Extremophiles (1999) 3:29–34

ORIGINAL PAPER

Hideto Takami · Kaoru Nakasone · Naotake Ogasawara
Chie Hirama · Yuka Nakamura · Noriaki Masui
Fumie Fuji · Yoshihiro Takaki · Akira Inoue
Koki Horikoshi

Sequencing of three lambda clones from the genome of alkaliphilic *Bacillus* sp. strain C-125

Received: April 17, 1998 / Accepted: June 23, 1998

Abstract The nucleotide sequences of three independent fragments (designated no. 3, 4, and 9; each 15–20 kb in size) of the genome of alkaliphilic *Bacillus* sp. C-125 cloned in a λ phage vector have been determined. Thirteen putative open reading frames (ORFs) were identified in sequenced fragment no. 3 and 11 ORFs were identified in no. 4. Twenty ORFs were also identified in fragment no. 9. All putative ORFs were analyzed in comparison with the BSORF database and non-redundant protein databases. The functions of 5 ORFs in fragment no. 3 and 3 ORFs in fragment no. 4 were suggested by their significant similarities to known proteins in the database. Among the 20 ORFs in fragment no. 9, the functions of 11 ORFs were similarly suggested. Most of the annotated ORFs in the DNA fragments of the genome of alkaliphilic *Bacillus* sp. C-125 were conserved in the *Bacillus subtilis* genome. The organization of ORFs in the genome of strain C-125 was found to differ from the order of genes in the chromosome of *B. subtilis*, although some gene clusters (*ydh*, *yqi*, *yer*, and *yts*) were conserved as operon units the same as in *B. subtilis*.

Key words Alkaliphile · *Bacillus* sp. C-125 · Genome analysis

Introduction

Alkaliphilic bacteria generally, cannot grow or grow poorly under neutral pH conditions, but grow well at a high pH, above 9.5. Since 1969, we have isolated a great number of alkaliphilic bacteria from various environments and have purified many alkaline enzymes. Over the past two decades, our studies have focused on the enzymology, physiology, and molecular genetics of alkaliphilic microorganisms (Horikoshi 1991). Industrial applications of these microbes have been investigated, and some enzymes have been commercialized. It is well recognized that these commercial enzymes have brought great advantages to industry (Horikoshi 1996). *Bacillus* sp. C-125 was isolated as a β-galactosidase (Ikura and Horikoshi 1979) and xylanase producer (Honda et al. 1985).

Recently, whole genome analysis of *Bacillus subtilis*, which is closely related to strain C-125 except for the alkaliphilic phenotype, has been completed in a project involving collaboration between Japan and the European Community (Kunst et al. 1997). The size and GC content of the C-125 chromosome are almost identical to those of *B. subtilis* (4.2 Mb and 43.7 mol%, respectively). Knowledge of the complete nucleotide sequence of the *B. subtilis* genome will definitely facilitate identification of common functions in bacilli, and such data will help us in analysis of the C-125 genome. As the first step in analysis of the C-125 genome, to compare the gene structure and organization with those of *B. subtilis*, we attempted to construct a λ phage library of the C-125 chromosome for large-scale nucleotide sequencing.

In this paper, we report the sequence determination and analysis of three large fragments (15–20 kb) of the genome of alkaliphilic *Bacillus* sp. C-125 cloned in a λ phage vector.

Communicated by T. A. Krulwich

H. Takami (✉) · K. Nakasone · C. Hirama · Y. Nakamura · N. Masui · F. Fuji · Y. Takaki · A. Inoue · K. Horikoshi
Deep-Sea Microorganisms Research Group, Japan Marine Science and Technology Center, 2-15 Natsushima, Yokosuka 237-0061, Japan
Tel. +81-468-67-3895; Fax +81-468-66-6364
e-mail: takamih@jamstec.go.jp

N. Ogasawara
Graduate School of Biological Sciences, Nara Institute of Science and Technology, 8916-5 Takayama, Ikoma 630-01, Japan

