Sequence analysis of the *Bacillus subtilis* chromosome region between the *serA* and *kdg* loci cloned in a yeast artificial chromosome

Alexei Sorokin, Vasco Azevedo,[†] Emmanuelle Zumstein,[‡]
Nathalie Galleron, S. Dusko Ehrlich and Pascale Serror

Author for correspondence: Alexei Sorokin. Tel: +33 1 34652533. Fax: +33 1 34652521.
e-mail: sorokine@biotec.jouy.inra.fr

Laboratoire de Génétique
Microbienne, Institut
National de la Recherche
Agronomique, Domaine de
Vilvert, 78352 Jouy en Josas
cedex, France

The standard strategies of genome sequencing based on λ -vector or cosmid libraries are only partially applicable to AT-rich Gram-positive bacteria because of the problem of instability of their chromosomal DNA in heterologous hosts like *Escherichia coli*. One complete collection of ordered clones known for such bacteria is that of *Bacillus subtilis*, established by using yeast artificial chromosomes (YACs). This paper reports the results of the direct use of one of the YAC clones from the above collection for the sequencing of the region cloned in it. The strategy applied consisted of the following: (i) construction of M13 banks of the partially purified YAC DNA and sequencing of 800 M13 clones chosen at random; (ii) directed selection of M13 clones to sequence by using marginal contig fragments as hybridization probes; (iii) direct sequencing of joining PCR fragments obtained by combinations of primers corresponding to the ends of representative contigs. The complete 104 109 bp insert sequence of this YAC clone was thus established. The strategy used allowed us to avoid resequencing the two largest, previously sequenced, contigs (13 695 and 20 303 bp) of the YAC insert. We propose that the strategy used can be applied to the sequencing of the whole bacterial genome without intermediate cloning, as well as for larger inserts of eukaryotic origin cloned in YACs. Sequencing of the insert of the YAC clone 15-6B allowed us to establish the contiguous sequence of 127 kb from *spolIA* to *kdg*. The organization of the newly determined region is presented. Of the 138 ORFs identified in the *spolIA-kdg* region, 57 have no clear putative function from their homology to proteins in the databases.

Keywords: *Bacillus subtilis* genome, yeast artificial chromosomes, combinatorial PCR

INTRODUCTION

The genomes of the bacteria *Haemophilus influenzae* and *Mycoplasma genitalium* have been completely sequenced (Fleishmann *et al.*, 1995; Fraser *et al.*, 1995) and several others, including *Escherichia coli* (Daniels *et al.*, 1992),

Bacillus subtilis (Kunst *et al.*, 1995; Ogasawara *et al.*, 1995) and *Mycobacterium leprae* (Honoré *et al.*, 1993), are currently being sequenced. The sequence of the genome of the lower eukaryote *Saccharomyces cerevisiae* is also expected to be available soon (Williams, 1995). In most cases, the sequencing strategies used have been based on the construction of ordered λ - or cosmid-based vector collections in *E. coli* (Daniels *et al.*, 1992; Glaser *et al.*, 1993; Honoré *et al.*, 1993; Oliver *et al.*, 1992; Dujon *et al.*, 1994; Wilson *et al.*, 1994). The sequencing of the *H. influenzae* genome by a random approach was made feasible by the construction and use of a λ -based library (Fleishmann *et al.*, 1995). In the case of the *B. subtilis* genome, and probably other AT-rich genomes of Gram-positive bacteria, many segments cannot be stably maintained in *E. coli*

[†] **Present address:** Departamento de Biologia Geral., Instituto de Ciencias Biológicas, UFMG, Caixa Postal 486, Belo Horizonte, Brazil.

[‡] **Present address:** Laboratoire de l'Environnement, INRA, Avenue des Etangs, 11100 Narbonne, France.

Abbreviations: contig, contiguous fragment; YAC, yeast artificial chromosome.

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(Glaser *et al.*, 1993; Ogasawara *et al.*, 1994). In order to overcome this difficulty we have developed a sequencing strategy based on yeast artificial chromosomes (YACs; Burke *et al.*, 1987). YACs have greatly facilitated genome mapping (Stalling *et al.*, 1994), but until now had not been used for direct sequencing.

An ordered collection of segments of the *B. subtilis* genome in YACs has been constructed (Azevedo *et al.*, 1993a). Here we report the sequencing of one of the YACs from the collection, designated 15-6B. The YAC carries a 104 kb insert from the region of the chromosome near 205° which was assigned to our laboratory within the framework of the European *B. subtilis* genome project (Kunst & Devine, 1991). The strategy used can presumably be applied to the megabase-scale YACs and to whole small genomes. The analysis of the nucleotide sequence is presented, focusing on the new genes for which a putative function can be postulated according to homologies with sequences in the databases. A putative transcriptional organization of the sequenced region is also proposed.

METHODS

Strains and growth conditions. Yeast cells containing artificial chromosomes were grown as described by Azevedo *et al.* (1993a). *E. coli* TG1 (K-12Δ(*lac-pro*) *supE thi hsdR* F' [*traD36 proAB lacI^q* Δ(*lacZ*)M15]) was used to make lawns on M13 selection plates and for growing phages in liquid medium. *E. coli* JJC128F' (*araD139* Δ(*ara-leu*)7696 *galE15 galK16* Δ(*lac*)X74 *hsdR hsdM⁺* Str^R F' [*lacI^q* Δ(*lacZ*)M15 *traD36*]), which reproducibly gave efficiency of electroporation by M13 DNA approaching 5×10^8 p.f.u. μg⁻¹, was used in cloning experiments. Electrocompetent cells of JJC128F' were prepared according to the protocol described by Dower *et al.* (1988) and stored at -80 °C. Plasmid pCGS966 (Smith *et al.*, 1990) was a gift from Dr D. Smith (Genome Therapeutics).

Isolation of YAC DNA. A derivative of the yeast strain 15-6B (Azevedo *et al.*, 1993a), called *rec9*, was constructed by transforming 15-6B with the *EcoRI*-*NdeI* fragment of pCGS966. Recombinants between the pCGS966 fragment and the left YAC15-6B arm, carrying the pBR322 replicon, were selected on a medium containing selective agents as described by Smith *et al.* (1990). A stable clone with an amplified (four copies) YAC, designated 15-6B*rec9*, was identified by direct screening of the yeast chromosomal DNA from resulting transformants. The identity of the insert in 15-6B*rec9* with the corresponding *B. subtilis* chromosome region was verified by Southern hybridization. Plugs containing yeast chromosomes were made as described by Anand *et al.* (1989). Electrophoresis was performed in 1.2% (w/v) agarose gels in 0.3 × TBE at 10 V cm⁻¹ at 18 °C (1 × TBE is 90 mM Tris/borate, 2 mM EDTA). The switching interval was from 0.3 to 6 s in forward migration and 0.1 to 2 s in reverse migration, for 20 h. After separation, YAC DNA was electroeluted in a dialysis membrane (Amicon) for 4 h at 10 V cm⁻¹. The solution containing YAC DNA was then concentrated fivefold by butanol treatment and DNA was treated with phenol/chloroform before ethanol precipitation. This protocol allowed us to obtain about 5–10 μg YAC DNA from 500 ml yeast cell culture.

M13 bank construction. The problem of constructing a representative and clean M13 library from purified YAC DNA

was analysed recently (Vaudin *et al.*, 1995). The authors suggested performing double purification of YAC by PFGE in order to avoid contamination by yeast DNA. We used another protocol, described below. Purified YAC DNA (0.5–10 μg) was partially digested by one of the following restriction enzymes: *AluI*, *TaqI*, *HpaII* or *HinfI*. The digested DNA was separated in agarose gel and segments between 500 and 1500 bp were purified as previously described (Sorokin *et al.*, 1993). Purified DNA was ligated with 50 ng *SmaI*- or *AclI*-cut and dephosphorylated M13mp19 vector in 20 μl ligase buffer (Boehringer), precipitated by 2-propanol using tRNA or glycogen as a carrier, rinsed with 70% ethanol, dried, dissolved in deionized water and used for electroporation of JJC128F' cells. The yield of phage plaques varied from 10⁵ to 10⁷ per μg M13 DNA, with a ratio of white to blue clones from 1 to 10.

Selection of M13 clones for random and directed sequencing.

White M13 plaques were propagated for 5 h in 48-well plates in 2 × YT medium on JJC128F' or TG1 cells as a host. After centrifugation on GPCR centrifuge (Beckman), 150 μl aliquots of the phage supernatants were distributed into 96-well plates by using a BIOMEC 1000 laboratory workstation (Beckman); 50 μl aliquots of the same supernatants were used to prepare filters corresponding to the stock plates. The resulting filters were used to identify the M13 clones which carried YAC DNA inserts. For this purpose we used purified YAC DNA, labelled with ³²P by using a random-prime labelling kit (Boehringer), as a probe. The hybridized clones were randomly chosen for sequencing. The M13 clones sequenced during the directed sequencing step were selected by hybridization with the inserts of the phages corresponding to the ends of contiguous fragments (contigs) obtained during random sequencing. These inserts were amplified by PCR and pooled before labelling and hybridization. When only small gaps (estimated to be less than 500 bp) between contigs remained to be sequenced, the phage inserts used as probes were pooled in four different mixtures and only the M13 clones hybridizing with two of these mixtures were used for sequencing.

Sequencing. ssDNA of M13 phages was prepared as previously described (Sorokin *et al.*, 1993). For reverse sequencing we used either M13 dsDNA prepared as described (Sorokin *et al.*, 1995) or DNA generated by PCR using biotinylated primers and magnetic beads according to the supplier's protocol (Dyna). Direct and reverse PCR sequencing was performed by using an Applied Biosystems PRISM direct or reverse sequencing kit on a Perkin Elmer 9600 thermal cycler or a Catalyst station from Applied Biosystems. ssDNA sequencing using Sequenase was performed as previously described (Sorokin *et al.*, 1993).

Oligonucleotide synthesis and PCR. Oligonucleotides were purchased from Eurogentec or synthesized on an Oligo 1000 DNA synthesizer (Beckman). PCR was performed by using M13 DNA or supernatants and *B. subtilis* or yeast chromosomal DNA in conditions previously described (Sorokin *et al.*, 1993).

Computing. The *xbap* and *xnip* programs (Dear & Staden, 1991) were used for gel assembling and consensus sequence analysis. The regions encoding proteins longer than 70 aa were further analysed for the presence of a sequence complementary to the 3' end of 16S rRNA of *B. subtilis*, which is 3' UCUUCCUCCACUAG... (McLaughlin *et al.*, 1981) upstream of a potential start codon (AUG, UUG or GUG) considering that the largest ORF is the most probably functional. In cases of doubt, the statistical tests, namely codon usage or uneven positional base frequencies, offered by the *xnip* program were also applied. FASTA (Pearson & Lipman, 1988) or BLAST (Altschul *et al.*, 1990) algorithms available on the NCBI e-mail server were used for rapid homology searches.

ORF nomenclature. In the framework of the *B. subtilis* Genome Project the names of the ORFs of the *lysA-ilvA* region should start with *yp-*. All the putative ORFs identified in this region were therefore named *ypxZ*, with *x* assigning them to a putative operon or divergon proposed on the basis of locations of *p*-independent transcription terminators, and *Z* distinguishing different ORFs in the same putative operon or divergon. The names of genes are used instead of, or along with, the *yp-* names when the function of an ORF has been determined experimentally or can be proposed as a result of a homology search. Three sequences (Bruand *et al.*, 1995; Sorokin *et al.*, 1995; Bower *et al.*, 1995; accession numbers U11289, U11687 and L38424) from the YAC15-6B region were released before the *yp-* nomenclature had been chosen. The ORF names in these releases started with *jo-* instead of *yp-*. They have been therefore correspondingly renamed here.

Sequence accession numbers. The sequences corresponding to the YAC15-6B insert are available from GenBank under accession numbers L09228 (positions 1–13695 in 15-6B), L47648 (12396–37282), M80926 (37015–39348), M80245 (39345–57318), L47709 (57126–80900) and L47838 (80824–104109). The entries L47648, L47709 and L47838 contain the newly determined sequence.

RESULTS AND DISCUSSION

Direct sequencing of a YAC

Sequencing strategy. Genomic sequencing generally comprises two main steps, one random and the other directed. The first is very efficient in the early stages of the project, and allows rapid generation of the bulk of the sequence, but it becomes inefficient later, since most of the randomly determined sequence is not novel. A switch to directed sequencing is therefore required to avoid an inordinate level of sequence redundancy. Our approach also used random and directed steps, carried out as follows.

Random sequencing was carried out on YAC segments subcloned in M13-derived vectors as described below. A number of contigs was thus established. Directed sequencing involved two phases. In the first, the clones from the M13 bank that allowed extension of the sequence were identified by hybridization, using as probes segments from the ends of the contigs. In the second, the contigs were ordered by combinatorial PCR and the gaps between them were closed by sequencing the appropriate PCR products.

Isolation of YAC DNA. To facilitate preparation of YAC DNA, we attempted to modify the copy number of YAC15-6B, using the approach described by Smith *et al.* (1990). For this purpose, pCGS966 was cleaved with *EcoRI* and *NdeI* and introduced by transformation into a yeast strain carrying YAC15-6B, selecting for expression of the thymidine kinase gene. The chromosomes of the transformants, cultivated under conditions inducing YAC amplification, were analysed by PFGE. Unexpectedly, YAC amplification was not induced in any of the clones. However, screening of clones grown on standard selective medium (Anand *et al.*, 1989) revealed that one of them, designated *rec9*, contained about four copies of the YAC. The reasons for this increase in copy number are not understood. Rearrangements had not taken place in the

B. subtilis moiety of the amplified YAC, as judged by Southern hybridization (not shown), and DNA of this amplified YAC was therefore used for sequence analysis. The DNA was purified by PFGE and used for the construction of M13 libraries. Hybridization of these collections with purified YAC DNA indicated that 20–50% of the M13 clones contained a DNA insert from the YAC. Other M13 phages forming white plaques presumably contained yeast chromosomal DNA.

Random and directed sequencing step. About 800 M13 clones of the YAC15-6B library were randomly chosen for sequencing. This generated ~90 kb of the consensus sequence, organized in 80 contigs, with a redundancy of 2.8. About 86% of the 104 kb YAC insert was thus sequenced. At this point we decided to switch to the direct sequencing. Two contiguous regions sequenced earlier (positions 1–13695 and 37015–57318; Sorokin *et al.*, 1993; Roels *et al.*, 1992; Henner *et al.*, 1990) were excluded from further analysis. We first attempted to extend the sequence of 40 of the largest contigs. For this purpose inserts of 80 M13 clones corresponding to the ends of the contigs were amplified by PCR, pooled in series of 20 and used as probes for hybridization with M13 clones that had not been previously sequenced. Sequencing of 300 positive clones extended the total contig length to ~96 kb, organized in 47 contigs. Iteration of this procedure revealed new hybridizing M13 clones, but did not allow us to extend the sequence significantly. This indicated that the appropriate M13 clones were absent or extremely rare in the M13 collection.

A similar conclusion was reached in attempts to identify the M13 clones carrying inserts that bridge two contigs and therefore hybridized with two different probe pools. Only five clones among 3000 tested joined the contigs. Given that the average distance between two contigs was ~250 bp (~12 kb missing sequence distributed among 47 contigs), and the average M13 insert size was about 500 bp, many more bridging clones should have been identified. This supports the assumption that some chromosomal regions were severely under-represented in the M13 collection.

Combinatorial PCR. To complete the YAC sequence we decided to synthesize the missing regions by PCR, using *B. subtilis* chromosomal DNA as template. Forty oligonucleotides corresponding to the ends of the 20 longest contigs were used for this purpose. PCR products were obtained for all missing regions, varying in size from 0.1 to 6 kb. They were used as probes for screening 2000 M13 clones which had not been sequenced previously; 258 hybridizing clones were detected. Sequencing of the clones allowed us to make only eight additional contig joins, and several new contigs were formed. This result confirmed that the M13 bank was biased, and suggested that some *B. subtilis* regions cannot be cloned in *E. coli* even in short segments on M13 phages. This conclusion is supported by our failure to clone the PCR fragments in *E. coli* directly. The sequence of the YAC was therefore completed by primer walking over the PCR products.

The length of the novel sequence is 70111 bp. Sequence

Fig. 1. Organization of the *B. subtilis* chromosome region between the *spoIIA* and *kdg* loci. Thick arrows correspond to ORFs, detected by sequence analysis. Thin arrows show putative transcripts. Small 't's show the positions of stem-loop structures which may function as transcription terminators. The insert in YAC15-6B starts from the middle of *ypuI* (position 14512) and ends in the middle of *ypwA* (position 118620). Nomenclature of ORFs is explained in the text. The regions from *spoIIA* to *serA* and from *spoIVA* to *aroE* have been discussed elsewhere (Sorokin et al., 1993; Henner et al., 1990). The ORFs with unknown functions from the first publication are renamed as follows: *orfX19* to *ypuA*; *orfX1* to *ypuC*; *orfX4* to *ypuD*; *orfX5* to *ypuE*; *orfX6* to *ypuF*; *orfX7* to *ypuG*; *orfX8* to *ypuH*; *orfX9* to *ypuI*; *orfX13* to *ypuL*; *orfX21* to *ypuN*. The involvement of the previously found *orfX11* and *orfX12* in spore maturation, giving the names *spmA* and *spmB*, was found recently (Popham et al., 1995). *resABCDE* were renamed from *orfX14–18* after establishing the respiratory function of this operon (Sun et al., 1995). *orfX20* was renamed *sigX* because this gene encodes a σ -factor (Lonetto et al., 1994). Other genes indicated above the scale bar encode the following: *spo*, sporulation genes; *lysA*, diaminopimelate decarboxylase; *ppiB*, peptidyl-prolyl isomerase; *sipS*, signal peptidase; *rib*, riboflavin biosynthesis operon; *dacB*, D,D-carboxypeptidase; *aroC*, 3-dehydroquinate dehydratase; *serA*, phosphoglycerate dehydrogenase; *fer*, ferredoxin; *recQ*, product homologous to the *E. coli* *recQ* gene product; *gud*, glutamate dehydrogenase; *cmk*, CMP kinase; *rpsA*, S1 ribosomal protein; *era*, product homologous to the *E. coli* *era* gene product; *gpsA*, glycerolphosphate dehydrogenase; *dbpA*, DNA-binding protein; *mtr*, 5-methyltryptophan resistance; *gerC*, germination deficiency; *ndk*, nucleotide diphosphate kinase; *cheR*, chemotactic methyl transferase; *aro*, general pathway of aromatic amino acid biosynthesis; *trp*, tryptophan biosynthesis; *hisH*, histidine biosynthesis; *tyrA*, tyrosine biosynthesis; *qcrABC*, respiration *bf*-complex; *dapB*, dihydropicolinate reductase; *papS*, poly(A) polymerase; *birA*, biotin biosynthesis regulation; *pan*, pantothenic acid biosynthesis; *dinG*, product homologous to the *E. coli* *dinG* gene product; *aspB*, aspartate aminotransferase; *asnS*, asparaginyl-tRNA synthetase; *dnaD*, a protein involved in the initiation of replication; *nth*, endonuclease III; *ponA*, penicillin-binding protein 1; *cotD*, spore-coat protein; *rnpB*, RNase P RNA component; *kdu*, *kdg*, enzymes of pectin and galacturonate catabolism. Numbering starts from the first base pair of the *SaI* site in the *spoVAF* gene, the same as for the entry with GenBank accession number L09228 (Sorokin et al., 1993).

redundancy is ~ 11 , which is rather high due to the unsuccessful efforts to find M13 clones by hybridization with contig extremities. We anticipate that YAC sequencing could be accomplished with a redundancy of 5–6, by using combinatorial PCR directly after the random sequencing step. The complete 104 109 bp sequence of the YAC15-6B insert was established by joining

the newly determined sequence to the known regions of 13695 and 20303 bp, mentioned above.

Analysis of the sequenced region

The sequenced region was analysed for the presence of ORFs encoding proteins larger than 70 amino acids and having an appropriate ribosome-binding site. The presence of structures characteristic of *p*-independent transcriptional terminators was also examined. The organization of the *spoIIA-kdg* region of the *B. subtilis* chromosome is presented in Fig. 1. The *spoIIA-serA* and *spoIVA-aroE* regions were described earlier (Sorokin *et al.*, 1993; Roels *et al.*, 1992; Henner *et al.*, 1990). In the *serA-kdgT* area, which is 90 kb long, 98 ORFs were detected. The average gene size is therefore 0.9 kb, a figure very similar to that previously reported (Glaser *et al.*, 1993; Ogasawara *et al.*, 1994). For 89 ORFs, that is 91% of those detected, the direction of transcription is colinear with the replication fork movement. The *ypfABCD*, *ypjD-birA*, *aspB-nth* and *ponA* regions were partially described in recent publications relevant to the functional characterization of the newly discovered genes (Sorokin *et al.*, 1995; Bower *et al.*, 1995; Bruand *et al.*, 1995; Popham & Setlow, 1995). The functional and structural characterization of the *cotD* and *rrnP* genes was also previously reported (Donovan *et al.*, 1987; Reich *et al.*, 1986). More detailed results of the database homology analysis are presented in Table 1 and discussed below.

Genetic organization of the sequenced region

***serA* and *ypaA*.** The *serA* and *ypaA* genes are organized in two divergent transcription units which share the regulatory region. *serA* was partially sequenced before and shown to be involved in serine biosynthesis (Sorokin *et al.*, 1993).

***ypbA (fer)* and *ypbC*.** The *ypbA (fer)* product is 79% identical to ferredoxin of *Bacillus stearothermophilus*, suggesting that *ypbA* encodes a ferredoxin. It shares the regulatory region with the divergent *ypbB-ypbF* operon. The protein encoded by *ypbC* shares high homology with RecQ of *E. coli*, which is believed to be involved in the SOS response (Irino *et al.*, 1986). Similarity of a region of these proteins (RecQ and YbpC) to RNA helicases of different species suggests that they contain an RNA-binding domain. The *ypbC* product may be involved in the SOS response, as suggested by the presence of a potential promoter overlapping with a damage-induction associated SOB-operator site: GAAC-N₄-GTTC (Cheo *et al.*, 1991). Other genes of this operon may therefore also be involved in the stress response. Since the divergently transcribed *ypbA* gene shares the regulatory region, it is conceivable that the stress response may be related to energy production or detoxification.

***ypcA (gud)*.** *ypcA (gud)* is the second glutamate dehydrogenase gene of *B. subtilis* identified by sequencing. The first, called *ipa-75d*, was detected near 330° (Glaser *et al.*, 1993). In contrast to *ipa-75d*, which was shown not to be expressed (Glaser *et al.*, 1993), *ypcA* mRNA synthesis

seems to be high and constitutive (P. Pujic, this laboratory, personal communication), indicating that: (i) YpcA is not the glutamate dehydrogenase studied by Kane *et al.* (1981), which has been shown to be repressed by carbohydrates and induced by glutamate, and (ii) YpcA might play a role in NH₄⁺ utilization. However, glutamate synthase seems to be the only enzyme essential for NH₄⁺ assimilation (Schreier, 1993).

***ypfC (cmk)* and *ypfD (rpsA)*.** The products of the genes *ypfC* and *ypfD* are homologous to the *E. coli* *mssA* and *rpsA* gene products, respectively, the latter encoding the S1 ribosomal protein. The similar organization of this region in *B. subtilis* and *E. coli* led us to suggest that these genes are counterparts (Sorokin *et al.*, 1995), although the function of the second gene in *B. subtilis* remains unclear. It has been shown recently that *mssA* of *E. coli* encodes cytidine monophosphate kinase and the gene was therefore renamed *cmk* (Fricke *et al.*, 1995). It was reported that, in contrast to *E. coli*, *B. subtilis* contains only one pyrimidine ribonucleoside monophosphate kinase, synthesizing both UMP and CMP (Walch & Ingraham, 1976). This is in good agreement with our finding that *ypfC* is essential (Sorokin *et al.*, 1995).

***ypgA*.** This gene has the highest homology with the hypothetical protein ORF6 from the *Erwinia herbicola* carotenoid biosynthesis gene cluster. It also shares a homologous region with flavocytochromes *b* and 2-hydroxy-acid oxidases, shown to be essential for the activity of these proteins (Diép Lê & Lederer, 1991). However, several amino acids essential for the binding of flavin are absent in *ypgA* and therefore no function for this gene can be suggested.

***ypbC (era)*.** The product of this gene shows homology with the *E. coli* Era protein, an essential GTPase with a regulatory function. These proteins are structurally similar to RAS proteins of eukaryotes (Ahnn *et al.*, 1986). In prokaryotes, they form a novel family of regulators, comprising at least five members (Ahnn *et al.*, 1986; Trach & Hoch, 1989; Yamashita *et al.*, 1993; Ogasawara *et al.*, 1994), but the function of none has yet been established. A distinctive feature of YphC from the proteins of this family is that it has two GTP-binding motifs (not shown). Studies of Era-depleted temperature-sensitive mutants of *E. coli* indicated that the protein is required for adaptation to thermal stress (Lerner & Inoye, 1991) and cell division (Gollop & March, 1991). The closest protein for which activities and tertiary structure are known in detail is EF-Tu (Weijland & Parmeggiani, 1993).

***ypbD (gpsA)*.** The protein encoded by *ypbD* has high homology to the NADH-dependent glycerol-3-phosphate dehydrogenases. Mutants devoid of this activity, designated *glyc*, were isolated and studied in two laboratories (Oh *et al.*, 1973; Freese & Oh, 1974; Lindgren & Rutberg, 1974). Three such mutations were mapped by Rutberg's group and high linkage was detected between *trpC*, *his* and *glyc* markers by transformation. Taking into account the known distance between *trpC* and *hisH* (5 kb), the approximate distance between these markers might be estimated as 10 kb, with the order *glyc-trpC-hisH*

Table 1. Results of comparison of ORFs with sequences in the PIR protein data base

ORF	End-points (bp)*	Size (aa)	PIR entry	Description (size, aa)	Homology (FASTA score)	Match (%)†
<i>serA</i>	14013–12436c	526	deecpg	Phosphoglycerate dehydrogenase <i>E. coli</i> (410)	324	30.5 (324)
<i>ypaA</i>	14510–15082	191	s02153	NADH dehydrogenase (ubiquinone) mitochondrion <i>Podospira anserina</i> (519)	130	28.6 (126)
<i>ypbA (fer)</i>	15370–15122c	83	FEBSFF	Ferredoxin <i>B. stearothermophilus</i> (81)	369	79.0 (81)
<i>ypbC (recQ)</i>	16687–18177	497	bvecrq	<i>recQ</i> protein of <i>E. coli</i> (610)	788	40.3 (380)
			s30278	RNA helicase <i>D. melanogaster</i> (402)	257	22.0 (369)
<i>ypcA (gud)</i>	21749–23029	427	a38168	Glutamate dehydrogenase <i>Clostridium difficile</i> (421)	1055	51.6 (419)
<i>ypdA</i>	23138–24112	325	s18998	Hypothetical protein <i>B. licheniformis</i> (122)	454	77.0 (122)
			s29117	Hypothetical protein <i>Clostridium pasteurianum</i> (308)	183	28.4 (296)
<i>ypfC (cmk)</i>	28377–29051	225	s29160	Cytidine monophosphate kinase (<i>cmk</i>) <i>E. coli</i> (227)	465	44.9 (216)
<i>ypfD (rpsA)</i>	29284–30432	383	r3ec1	Ribosomal protein S1 <i>E. coli</i> (557)	662	39.0 (333)
			a26118	Polynucleotide phosphorylase <i>E. coli</i> (711)	214	24.2 (236)
			s13643	PRP22 <i>S. cerevisiae</i> (1145)	172	21.2 (255)
			c36718	Pyruvate dehydrogenase E1 component <i>B. subtilis</i> (325)	121	19.2 (281)
<i>ypgA</i>	30856–31494	213	M87280‡	ORF6 of carotenoid biosynthesis cluster <i>Erwinia herbicola</i> (492)	7.3e ⁻²³	46.0 (92)
<i>ypbC (era)</i>	33581–34891	437	rgecgt	GTP-binding protein Era <i>E. coli</i> (301)	226	28.2 (226)
<i>ypbD (gpsA)</i>	34909–35946	345	a26687	Glycerol-3-phosphate dehydrogenase <i>Mus musculus</i> (349)	335	31.1 (347)
<i>ypiC (qcrA)</i>	60000–60503	168	a35580	Rieske iron-sulfur protein <i>Nostoc</i> sp. (179)	159	26.2 (145)
			s16572	Plastoquinol-plastocyanin reductase <i>Synechocystis</i> sp. (192)	177	28.3 (145)
<i>ypiD (qcrB)</i>	60505–61077	191	cbk16p	Cytochrome <i>b₆</i> <i>Chlorella protothecoides</i> (215)	404	42.3 (182)
<i>ypiE (qcrC)</i>	61215–61982	256	wmsp17	Cytochrome <i>b</i> <i>Spinacia oleracea</i> (382)	174	29.1 (117)
<i>ypjA</i>	62129–62686	186	b31383	Hypothetical 83K protein (<i>hrp</i> locus) <i>Pseudomonas syringae</i> (731)	107	24.7 (81)
<i>ypjE (dapB)</i>	64950–65753	268	rdecpd	Dihydrodipicolinate reductase <i>Brevibacterium lactofermentum</i> (248)	480	44.9 (205)
<i>ypjH</i>	66881–68014	372	a61112	Trans-acting transcription factor SPT14 <i>Saccharomyces cerevisiae</i> (452)	288	21.6 (380)
			a46217	Phosphatidylinositol glycan class A, PIG-A <i>Homo sapiens</i> (484)	186	16.7 (371)
			s25526	Sucrose-phosphate synthase <i>Oryza sativa</i> (452)	132	24.7 (219)
			s15296	Hypothetical protein <i>Salmonella typhimurium</i> (406)	166	25.0 (324)
<i>ypjI (papS)</i>	68019–69212	398	s11887	<i>penB</i> <i>Escherichia coli</i> (468)	344	38.9 (221)
			rnecta	tRNA nucleotidyltransferase <i>E. coli</i> (412)	224	30.4 (224)
<i>birA (birA)</i>	69213–70190	326	bvecbf	<i>birA</i> bifunctional protein <i>E. coli</i> (321)	342	26.7 (322)
<i>ypkA (panB)</i>	70420–71253	278	P31057§	Ketopantoate hydroxymethyltransferase <i>E. coli</i> (264)	630	47.1 (263)
<i>ypkB (panC)</i>	71255–72115	287	P31663§	Pantothenate synthetase <i>E. coli</i> (283)	566	40.8 (282)
<i>ypkC (panD)</i>	72117–72500	128	P31664§	Aspartate 1-decarboxylase <i>E. coli</i> (126)	254	48.3 (116)
<i>ypjA (dinG)</i>	72626–75421	932	a42717	<i>dinG</i> protein <i>E. coli</i> (716)	366	22.4 (696)
<i>aspB (aspB)</i>	76251–77432	394	a38621	Aspartate aminotransferase <i>Bacillus</i> sp. (392)	1428	71.3 (387)
<i>ypnA (asnS)</i>	77576–78868	431	syecnt	Asparaginyl-tRNA synthetase <i>E. coli</i> (466)	624	41.1 (265)
<i>dnaD (dnaD)</i>	78961–79659	233		No homologies	< 70	
<i>ypoB (nth)</i>	79696–80355	220	a32412	Endonuclease III <i>E. coli</i> (211)	439	46.2 (208)
<i>ponA (ponA)</i>	83648–80904c	915	s28033	Penicillin-binding protein 1a <i>Streptococcus pneumoniae</i> (719)	1083	39.1 (586)
<i>yppB</i>	84290–83670	207	y23k_stror§	Hypothetical 23.1 kDa protein in <i>ponA</i> 5' region <i>Streptococcus oralis</i> (198)	477	50.3 (179)

Table 1. (cont.)

ORF	End-points (bp)*	Size (aa)	PIR entry	Description (size, aa)	Homology (FASTA score)	Match (%)†
<i>ypqB</i>	87653–88159	169	wqeb3t	Phosphotransferase system enzyme II, glucose specific <i>Salmonella typhimurium</i> (530)	357	40.1 (167)
<i>yprA</i>	88262–90511	750	s00986	Initiation factor eIF-4AI <i>Mus musculus</i> (407)	121	20.0 (140)
<i>cotD</i>	92081–92308	76	d27393	<i>cotD</i> protein <i>B. subtilis</i> (75)	418	100 (75)
<i>ypuA</i>	95679–97604	642	ding_ecoli§	ATP-dependent helicase DinG <i>E. coli</i> (716)	145	33.3 (135)
<i>ypvA</i> (<i>kduD</i>)	98409–97645c	255	s17711	Deoxygluconate oxidoreductase <i>Erwinia chrysanthemi</i> (253)	659	55.0 (251)
<i>ypvB</i> (<i>kduI</i>)	99238–98411c	276	s17710	Deoxyuronate isomerase <i>Erwinia chrysanthemi</i> (278)	753	46.0 (278)
<i>ypvC</i> (<i>kdgR</i>)	99460–100479	340	s15318	<i>ccpA</i> protein <i>B. subtilis</i> (334)	425	27.8 (334)
<i>ypvD</i> (<i>kdgK</i>)	100517–101491	325	jq0782	Fructokinase <i>Vibrio alginolyticus</i> (307)	251	27.5 (287)
<i>ypvE</i> (<i>kdgA</i>)	101493–102083	197	adecoq	2-Keto-4-hydroxyglutarate aldolase <i>E. coli</i> (213)	279	36.4 (165)
<i>ypvF</i> (<i>kdgT</i>)	102128–103120	331	jq0113	2-Keto-4-deoxygluconate permease <i>Erwinia chrysanthemi</i> (398)	894	51.4 (315)

* Positions in the YAC15-6B insert.

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

‡ GenBank entry, homology detected by BLASTA search.

§ SwissProt entry.

(Lindgren & Rutberg, 1974). This matches well with our sequence data, supporting the possibility that *ypbD* corresponds to the *glyc* gene. The updated name for this gene, proposed by Dr R. Rutberg, is *gpsA*.

***ypjCDE* (*qcrABC*).** The proteins encoded by *ypjCDE* are homologous to the Rieske iron-sulphur protein and the cytochrome *b* subunit of the *bc₁* or *bf* respiration complexes. The *bf* complex should contain cytochrome *b*, which consists of two subunits, and an *f*-type cytochrome (Trumpower, 1990). No cytochrome *f*-encoding gene was detected in the *ypj* operon, nor elsewhere in the sequenced region. A more detailed characterization of these genes and the regulation of their transcription has been reported recently (Yu *et al.*, 1995; Sun *et al.*, 1995). We therefore used the names for these genes proposed by Yu *et al.* (1995).

***ypjE* (*dapB*).** This gene encodes a protein highly homologous to the *E. coli* dihydropicolinate reductase, an enzyme involved in the early steps of lysin biosynthesis. An enzyme with such activity was isolated from sporulating *B. subtilis* cells (Kimura, 1975). Since the protein isolated by Kimura (1975) is different from that encoded by *ypjE*, as judged by molecular mass (18.5 kDa and 29.5 kDa, respectively), it is possible that *B. subtilis* uses two enzymes for this pathway. This is in agreement with the finding of strikingly different dihydropicolinate reductases in *Bacillus cereus* and *B. megaterium* (Kimura & Goto, 1977), leading to the notion of the existence of two different types of this enzyme in bacilli (Kimura *et al.*, 1978).

***ypjH*.** The *ypjH* gene product has homology with the yeast SPT14 and the human PIG-A proteins, which were

recently shown to be involved in glycosylphosphatidylinositol (GPI) synthesis (Schönbachler *et al.*, 1995). GPI is involved in anchoring of some proteins to the plasma membrane of eukaryotes. The presence of such a gene in *B. subtilis* suggests the existence of GPI-anchored proteins in this bacterium.

***ypjI*.** About half of the protein encoded by *ypjI* is highly homologous to the *E. coli* mRNA polyadenylase and tRNA nucleotidyltransferase. Multiple alignments of these proteins (not shown) indicate that it is more probable that they have a common motif, possibly involved in RNA-binding, rather than a common function.

***ypkABC* (*panBDC*).** These three ORFs specify proteins highly homologous to the enzymes of the pantothenic acid biosynthesis pathway, encoded in *E. coli* by the *panB*, *panC* and *panD* genes. A functional *panB* gene was mapped near 60° on the *B. subtilis* chromosome, next to *panE*, which also encodes an enzyme implicated in this pathway (Baigori *et al.*, 1991).

***ypjA* (*dinG*).** The 22% homology of the *ypjA* gene product to the DinG of *E. coli* suggests similar functions. The high molecular mass (103 kDa) allowed us to find the corresponding product, having a pI of 6.2, to be detected on two-dimensional protein gels as a well-separated spot (Völker *et al.*, 1994). This feature should allow easy monitoring of protein synthesis during induction of DNA damage, which might be a way of revealing its specific function.

***aspB*.** The gene encoding aspartate aminotransferase was previously localized in this part of the *B. subtilis* chromosome by transformation (Hoch & Mathews, 1972). The

ypaA tgcacatattccataaataAAAAAGCTCCCGtCGGGAGCTTTTTtagttactctatt
 ypfB agtaataaataatggggcgttttctcAAACGatagaaCGTcTTttttatgtcgtctt
 ypbH ttcataagaatgcaacgggAAAACTGCTGATTCAGCAGTTTTtttgcgttaaaacatg
 ypcA (gud) atttgcataaataAAAAATCTCCTATgataaATAGGAGATTTTTtttgcggaata
 ypdC atgcaagatagAAGCCGCAcagcaaccGTGCGGCTTTTTgtgagttttatgtc
 ypeB lataaataacaggAGGAAAGGACtaaatGCTTTTCCTTTTTtttcatcgttctaata
 ypfC (cmk) caaaaglcggcgtgaggaTGCCAAATGCCAActttttgaattttcaacgaagatagaa
 ypfD atttccacAAAAATCGGCGAACTTcaagcAGTTGCGCGATTTTTtttcaatgcaatgact
 ypgA tgcgataaatgatAAAAGCCCAAAACCAatgTGGTTTGGGCTTTTTgtatattgtttt
 yphB ataataaatgagttAAAGCCCTTccttGAAGGGTcctttcataaaagtgaatgaatg
 yphF ggaatgAACCTTTTCCTCTtgcac acaatAGGGAGAAAGGTTtttt
 spoIVa ctgtaataccggtagAACCTCTttatagaatggGAGGTCTttttcttgc
 dbpA cttttattttcacGAAAAAGCCCTTtcttAAGGGGCTTTTTcatattcaa
 mtrB TGGCTGTcCCCCGTGTTaaagcaaatgctcgcaAACAGCGGGaggatACAGCCAattc
 ndr aataaagcaacagagCCGGCcaatataacgcccGCCGGttttgtctgtttt
 aroH tgagacattatgtttatttACCCAAAAGAAgctTTCTTTTGGGTttattgttat
 aroE AAAAAATCTGTAAGTtttACTTCAGGATTTTTtttgcagatcataatg
 ypiE (qcrC) ttattatcAAAAGCTGAACCGGCTGACGCTTTTTttatagacattat
 ypiB taagatgaggGGCGGCGgtcagGCCGCCtttttctatta
 birA aaagtgaagaatCCTTCTCCTTGAAGGGAAGGtttttggcgtgcagaa
 ypkC (panD) aattttgtagAAGAAAGCCCCtttatoGGGGGtTTCTTTTaagatttg
 ypiA (dinG) gggggaataaataAAAAAGCCGCAaaAGCGCTTTTTgcgggtgaggatg
 aspB agtcaacagatcAAAAAGCGGCTgacagaaaAGCGCTTTTTTatctgcat
 asnS aatgaacaaaAAAAAGTCTCCTgcaagGAGACTTTTTTgcagtaaaagg
 ypoC atgaatcgaAAAAACCTCTgttaaaagGGAGGTTTTttatgtg
 ponA ttaaacAAAAAGCGCgtcactttgggggtgagGCTTTTTTggtacac
 yppE attgcaagAGAAAGACAGCGCGgttaaaGGCTGTCTTCTttcgttttata
 yppG tgaatgAAGCATGAAGAAAGCTTCATTCATGCTTtttttggaaag
 ypqB taaagcgaaatAAGCAGGGCGgtatGCCCTTGCTTTTTttttcaacgt
 yprB tAAAAATTC ccCGGGAAG gcgcaAAAGac gaCTTgTTTC gCCatGAA TTTTtttgc
 cotD gcacaacAAATGGGGCCcaggggaGCCCATTTtttaagcaggatga
 rnpB tgaatacAAGCTCTCCgtataaGGAGGACTTTtatcttgaagagagaaa
 ypsC gaaaacgcataaAAAAAGACGCTcgtcatGCCCTTCTTctatctgacccc
 yptA aatcgtgaagtaactgcAAAAAAGTGGCAITGCCAAGTTTTTtttga
 ypuA atgagtaatgAAAAAGCTGACGAtgTTCGTGAGCTTTTTTattgt
 ypvF (kdgT) actgaataAAAAAGGCTGCCacaaacataaccGGCAGCCTTTTatacaaac

Fig. 2. Structure of putative *p*-independent transcription terminators. On the left are the names of ORFs preceding the putative terminators. Inverted repeats are indicated by capital letters.

very high similarity of its product to the structure of the enzyme from *Bacillus* sp. strongly suggested its function.

ypnA (asnS). The *ypnA* gene product is highly homologous to asparaginyl-tRNA-synthetase but its function needs to be experimentally tested. In the cases of tyrosyl- and threonine-tRNA-synthetase two active genes were found, having concerted regulation and complementing each other's activity (Glaser *et al.*, 1990; Putzer *et al.*, 1992; Grundy & Henkin, 1993).

ypqB. The protein encoded by this gene is homologous to the histidyl domain of phosphotransferase system enzyme II of *Salmonella typhimurium*. According to the nomenclature suggested by Saier & Reizer (1992), this domain is the first phosphorylation site of the IIA domain. This finding suggests similarities between the *B. subtilis* system and the glucose permease of *E. coli* or the sucrose permease of *S. typhimurium*.

yprA. Substantial homology of part of the YprA protein to the eukaryotic initiation factor eIF-4A from different species and also to an RNA-helicase of *Drosophila melanogaster* suggests that *yprA* encodes an RNA-binding protein.

rnpB. The *rnpB* gene that encodes the RNA component of RNase P was cloned and sequenced earlier (Reich *et al.*, 1986).

ypvABCDEF (kduDI-kdgrKAT). Several genes involved in pectin catabolism were isolated and sequenced from

Erwinia chrysanthemi (Condemine & Robert-Baudouy, 1991; Hugouvieux-Cotte-Pattat & Robert-Baudouy, 1994). The characterized genes from *E. chrysanthemi* also include the regulator of this system, *kdgR* (Reverchon *et al.*, 1991). The relevant aldolase, the gene of which in Gram-negative bacteria is called *kgdA* or *eda*, is one of the two enzymes of the Entner-Doudoroff pathway of gluconate assimilation, which does not play an essential role in *Bacillus* species (Goldman & Blumenthal, 1963). Our homology search revealed six genes encoding a system for polygalacturonate catabolism, including aldolase and the putative regulator. Interestingly, the product of the regulatory gene has highest homology with the apparent catabolite repressor of *B. subtilis* CcpA.

Transcriptional features of the sequenced region

The transcription signals which can be reliably predicted in bacteria from sequencing data are the *p*-independent transcription terminators (Daniels *et al.*, 1992; Glaser *et al.*, 1993). Identification of transcription terminators was shown to be useful for prediction of the transcription map which matches well with the experimentally defined one (Azevedo *et al.*, 1993b). Fig. 2 lists the 36 stem-loop structures which may work as *p*-independent transcription terminators that were detected by analysis of the sequence of the region between the *serA* gene and the *kdg* operon. This list also contains terminators from the *spoIVA*–*aroE* region, which was sequenced by others and was not discussed above, but is relevant to this study. Experimental studies of transcription of *ypfC*–*ypfD* (Sorokin *et al.*, 1995), *spoIVA* (Roels *et al.*, 1992; Stevens *et al.*, 1992), the *trpE* regulatory region (Henner & Yanofski, 1993), *qcrABC* (Sun *et al.*, 1996; Yu *et al.*, 1995), *cotD* (Zheng & Losick, 1990) and *rnpB* (Reich *et al.*, 1986) have already been reported. We can therefore consider the corresponding terminators as experimentally proven to be active. Some features of the promoter regions deduced from the sequence and which are potentially relevant to the regulation are discussed below.

ORFs *yphC* and *yplA* encode proteins that might be involved in the SOS response (see above). This implies that the corresponding promoters belong to the SOB regulon, which is characterized by the presence of the GAAC-N₄-GTTC structure in the promoter regions (Cheo *et al.*, 1991). Promoter structures and the SOB consensus sequence can easily be detected upstream of *yphC* and *yplA* (Fig. 3a, b). Although not so evident, a similar sequence exists also in the *yprA* leader region (Fig. 3c).

Surprisingly, the *ypjD*–*birA* operon encodes several genes having apparently different functions (see above, and Bower *et al.*, 1995). This might be due to the fact that the biotin biosynthesis regulator, BirA, is a negative regulator of biotin biosynthesis and at the same time a positive regulator of the *ypjD*–*birA* operon. Thus, the biotin regulator might work as a switch between carboxylating enzymes containing biotin and lysine biosynthesis through tetrahydrodipicolinate. This is probably needed to regulate the concentration of oxaloacetate in the cells

(a)	
L H E P A F M I N D Y V S A E D Q L Q I	ypbB 31080
tctccatgagcgtcggtttatgattaatgactatgtatcagcagaagaccagcttcaaat	
-35 -10	
A E F A K R M R T N K I K Q I R D G L E	ypbB 31140
cgcagagtTTGCCAaacggatgctggacgaaTAAATcaaacaaattcgcgacgggtgGA	
Q R F S Y F Q I R L A L T K Q V Q Q Y D	ypbB 31200
ACagcgGTTcagctactttcaaatcagactggcattgacaaagcaggtgcagcaatatga	ypbC 31260
SOB	
T K L Q Q T L Y Q F F G F T S F K K G Q	ypbC 31260
ctaaattacagcaaacggttatatcagtttttgggttttacttcttataaaaagggcagc	
(b)	
A V L N D Q N K I E Q M L G N E P A R T	panD 87000
gctgttcgaatgatcaaaacaaattgaacaaatgctggggaacgaaccagccgtaca	
I L *	panD 87060
attttgtagaagaaaagccccctttatcggggtttttctttaagattttgagagtaaaa	
-35 -10	
cggttttgcccgtccatttccgtacaaaacGTGTTAacttttgcgtatgcAAGAAATg	87120
M N K Q R F V V I D V E T T G	yplA 87180
aggtgtccatttttaataagaagCAACggttcGTTGttatagatgtagagacaacaggga	
SOB	
(c)	
M V S A G E K A A R K G E S K L F T I K	ypqB 102660
tggtatcgccaggcgcaaaaggctgctcgttaaaggcgaatcaaaagctctttacaataaaag	
A K *	ypqB 102720
cgaaataagcagggtcgatgctctgtttttttattcaactgtataaaatTTGCACctag	
-10	
ccatgcaaaacAATATTcggttaactgtacttgacaaagaggtgtaagaatgaaaaaga	yprA 102780
M K K	
K S L T E L I S D L K G N E N V V N W H	yprA 102840
aatcactgactGAACccaTTTCgtatttaaaaggaaatgaaacggtgtgtaattggcatg	
SOB	

Fig. 3. Regulatory regions of the *ypbC* (a), *yplA* (b) and *yprA* (c) genes. Capital letters within the nucleotide sequence indicate the regions homologous to the *B. subtilis* SOB box (GAAC-N₄-GTTG) or the -35 (TTGACA) and -10 (TATAAT) boxes of the σ^A promoters. Numbering as for Fig. 1.

agtgcgcgacaagggtataaaacaaactgtctcgtccgagcaaacgccagccaataaaaaa	78780
T G V L T Y V F M	ypjC
aatcggaatgttcaacaccaggttctgatataaggggctgatgtggaagggcataaag	78840
caaaagtgtataacctgtaaaacccctcagccagatgttctgcatattgaaatgaac	78900
caagccaattgaaaaattgcgcctcggattaaataaaaaatattgttttttaattctaat	78960
ttctccaagcatcacacaaaaccccttttcttccattagatagcgtttatattatagtg	79020
-35 -10	
gaTGaaaAagAAACAgCAaAcGTGcTTAAaCatTTGTCAaacgtatctgaattagcTAA	79080
V S D K T M K D I Q	ypjD 79140
CATgtaagaggatgataggaggttttcgttcagtgagtgataaaacaatgaaagacataca	

Fig. 4. Regulatory region of the *ypjD* gene. Structures resembling σ^A promoter regions (TTGACA and TATAAT) and homologous to the apparent BirA-operator site of *B. sphaericus* (AATGTGTTAACTAAAACTATAGTTGGTTAAC) are shown in capital letters. Numbering as for Fig. 1.

growing on metabolized sugars, since oxaloacetate produced by pyruvate carboxylation cannot enter rapidly into the citric acid cycle in such conditions (acetyl-CoA is needed) and must go to the biosynthesis of amino acids. An indirect support for this hypothesis might be that similar structures are present downstream of the *Bacillus sphaericus* biotin operon promoter and upstream of the potential *ypjD*-*birA* operon promoter (Fig. 4).

The two operons in the *kdgRKAT-kduDI* divergon resemble the classical *lac* operon case from Gram-negative bacteria (Jacob & Monod, 1961). The putative regulator KdgR is highly homologous to the CcpA catabolite regulator of *B. subtilis* and therefore belongs to the LacI family of regulators. Preliminary data indicated that KdgR and CcpA are both negative regulators of transcription of the *kdgRKAT* operon (P. Pujic, personal communication). It would be interesting to identify the structural features which specify the difference in the interaction of KdgR and CcpA with the *kdg* and other regulatory regions.

Conclusions

We have described the application of a sequencing strategy of a YAC insert by direct subcloning into M13 and by using random and directed selection of the templates to be sequenced. Since the *B. subtilis* genome contains sequences which are difficult or impossible to clone in *E. coli*, the direct sequencing of PCR products appears to be the most efficient directed sequencing approach. An optimal practical strategy can be proposed: (i) random sequencing, which becomes ineffective when the redundancy of accumulated raw sequence data approaches 3-4; and (ii) directed sequencing of PCR products corresponding to the gaps between contigs, ordered by combinatorial PCR strategies. This second step allows us to overcome the bias of the M13 libraries due to the 'unclonability' of some genome fragments in bacteriophage M13 (Glaser *et al.*, 1993; Sorokin *et al.*, 1993; Ogasawara *et al.*, 1994). We do not see any obvious arguments against the application of this approach to the sequencing of a whole small genome (less than 10 Mb). Long-target PCR (Barnes, 1994; Cheng *et al.*, 1994) greatly extends the possibilities for the use of combinatorial PCR. The feasibility of such an approach for the sequencing of a complete bacterial genome is supported by the data presented on YAC15-6B and by the sequencing of the *B. subtilis* genome regions contained in the YAC10-9 and YAC15-132 clones of the same collection, which is now being carried out in this laboratory. Our results also demonstrate that YAC-based collections of large genomes, for example the human genome, can be used directly for sequencing of regions of interest.

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