Organization of the European *Bacillus subtilis* genome sequencing project

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Introduction

Several genome sequencing projects of model organisms have recently been initiated, including several prokaryotes such as *Escherichia coli* (Sofia *et al.*, 1994), *Bacillus subtilis*, *Mycobacterium leprae* (Bergh & Cole, 1994) and *Mycoplasma genitalium* (Peterson *et al.*, 1993). The genome sequencing of three model organisms is presently supported by the European Commission (EC) under the Biotechnology programme: *Saccharomyces cerevisiae* (Oliver *et al.*, 1992; Vassarotti & Goffeau, 1992; Dujon *et al.*, 1994), *Arabidopsis thaliana* (Magnien *et al.*, 1992) and *B. subtilis* (Glaser *et al.*, 1993; Sorokin *et al.*, 1993; Azevedo *et al.*, 1993b; Daniel & Errington, 1993; Scotti *et al.*, 1993). The genome sequencing of a fourth model organism, *Caenorhabditis elegans*, was initiated as a British–American collaborative project (Wilson *et al.*, 1994).

The advantages of such a systematic approach in the case of *B. subtilis* have recently been described (Glaser *et al.*, 1993; Sorokin *et al.*, 1993). It allows us to get a complete blueprint of the genetic content of an organism and it contributes key information to the understanding of molecular evolution. Gram-negative and Gram-positive bacteria diverged more than 2 billion years ago (Woese, 1987), underscoring the importance of sequencing in parallel the genomes of both *E. coli* and *B. subtilis*.

The participants

In September 1989 a consortium of five European laboratories started a joint project aimed at developing the physical map, constructing appropriate DNA libraries and launching on a pilot scale the systematic sequencing of the *B. subtilis* genome (Kunst & Devine, 1991). These

laboratories, listed as numbers 1–5 in Table 1, were supported by the European Commission (EC) for an 18 month period in the framework of the SCIENCE programme. The coordinator during this stage of the project was R. Dedonder (Institut Pasteur, Paris, France), who is presently participating in the project as a scientific adviser.

In August 1993 four additional laboratories (numbers 6–9, Table 1) joined the project in the frame of the BIOTECHNOLOGY programme of the EC. The laboratory of D. Karamata (laboratory number 10, Table 1) participates in this project as a non-European Union member using funds obtained from the Swiss government. A new contract allowing participation of seven additional laboratories has recently been finalized with the EC (laboratory numbers 11–17, Table 1). A Finnish–American Company, Genencor (San Francisco, USA) participates in the European project using its own funds.

Different DNA regions were assigned to these laboratories (see below); each laboratory is in charge of both cloning and sequencing of its assigned DNA region and chooses its own strategy to achieve this goal.

Thus the original project has grown from a core of five laboratories into a major European sequencing initiative carried out in close cooperation with the Japanese project involving the participation of seven laboratories (Ogasawara *et al.*, 1995). Since detailed genetic and physical maps are available for the *B. subtilis* chromosome (Amjad *et al.*, 1990; Itaya & Tanaka, 1991; Anagnostopoulos *et al.*, 1993), it was possible to assign precisely DNA regions flanked by well identified genetic markers or – whenever possible – by already sequenced genes to the different participating laboratories as indicated in Fig. 1. This international subdivision of the genome has been achieved by direct negotiation between the scientists who had obtained funds to carry out sequencing projects, taking into account individual scientific interests in specific DNA

* Administrative coordinator of the project.

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Table 1. Contact addresses of the European laboratories participating in the *B. subtilis* genome sequencing project

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9	G. Grandi	Eniricerca S.P.A., Via Maritano, 26, San Donato Milanese, 20097 Milan, Italy Fax: +39 2 520 4422 e-mail: ergrandi@galactica.it
10	D. Karamata & C. Mauel	Institut de Génétique et de Biologie Microbiennes, 19 rue César Roux, 1005 Lausanne, Switzerland Fax: +41 21 3 206 078 e-mail: CMauel@IGBM.UNIL.CH
11	J. Haiech & F. Denizot	CNRS, BP 71, Laboratoire de Chimie Bactérienne, 31 Chemin Joseph Aguiar, 13402 Marseille Cedex 09, France Fax: +33 91 71 89 14 e-mail: DENIZOT@LCB.CNRS-MRS.FR
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Table 1 (cont.)

Laboratory number	Principal investigator(s)	Contact address
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15	B. Joris	Université de Liège, Centre d'Ingénierie des Protéines, Institut de Chimie B6, Sart Tilman, B-4000 Liège, Belgium Fax: +32 41 66 33 64 e-mail: ALF@NMR.CIP.ULG.AC.BE
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Fig. 1. The DNA regions assigned to the laboratories participating in the international *B. subtilis* genome sequencing project (see Table 1). The names of the principal investigators of these laboratories are indicated and the assigned DNA regions are presented as black bars flanked by the indicated genetic markers.

Table 2. *B. subtilis* genes identified and their putative function

The classification is similar to the one proposed by Riley (1993).

I. Metabolism of small molecules

(intermediary metabolism, biosynthesis and degradation of small molecules including sugars, cofactors, fatty acids, antibiotics, electron transport)

Gene name	Similarities	Reference
<i>ipa-1r</i>	Ketoacyl reductase	Glaser <i>et al.</i> (1993)
<i>ipa-3r</i>	Acyl carrier protein	
<i>ipa-26d (thiC)</i>	Protein involved in thiamine metabolism	
<i>ipa-58r</i>	Aldehyde dehydrogenase	
<i>rocA</i>	Aldehyde dehydrogenase	
<i>ipa-82d</i>	Glucose dehydrogenase	
<i>ipa-85d</i>	Aspartate aminotransferase	
<i>ipa-86r</i>	Acetoacetyl-CoA reductase	
<i>qoxA,B,C,D</i>	Subunits of quinol oxidase	Sorokin <i>et al.</i> (1993)
<i>ribA,B,G,H</i>	Riboflavin synthesis	
<i>lysA</i>	DAP decarboxylase	
<i>aroC</i>	3-Dehydroquinase	
<i>serA</i>	Phosphoglycerate dehydrogenase	O'Reilly & Devine (1994)
<i>orfX14</i>	Cytochrome <i>c</i> biogenesis protein	
<i>argC-F</i>	Arginine biosynthesis	Santana <i>et al.</i> (1994)
<i>atp</i>	F ₀ F ₁ ATP synthase	
<i>ipa-88d</i>	Phosphotransacetylase	Glaser and others (unpublished)
<i>glyC</i>	Glycine (serine) hydroxymethyltransferase	
<i>upp</i>	Uracil phosphoribosyltransferase	Scotti <i>et al.</i> (1993)
<i>pksX</i>	Polyketide synthase	

II. Broad regulatory functions

(including putative regulatory proteins with unidentified targets)

Gene name	Similarities	Reference
<i>ipa-24d, ipa-89d</i>	Regulatory proteins of the LysR family	Glaser <i>et al.</i> (1993)
<i>orfX17</i>	PhoP regulatory protein	Sorokin <i>et al.</i> (1993)
<i>orfX18</i>	PhoR regulatory protein	Sorokin <i>et al.</i> (1993)

III. Macromolecular metabolism

(protein, RNA and DNA metabolism)

Gene name	Similarities	Reference
<i>tyrZ</i>	Tyrosyl-tRNA synthase	Glaser <i>et al.</i> (1993)
<i>ipa-57d</i>	Uracil-DNA glycosylase	Glaser <i>et al.</i> (1993)
<i>ppiB</i>	Peptidyl prolyl isomerase	Sorokin <i>et al.</i> (1993)

regions. The decision has also been taken to concentrate all efforts on the same strain: *B. subtilis* strain 168.

Cloning and sequencing

The first results obtained gave us some insight into the requirements for the cloning and analysis of large genome segments. It now appears that the main bottleneck does not occur at the sequencing level but rather at two other levels: the cloning step and data interpretation.

Different strategies were successfully applied to obtain appropriate ordered sublibraries covering portions of the

genome: (i) cloning in plasmid, λ and YAC vectors; (ii) ordering of λ clones using YAC recombinant vectors; (iii) use of integrative plasmids and marker rescue in genome walking experiments; and (iv) *in vitro* amplification using PCR.

Since *B. subtilis* fragments cloned in *E. coli* are often unstable, attempts to construct complete libraries of overlapping λ or cosmid clones failed. However, *B. subtilis* DNA fragments which were not present in a constructed λ library could be cloned by chromosome walking (Kunst & Devine, 1991; Glaser *et al.*, 1993). This alternative strategy is based on either plasmid rescue or marker

Table 2 (cont.)

IV. Cell structure
(surface structures and polysaccharides, flagella)

Gene name	Similarities	Reference
<i>ipa-5r</i>	Alanine activating enzyme	Glaser <i>et al.</i> (1993)
<i>ipa-12d</i>	LPS 1,3-galactosyltransferase	
<i>ipa-36d</i>	Galactose-1-P uridyltransferase	
<i>ipa-62r</i>	Glutenin	
<i>ipa-71d</i>	Sugar-nucleotidyltransferase	
<i>ipa-72d</i>	UDP-N-acetylglucosamine epimerase	
<i>ipa-73d</i>	Glucose-1-P thymidyltransferase	Yanouri <i>et al.</i> (1993)
<i>phpB</i>	Penicillin binding protein	
<i>orfX10 (dacB)</i>	Penicillin binding protein	Sorokin <i>et al.</i> (1993)
<i>orfX16</i>	Chloroplast protein	Sorokin <i>et al.</i> (1993)
<i>murE, mraY, murD</i>	N-Acetylmuramic acid-peptide synthesis	Daniel & Errington (1993)
<i>flfF,G,H,I</i>	Flagellar biosynthesis	Albertini <i>et al.</i> (1991)
<i>flfJ,K,L, flgG</i>	Flagellar biosynthesis	

V. Cellular processes
(transport/binding proteins, cell division, chemotaxis, protein secretion)

Gene name	Similarities	Reference
<i>ipa-16d</i>	PTS Enzyme II β -glucoside	Glaser <i>et al.</i> (1993)
<i>rocC</i>	Amino acid permease	
<i>ipa-42d</i>	FtsW, SpoVE	
<i>sipS</i>	Signal peptidase	Sorokin <i>et al.</i> (1993) and van Dijk <i>et al.</i> (1992)
<i>orfX20</i>	Iron transport, FecI regulatory protein	Sorokin <i>et al.</i> (1993)
<i>orfX21</i>	Iron transport, FecR regulatory protein	Sorokin <i>et al.</i> (1993)
<i>orf6</i>	Methyl-accepting chemotaxis receptor protein	Albertini <i>et al.</i> (1991)

VI. Sporulation

Gene name	Similarities	Reference
<i>spoVAF</i>	Spore germination protein I	Sorokin <i>et al.</i> (1993)
<i>spoIVA</i>		Stevens <i>et al.</i> (1992)
<i>spoVD</i>		Daniel <i>et al.</i> (1994)

rescue. In the first method, an *E. coli* plasmid, a pDIA5304 derivative containing a *B. subtilis* DNA fragment, is integrated through homologous recombination in the *B. subtilis* chromosome, thus conferring chloramphenicol resistance to the host. This recombinant plasmid DNA, as well as adjacent *B. subtilis* DNA sequences, is then excised from the chromosome using a restriction enzyme with a single site within the polylinker of the plasmid and infrequent sites in *B. subtilis* chromosomal DNA. After circularization with T4 DNA ligase, the recombinant plasmid containing adjacent DNA sequences is cloned in *E. coli* strain TP611 (Glaser *et al.*, 1993). In this strain ColE1 plasmids are maintained at a low copy number, thus avoiding high levels of expression of *B. subtilis* genes. However, cloning of some *B. subtilis* DNA fragments in this host proved unsuccessful. In these cases, a marker rescue method is applied which consists of integrating a selective marker into the *B. subtilis* chromosome *via* a

double cross-over event. This marker, as well as adjacent DNA sequences, is then excised from the chromosome and directly cloned in *B. subtilis* using a replicative plasmid. Finally, in a limited number of cases *B. subtilis* genes were cloned by direct complementation of genetic markers.

The YAC library constructed by S.D. Ehrlich and coworkers covered more than 98% of the *B. subtilis* chromosome (Azevedo *et al.*, 1993a), indicating that the YAC recombinant library covers a larger part of the *B. subtilis* chromosome. The drawback of this method, however, is that the yield of YAC recombinant DNA after purification is usually low.

The European laboratories, numbered from 1 to 10, are each sequencing at least 20 kb of *B. subtilis* genomic DNA every year. The other European groups, numbered from 11 to 18, which have recently joined the consortium, are

also going to contribute at the same level. At this stage of the European project several fragments of more than 20 kb have already been sequenced, including contigs of 127 kb, 105 kb, 60 kb, 50 kb and 42 kb. The average error rate is presently estimated to be lower than 1 per 2000 nucleotides. Appropriate quality controls are implemented to further improve this score. The estimated amounts of *B. subtilis* genomic DNA sequenced by the piecemeal approach or by the systematic approach in the framework of the European project are at present 600 and 500 kb, respectively. By the end of 1996 the European consortium is expected to have contributed 1.5 Mb.

Identified genes

A list of putative genes, which were identified during this initial phase of the European project and which encode putative proteins showing similarities with proteins present in data banks, is presented in Table 2. Additional genes which have recently been identified are also presented in several publications in this issue.

Data handling

A depository representing a collection of verified DNA sequences by the laboratories of the European consortium has been established at the Institut Pasteur. In addition, a specialized database is presently being built (Moszer *et al.*, 1995). This database contains sequences in the depository as well as non-duplicate data concerning *B. subtilis* 168 which are already present in available libraries. Concomitantly, a task-driven, object-oriented database is being constructed allowing fast analysis of unknown sequences (Médigue *et al.*, 1993).

Release of the sequences

Sequences in the depository at the Institut Pasteur are kept confidential for a period of 9 months following submission until verification has been completed satisfactorily. This process includes verification, under the responsibility of two of us (A.D. and F.K.), and resequencing up to 15% of the submitted sequence by a second laboratory of the network to minimize errors.

An agreement has been reached among the members of the network allowing them to forward, immediately after submission to the depository, the determined DNA sequences to other members of the European-Japanese consortium.

Finally, one of us (A.V.) will transmit a document containing similarities of putative *B. subtilis* proteins with proteins present in international data banks to European industries every 3 months to facilitate contacts and to establish possible bilateral interactions.

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