Materials and methods

Bacterial strains, phage, plasmid, and media

Alkaliphilic *Bacillus* sp. C-125 was used as a standard alkaliphilic *Bacillus* strain. *E. coli* XL1-Blue MRA was used as the host strain for preparation of the DNA library from *Bacillus* sp. C-125. λDASHII (Stratagene, La Jolla, CA,

USA), M13mpRF, and pUC18 (Pharmacia Biotech, Tokyo, Japan) were used as vectors for preparation of the library. Strain C-125 was grown aerobically at 37°C in N-II medium (pH 7.5) consisting of 1% soluble starch, 0.5% polypeptone, 0.5% yeast extract, 0.1% K_2HPO_4, 0.02% $MgSO_4 \cdot 7H_2O$, and 2% NaCl (Takami et al. 1992). *E. coli* was grown under conditions described previously (Sambrook et al. 1989).

Preparation of DNA libraries from alkaliphilic *Bacillus* sp. C-125

Chromosomal DNA was isolated from *Bacillus* sp. C-125 as described previously (Saito and Miura 1963). DNA fragments partially digested with *Sau*3AI and treated with bacterial alkaline phosphatase (BAP) were ligated to a λDASHII/*Bam*HI vector. Three λ clones (λ no. 3, no. 4, and no. 9) were randomly selected for sequencing analysis among the original recombinant phages prepared using the in vitro packaging system from Stratagene. The DNA fragments cloned in λ phage were amplified by long accurate polymerase chain reaction (PCR) using LA PCR Kit Ver. 2 (Takara Shuzo, Kyoto, Japan). A 7.5 μg aliquot of PCR fragments was sonicated for 10–20 s with a Branson Sonifier (Heat Systems, Farmingdale, NY, USA). The sonicated DNA fragments were blunt-ended using a DNA blunting kit (Takara Shuzo) and fractionated by 1% agarose gel electrophoresis. DNA fragments 1–2 kb in length were excised from the gel and eluted by the freeze-squeeze method (Thuring et al. 1975). The DNA recovered was ligated to the *Sma*I site of vector M13mp19 or pUC18 that had been previously treated with BAP, and introduced into competent XL1-Blue cells by the standard method (Sambrook et al. 1989).

DNA sequencing

M13 phage clones isolated were grown in 2× YT medium for 8 h in the host XL1-Blue. The M13 single-strand DNA was purified by means of a QIAprep 96 M13 kit (QIAGEN, Santa Claria, CA, USA). The DNA fragment inserted into pUC18 was amplified by PCR using M13-20 and reverse primers. PCR fragments, treated with exonuclease I and shrimp alkaline phosphatase (Pharmacia Biotech, Tokyo, Japan) to eliminate excess primers in the PCR reaction mixture, were used for sequencing analysis as template DNA. Sequencing was performed with a DNA sequencer ABI PRISM 377 using a Taq Dye Primer and Dye Terminator Cycle Sequencing Kit (Perkin-Elmer, Nor walk, CT, USA). Several primers were prepared and used to sequence the DNA covering the gap region.

Computer analysis

DNA sequences determined by means of the ABI sequencer were assembled into contigs using a personal computer with AutoAssembler Ver 1.40 (Perkin Elmer). The sequences were analyzed for the locations of possible ORFs using the GeneWorks program (version 2.5.1N) from IntelliGenetics (Campbell, CA, USA). The deduced amino acid sequences of the identified ORFs were compared with sequences reported previously in a search of the BSORF database (http://bacillus.tokyo-center.genome.ad.jp:8008/) and the nonredundant protein databank using the FASTA and BLAST network service (GenomeNet WWW server, http://www.genome.ad.jp). The sequences of the three DNA fragments sequenced in this report were deposited at DDBJ with the accession numbers AB011836 for fragment no. 3, AB011838 for fragment no. 4, and AB011837 for fragment no. 9.

Results and discussion

ORF analysis of sequenced fragment no. 3

The determined sequence of fragment no. 3 was searched for ORFs beginning with an ATG, GTG, or TTG start codon and comprising more than 100 codons. Thirteen putative ORFs were identified on the basis of the presence of a preceding Shine-Dalgano (SD) sequence in each instance (Fig. 1A and Table 1). The SD sequence was complementary to one found at the 3′-end (UCUUUCCUCCACUAG . . .) of the 16S rRNA of alkaliphilic *Bacillus* sp. C-125 (Takami et al. 1997). The initiation codons for one of the ORFs was TTG, two of them were GTG, and the other 10 ORFs started with ATG (Table 1). The deduced amino acid sequences of these ORFs were used for homology analysis in searches of the BSORF, SWISS-PROT, and PIR protein databases using the FASTA program. Seven ORFs showed similarities to unknown *B. subtilis* proteins.

Two ORFs, ORF 2 and 3, were similar to the products of the *yerN* and *yerM* genes of *B. subtilis*, with 87% and 84% identity, respectively, and these genes seemed to constitute an operon with *opuE* at position 727–730 kb of the *B. subtilis* chromosome (Fig. 1a and Table 1). The ORF 6 product was similar to the *B. subtilis rapG* product (33% identity) that functions as a response regulator aspartate phosphatase. In addition, the ORF 9 and ORF 12 products were similar to the *B. subtilis ahpF* and *rocF* products, which function as alkyl hydroperoxide reductase NADH dehydrogenase and arginase, respectively, as shown in Table 1. ORF 5 showed 25% identity to the hypothetical protein of *Methanococcus jannaschii* (Bult et al. 1996). The ORF 13 product showed no significant similarity to any other proteins so far reported. On the other hand, ORF 1 showed 26% similarity to a transposase produced by the gram-negative bacterium *Bordetella parapertussis* (Van der Zee et al. 1993). This finding suggests that this kind of transposable element may be involved in gene transposition in the genome of alkaliphilic *Bacillus* sp. C-125, considering that the gene organization in this region differs totally from that of *B. subtilis*.

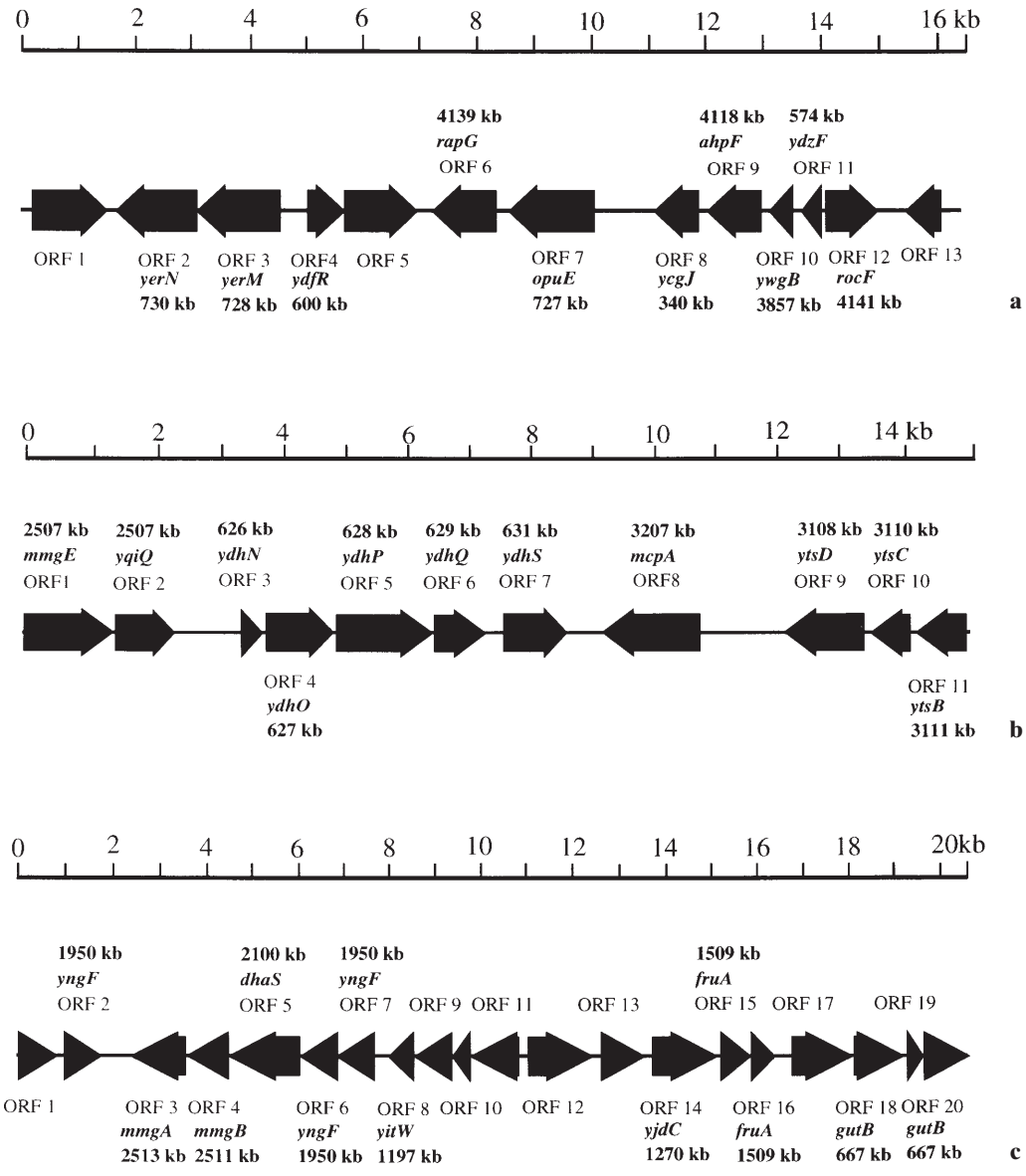

Fig. 1a–c. ORF organization of the genomic fragments of alkaliphilic *Bacillus* sp. C-125 cloned in λ phage. **a** DNA fragment no. 3; **b** DNA fragment no. 4; **C** DNA fragment no. 9. The annotated ORFs are shown with the ORF position in the *B. subtilis* chromosome

ORF analysis of sequenced fragment no. 4

The determined sequence of fragment no. 4 was searched for ORFs comprising more than 100 codons, and 11 putative ORFs were identified (Fig. 1b and Table 2). The initiation codons for two of these ORFs were TTG and GTG, and the other 7 ORFs started with ATG (Table 2). Seven ORFs from ORF 3 to ORF 7 showed significant similarities to the products of the *ydhN*, *ydhO*, *ydhP*, *ydhQ*, and *ydhS* of *B. subtilis*, which may constitute a large operon involved in degradation and incorporation of mannan (Sadaie et al. 1997). ORF 8 showed comparatively low similarity (28%) to the *mcpA* gene product of *B. subtilis* among the ORFs in this region. Three ORFs from ORF 9 to 11, which are similar to the series of *yts* (*ytsD*, *ytsC*, and *ytsB*) gene products of *B. subtilis*, were also conserved in the chromosome of strain C-125. ORF 1 was found to be similar to the product of the *mmgE* gene of *B. subtilis*, with 74% identity. The ORF 2 was similar to the product of *yqiQ* of *B. subtilis* that is adjacent to *mmgE*.

ORF analysis of sequenced fragment no. 9

There were 20 ORFs comprising more than 100 codons in the sequenced fragment no. 9 (Fig. 1c and Table 3). The initiation codons for 3 of these ORFs were TTG, 1 of them was GTG, and the other 16 ORFs started with ATG (Table 3). Seven ORFs (ORFs 1, 9, 10, 11, 12, 13, and 20) did not show significant similarity to any protein in the protein databases. Three ORFs, ORF 2, ORF 6, and ORF 7, were found to be similar to the *yngF* product whose function seems to be enoyl CoA hydratase. The products of ORFs 3 and 4 showed about 40% identity to the products

Table 1. ORF analysis of 16.8 kb fragment of *Bacillus* sp. C-125 genome in λ phage clone no. 3

ORF	Characteristics	SD/initiation codon
1. (nt 201–1523; length 621; 207 aa)	Similar to *Bordetella parapertussis* transposase for insertion sequence element (26% similarity) (406 aa)	AAAAGGCTCTACAATAA**ATG**
2. (nt 3109–1682; length 1428; 476 aa)	Similar to *B. subtilis yerN* (87% identity) (nt 730192–731445; 417 aa; function unknown)	AGGGGTGAAAGCAG**ATG**
3. (nt 4580–3126; length 1473; 485 aa)	Similar to *B. subtilis yerM* (84% identity) (nt 728544–730001; 485 aa; function unknown)	GGAAcGAGCTTA**ATG**
4. (nt 5118–5726; length 642; 203 aa)	Similar to *B. subtilis ydfR* (40% identity) (nt 600620–599943; 225 aa; function unknown)	AAAGGAGGAACAACCT**TTG**
5. (nt 5743–7014; length 1272; 424 aa)	Similar to hypothetical protein MJ0305 *Methanococcus jannaschii* (25% identity) (395 aa)	AGGATGATAGACGA**ATG**
6. (nt 8418–7306; length 1113; 371 aa)	Similar to *B. subtilis rapG* (33% identity) (nt 4139469–4140566; 365 aa; response regulator aspartate phosphatase)	AAAAGGggAGGGGTAAACG**GTG**
7. (nt 10174–8672; length 1503; 501 aa)	Similar to *B. subtilis opuE* (53% identity) (nt 727824–726346; 492 aa; proline transporter)	AGGAGTGAGTGAACT**GTG**
8. (nt 12022–11258; length 765; 252 aa)	Similar to *B. subtilis ycgJ* (35% identity) (nt 340871–340185; 228 aa; function unknown)	AAAAtGGAGGGGG**ATG**
9. (nt 13118–12204; length 933; 305 aa)	Similar to *B. subtilis ahpF* (25% similarity) (nt 4118735–4120264; 509 aa; alkyl hydroperoxide reductase (large subunit) NADH dehydrogenase EC 1.6.99.3)	GGAGGTGCTTAAACGT**ATG**
10. (nt 13700–13305; length 396; 132 aa)	Similar to *B. subtilis ywgB* (27% identity) (nt 3857686–3857216; 156 aa; function unknown)	AGGTGAACACTGCATC**ATG**
11. (nt 14212–13874; length 339; 113 aa)	Similar to *B. subtilis ydzF* (54% identity) (nt 574149–573820; 109 aa; function unknown)	AGGAAAGTGTGAAT**ATG**
12. (nt 14319–15230; length 912; 304 aa)	Similar to *B. subtilis rocF* (29% identity) (nt 4141809–4140919; 296 aa; arginase)	AAAGGAGTGTCAATC**ATG**
13. (nt 16339–15737; length 603; 201 aa)	Unknown	AAAGGAGGCGTACATT**ATG**

The beginning and end nucleotide numbers of each ORF in the DNA fragment of the chromosome of alkaliphilic *Bacillus* sp. C-125 and the size of the putative protein are shown. The putative initiation codons and SD sequences are shown in bold and underlined, respectively. The protein homologous to each ORF is shown with the size of the protein and similarity %. In the case of the *B. subtilis* protein, the beginning and end nucleotide numbers for each protein are also shown

Table 2. ORF analysis of 15 kb fragment of *Bacillus* sp. C-125 genome in λ phage clone no. 4

ORF	Characteristics	SD/initiation codon
1. (nt 21–1418; length 1398; 466 aa)	Similar to *B. subtilis mmgE* (74% identity) (nt 2508875–2507457; 472 aa; function unknown)	AGAGAACGACGGATCTT**TTG**
2. (nt 1439–2338; length 900; 300 aa)	Similar to *B. subtilis yqiQ* (73% identity) (nt 2507439–2506534; 301 aa; function unknown)	AGGAGGACACGAAA**ATG**
3. (nt 3458–3784; length 327; 109 aa)	Similar to *B. subtilis ydhN* (59% identity) (nt 626649–626981; 110 aa; function unknown)	AGGAGGACGTTA**ATG**
4. (nt 3813–4955; length 1143; 381 aa)	Similar to *B. subtilis ydhO* (78% identity) (nt 627000–628328; 442 aa; function unknown)	GGAGcGAACGTCCCA**ATG**
5. (nt 4965–6473; length 1509; 503 aa)	Similar to *B. subtilis ydhP* (80% identity) (nt 628346–629743; 465 aa; β-glucosidase (EC3.2.1.21))	AAAGGGAT**ATG**
6. (nt 6515–7312; length 798; 266 aa)	Similar to *B. subtilis ydhQ* (70% identity) (nt 629886–630599; 237 aa; function unknown)	GGAGAATA**ATG**
7. (nt 7674–8618; length 945; 315 aa)	Similar to *B. subtilis ydhS* (60% identity) (nt 631524–632471; 315 aa; mannose-6-phosphate isomerase (EC 5.3.1.8))	GGAGACTGT**ATG**
8. (nt 10726–9191; length 1536; 512 aa)	Similar to *B. subtilis mcpA* (28% similarity) (nt 3207224–3205239; 661 aa; methyl-accepting chemotaxis protein)	AAAGGATTGATCAA**GTG**
9. (nt 13323–12088; length 1236; 412 aa)	Similar to *B. subtilis ytsD* (64% similarity) (nt 3110418–3108478; 646 aa; function unknown)	AGGTGTGTTAG**GTG**
10. (nt 14063–13458; length 606; 202 aa)	Similar to *B. subtilis ytsC* (80% identity) (nt 3111169–3110408; 253 aa; function unknown)	AGGAGTGAAAATAC**ATG**
11. (nt 14965–14183; length 783; 261 aa)	Similar to *B. subtilis ytsB* (64% similarity) (nt 3112275–3111271; 334 aa; function unknown)	AGGaAtGGGACAAAGAT**TTG**

The beginning and end nucleotide numbers of each ORF in the DNA fragment of the chromosome of alkaliphilic *Bacillus* sp. C-125 and the size of the putative protein are shown. The putative initiation codons and SD sequences are shown in bold and underlined, respectively. The protein homologous to each ORF is shown with the size of the protein and similarity %. In the case of the *B. subtilis* protein, the beginning and end nucleotide numbers for each protein are also shown

Table 3. ORF analysis of 20 kb fragment of *Bacillus* sp. C-125 genome in λ phage clone no. 9

ORF	Characteristics	SD/initiation codon
1. (nt 51–836; length 786; 262 aa)	Unknown	AAAGcAGGCGTCATT**ATG**
2. (nt 1001–1777; length 777; 259 aa)	Similar to *B. subtilis yngF* (56% identity) (nt 1951265–1950483; 260 aa; enoyl CoA hydratase)	AGGAGGACAATCG**ATG**
3. (nt 3521–2412; length 1110; 370 aa)	Similar to *B. subtilis mmgA* (38% identity) (nt 2513271–2512090; 391 aa; acetyl-CoA acetyl transferase)	AGGGGGCTAGTA**ATG**
4. (nt 4387–3539; length 849; 283 aa)	Similar to *B. subtilis mmgB* (42% identity) (nt 2511940–2511206; 244 aa; 3-hydroxybutyril-CoA dehydrogenase EC 1.1.1.157)	AGGaAGGTTGCT**ATG**
5. (nt 5919–4456; length 1464; 488 aa)	Similar to *B. subtilis dhaS* (44% identity) (nt 2099836–2101323; 495 aa; aldehyde dehydrogenase)	AAAGGAGCGATTCCA**TTG**
6. (nt 6738–5959; length 780; 260 aa)	Similar to *B. subtilis yngF* (32% identity) (nt 1951265–1950483; 260 aa; enoyl CoA hydratase)	AGGAGAGTAAAG**ATG**
7. (nt 7514–6744; length 771; 257 aa)	Similar to *B. subtilis yngF* (39% identity) (nt 1951265–1950483; 260 aa; enoyl CoA hydratase)	AGGAGATGG**ATG**
8. (nt 8336–7833; length 504; 168 aa)	Similar to *B. subtilis yitW* (35% similarity) (nt 1191727–1192035; 102 aa; function unknown)	AGGGGGAGTGAAT**GTG**
9. (nt 9128–8352; length 777; 259 aa)	Unknown	AAGAGGCTAAAACGA**ATG**
10. (nt 9521–9165; length 357; 119 aa)	Unknown	AAAGGAGAAGGATG**ATG**
11. (nt 10528–9548; length 981; 327 aa)	Unknown	AAAAGGAGTTGTCGAT**ATG**
12. (nt 10779–12113; length 1335; 445 aa)	Unknown	AGGAGGGTGCAATTG**ATG**
13. (nt 12302–13195; length 894; 298 aa)	Unknown	AAAcAGGGTGGGATGAATC**TTG**
14. (nt 13404–14735; length 1332; 444 aa)	Similar to *B. subtilis yjdC* (24% similarity) (nt 127012–1272048; 648 aa; function unknown)	AGGgAGGTC**ATG**
15. (nt 14839–15453; length 615; 205 aa)	Similar to *B. subtilis fruA* (33% similarity) (nt 1508661–1510568; 635 aa; phosphotransferase system (PTS) fructose-specific enzyme IIBC component)	AAAAGAacAGGTC**ATG**
16. (nt 15480–15959; length 480; 160 aa)	Similar to *B. subtilis fruA* (29% similarity) (nt 1508661–1510568; 635 aa; phosphotransferase system (PTS) fructose-specific enzyme IIBC component)	AGAGGTGAACTG**ATG**
17. (nt 16333–17589; length 1257; 419 aa)	Similar to *E. coli* PTS system galactitol-specific IIC component (43% identity) (451 aa)	AAAGGGGGAACACC**ATG**
18. (nt 17632–18660; length 1029; 343 aa)	Similar to *B. subtilis gutB* (35% identity) (nt 667183–668244; 353 aa; sorbitol dehydrogenase EC 1.1.1.14)	AAtGGAGGATCGCATG**ATG**
19. (nt 18775–19074; length 300; 100 aa)	Unknown	AAGAGGCA**TTG**
20. (nt 19119–20036; length 918; 306 aa)	Similar to *B. subtilis gutB* (39% identity) (nt 667183–668244; 353 aa; sorbitol dehydrogenase EC 1.1.1.14)	AGGAGTGACAACCA**ATG**

The beginning and end nucleotide numbers of each ORF in the DNA fragment of the chromosome of alkaliphilic *Bacillus* sp. C-125 and the size of the putative protein are shown. The putative initiation codons and SD sequences are shown in bold and underlined, respectively. The protein homologous to each ORF is shown with the size of the protein and similarity %. In the case of the *B. subtilis* protein, the beginning and end nucleotide numbers for each protein are also shown

of *mmgA* (acetyl CoA acetyltransferase) and *mmgB* (3-hydroxybutyryl-CoA dehydrogenase) of *B. subtilis*, respectively, which should be adjacent to *mmgE* observed in clone no. 4. (Fig. 1c). ORFs 15 and 16 encoded products similar to the product of *fruA*, the fructose-specific enzyme IIBC that functions as a component of the phosphotransferase system (PTS). On the other hand, the product of ORF 17 showed 43% identity to galactitol-specific IIC, a component of the *E. coli* PTS system, but showed no significant similarity to *B. subtilis* proteins (Fig. 1c and Table 3). In addition, there were 2 ORFs (ORFs 18 and 20) at the terminus of no. 9 fragment (Fig. 1c and Table 3) that were found to be similar to the product of *gutB* of *B. subtilis* whose function is sorbitol dehydrogenase. Thus, the gene organization of this region of the genome of strain C-125 was different from that of *B. subtilis*, and there were many ORFs of unknown function that are not present in the *B. subtilis* chromosome.

We have determined the nucleotide sequences of three independent fragments of the chromosome of alkaliphilic *Bacillus* sp. C-125 and compared them with the sequence of the *B. subtilis* chromosome. The ORFs identified in these fragments were found to be similar to genes in the *B. subtilis* chromosome but were organized in a different order. These results will be helpful in elucidation of the basis of alkaliphily in *Bacillus* and also may further contribute to future identification of the function of some *B. subtilis* genes.

Acknowledgments We thank Dr. Y. Kasahara for his useful suggestions and discussions. Thanks are also due to Mr. Nakade for his technical assistance. We thank Prof. H. Mori for his kindness.

References

Bult CJ, White O, Olsen GJ, Zhou L, Fleischmann RD, Sutton GG, Blake JA, FitzGerald LM, Clayton RA, Gocayne JD, Kerlavage AR, Dougherty BA, Tomb JF, Adams MD, Reich CI, Overbeek R, Kirkness EF, Weinstock KG, Merrick JM, Glodek A, Scott JL, Geoghagen NSM, Venter JC (1996) Complete genome sequence of the methanogenic archaeon, *Methanococcus jannaschii*. Science 273:1058–1073

Honda H, Kudo T, Ikura Y, Horikoshi K (1985) Two types of xylanases of alkalophilic *Bacillus* sp. no. C-125. Can J Microbiol 31:538–542

Horikoshi K (1991) Microorganisms in alkaline environments. Kodansha, Tokyo

Horikoshi K (1996) Alkaliphiles – from an industrial point of view. FEMS Microbiol Rev 18:259–270

Kunst F, Ogasawara N, Moszer I, Albertini AM, Alloni G, Azevedo V, Bertero MG, Bessières P, Bolotin A, Borchert S, Borriss R, Boursier L, Brans A, Braun M, Brignell SC, Bron S, Brouillet S, Bruschi CV, Caldwell B, Capuano V, Carter NM, Choi SK, Codani JJ, Connerton IF, Danchin A, et al. (1997) The complete genome sequence of the gram-positive bacterium *Bacillus subtilis*. Nature (London) 390:249–256

Sadaie Y, Yata K, Fujita M, Sagai H, Itaya M, Kasahara Y, Ogasawara N (1997) Nucleotide sequence and analysis of the *phoB-rrnE-groEL* region of the *Bacillus subtilis* chromosome. Microbiology 143:1861–1866

Saito H, Miura K (1963) Preparation of transforming deoxyribonucleic acid by phenol treatment. Biochim Biophys Acta 72:619–629

Sambrook J, Frisch EF, Maniatis T (1989) Molecular cloning: a laboratory manual, 2nd edn. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

Takami H, Kobayashi T, Aono R, Horikoshi K (1992) Molecular cloning, nucleotide sequence and expression of the structural gene for a thermostable alkaline protease from *Bacillus* sp. no. AH-101. Appl Microbiol Biotechnol 38:101–108

Takami H, Inoue A, Fuji F, Horikoshi K (1997) Microbial flora in the deepest sea mud of the Mariana Trench. FEMS Microbiol Lett 152:279–285

Thuring RW, Sanders JMP, