcaacc ATG
<i>yebB</i>	11973 > 13200	46.0	46.2	GAAGGGAGtcaAatagct TTG
<i>yebC</i>	13415 > 14216	43.0	39.2	GAtGGAGtTGcTgac ATG
<i>yebD</i>	14377 > 14596	40.2	40.3	AAGGAGGaTGgac ATG
<i>yebE</i>	14765 > 15001	40.0	51.6	AGGgGGaTGAataa ATG
<i>yebF</i>	15046 > 15309	47.1	52.8	GGcGcTtggctatt ATG
<i>yebG</i>	15312 > 15507	39.9	45.5	AGGAGaattaaagaata ATG
<i>purE</i>	15832 > 16319	50.1	47.9	AGAAGGtGGgGAacaga ATG
<i>purK</i>	16314 > 17450	47.1	49.4	AGAAAGcAGTGATCagc TTG
<i>purB</i>	17449 > 18742	45.6	45.6	GGAGGacaagcaga ATG
<i>purC</i>	18818 > 19540	49.1	46.4	AGGAGGccttcc GTG
<i>purQ</i>	19787 > 20467	47.4	47.6	AGGAGGTagtgcaca GTG
<i>purL</i>	20454 > 22679	48.3	45.4	GGAGGGAAactc ATG
<i>purF</i>	22658 > 24085	49.7	51.9	GAAAAAAGctatccc ATG
<i>purM</i>	24189 > 25226	49.2	48.1	AAAGGAGTGAatagg ATG
<i>purN</i>	25226 > 25810	47.3	47.4	GGcGGTGcggcactt ATG
<i>purH(J)</i>	25810 > 27345	48.1	48.3	aGAGGTGAaaaggc ATG
<i>purD</i>	27364 > 28629	48.7	46.0	AAGGGGaTGAaaacgac GTG
<i>yecA</i>	29236 > 30483	48.5	49.5	AGAgAGGgGGcatc ATG
<i>yecB</i>	31188 > 32470	46.7	51.6	AaActGGcGGaatat GTG
<i>yecC</i>	32498 > 32989	47.7	50.9	AAaGAGGct ATG
<i>yecD</i>	33063 > 33638	41.2	37.8	GGAGcTGccCgattat ATG

* ORFs with known function are shown in bold letters.

putative Shine–Dalgarno (SD) sequence (McLaughlin *et al.*, 1981) 5–20 bp upstream from the putative start codon ATG, TTG or GTG. The shortest ORF is *yebG* with 66 codons, the longest is *yebA* with 655 codons. Considerable variation in codon usage (GC-bias) ranging from 37.8% (*yecD*) to 51.6% (*yecB*) was detected (Table 1). With the

exception of the first three (*yeaA*, *cotA*, *gabP*) the ORFs are directed towards the terminus of replication. Factor-independent terminator structures 3' to the translation stop signal(s) were found after *cotA* (ΔG –13.6 kcal), *gabP* (ΔG –4.8 kcal), *yeaB*–*yeaC*–*yeaD*–*yebA*–*guaA* (ΔG –15.0 kcal), *yebB* (ΔG –23.4 kcal), *yebC* (ΔG –21.6 kcal),

Table 2. Similarity of the predicted ORF products with entries of the databases

ORF	size (aa)	Database accession no.*	Function/similarity†	BLAST score	Match (%)‡
<i>cotA</i>	510	PIR: P07788	<i>Spore coat protein A (B. subtilis)</i>	196	100 (37)
		GB: U04283	Phenoxazine synthase (<i>Streptomyces antibioticus</i>)	182	35.1 (313)
		PIR: B48521	Bilirubin oxidase (<i>Myrothecium verrucaria</i>)	146	28.3 (431)
		SP: P26648	SufI protein (<i>E. coli</i>)	177	27.3 (300)
<i>gabP</i>	469	SP: P46349	<i>γ-Amino butyrate permease (B. subtilis)</i>	2530	100 (469)
		SP: P25527	<i>γ-Amino butyrate permease (E. coli)</i>	737	48.6 (444)
<i>yeaB</i>	290	SP: P46348	<i>Hypothetical protein YDXT (B. subtilis)</i>	1367	100 (276)
		SP: P32159	Hypothetical 32.9 kDa protein YIIP (<i>E. coli</i>)	146	22.0 (195)
		SP: P13512	Cation efflux system Czd protein (<i>Alcaligenes eutrophus</i>)	101	22.0 (99)
<i>yeaC</i>	320	GB: D64003	Hypothetical protein SYCSLLE_70 (<i>Synechocystis</i> sp.)	353	50.1 (280)
		PIR: S43189	Hypothetical protein_2 (<i>Pseudomonas aeruginosa</i>)	308	54.1 (170)
		SP: P29901	MoxR protein (<i>Paracoccus denitrificans</i>)	130	25.7 (272)
<i>yebA</i>	655	GB: X89598	Photoreceptor CNG-channel protein (<i>Gallus gallus</i>)	91	36.0 (44)
		SP: P29974	Photoreceptor cGMP-channel protein (<i>Mus musculus</i>)	90	38.0 (44)
<i>guaA</i>	513	SP: P29727	<i>GMP synthetase (B. subtilis)</i>	2658	98.0 (513)
		SP: P44335	GMP synthetase (<i>Haemophilus influenzae</i>)	557	54.5 (489)
<i>yebB</i>	440	SP: P31440	Hypothetical 49.9 kDa protein YICO (<i>E. coli</i>)	377	41.2 (440)
		SP: P31466	Hypothetical 46.9 kDa protein YIEG (<i>E. coli</i>)	357	40.2 (440)
<i>purE</i>	162		<i>AIR carboxylase I (B. subtilis)</i>		
<i>purK</i>	379		<i>AIR carboxylase II (B. subtilis)</i>		
<i>purB</i>	431		<i>Adenylosuccinate lyase (B. subtilis)</i>		
<i>purC</i>	241		<i>Phosphoribosylaminoimidazole-succinocarboxamide synthetase (B. subtilis)</i>		
<i>purQ</i>	227		<i>FGAM synthetase I (B. subtilis)</i>		
<i>purL</i>	742		<i>FGAM synthetase II (B. subtilis)</i>		
<i>purF</i>	476		<i>Amidophosphoribosyltransferase (B. subtilis)</i>		
<i>purM</i>	346		<i>AIR synthetase (B. subtilis)</i>		
<i>purN</i>	195		<i>GAR formyltransferase (B. subtilis)</i>		
<i>purH(J)</i>	512		<i>Phosphoribosylaminoimidazole-carboxamide formyltransferase/IMP cyclohydrolase</i>		
<i>purD</i>	422		<i>GAR synthetase</i>		
<i>yecA</i>	433	SP: P25527	<i>γ-Amino butyrate permease (E. coli)</i>	119	21.8 (308)
		SP: P40812	L-Asparagine permease (<i>Salmonella typhimurium</i>)	115	20.1 (334)
<i>yecB</i>	427	SP: P39761	Adenine deaminase (<i>B. subtilis</i>)	118	22.2 (436)

* SP, SwissProt; GB, GenBank.

† Identical proteins (100% match) are shown in italics.

‡ The number of amino acids over which the percentage match was determined is shown in parentheses.

yebD-yebE-yebF-yebG (ΔG -13.8 kcal), the *pur* gene cluster (ΔG -21.6 kcal), *yecA* (ΔG -20.2 kcal) and *yecB* (ΔG -16.8 kcal). These results indicate that both polycistronic and monocistronic transcripts are present (Fig. 2).

Similarity analysis of the putative gene products to known proteins

Results of similarity analyses are summarized in Table 2. Besides the known genes of the *pur* operon, genes *cotA*, *gabP* and *guaA* were identified within the 34 960 bp contig. Five putative gene products show homology to other known or hypothetical proteins in the data banks.

***cotA*.** *cotA*, mapped at 52°, encodes the 65 kDa coat polypeptide which is identical to the Pig protein. The molecular mass deduced from the putative amino acid sequence is 58 kDa. The *CotA* protein transcribed by $E\sigma^K$ is responsible for the brown colour of sporulating cells but is not required for spore formation (Donovan *et al.*, 1987). The high homology to phenoxazinone synthase, bilirubin oxidase (EC 1.3.3.5) and the manganese oxidizing protein from *Leptothrix discophora* suggests a putative function as an oxidase. Homology was also found to the *sufI* gene product of *E. coli* which acts as a suppressor of the *ftsI* allele involved in cell division and chromosome segregation.

gabP. This gene encodes γ -aminobutyrate permease as a monocistronic transcript. The primary sequence suggests the presence of 12 transmembrane helices (program RAOARGOS). The 48.6% identity with GabP from *Escherichia coli* confirms the function as GABA permease.

Two of the ORFs (*yebB* and *yebA*) identified between *gabP* and *guaA* encode proteins containing transmembrane-spanning helices (Table 2).

guaA. *guaA*, mapped at 54°, encodes GMP synthetase (Mäntsälä & Zalkin, 1992). The enzyme is involved in purine nucleotide biosynthesis (XMP $\rightarrow$ GMP). Re-sequencing revealed six differences in the nucleotide sequence which lead to changes in amino acid sequence. Most striking are H₁₆₈ $\rightarrow$ Y, S₁₉₆ $\rightarrow$ C and R₂₆₂ $\rightarrow$ A, since these substitutions improve the similarity to other GMP synthetases.

purE-D. This region, mapped at 55°, contains a cluster of 12 genes that encode enzymes for *de novo* synthesis of purine nucleotides. The cluster is a single transcription unit and is organized into three groups of overlapping genes followed by the last gene: *purEKB-purC(orf)QLF-purMNH(J)-purD* (Ebbole & Zalkin, 1987).

Mutants resistant to 8-azaguanine (*phnG* mutants) appear to be defective in hypoxanthine and guanine transport (Saxild & Nygaard, 1987). They were mapped between *guaA* and the *pur* gene cluster (Saxild & Nygaard, 1988). Two of the ORFs identified in this region encoded membrane proteins which might be involved in transport of these compounds: *yebB* encodes a transmembrane protein with five membrane-spanning helices; and *yebC* encodes a transmembrane protein with one membrane-spanning and one membrane-associated helix motif. However, since similarity to proteins with known function was not detectable (Table 2), complementation analysis with DNA fragments spanning *guaA* and *purE* will be necessary to identify the *phnG* locus in this area.

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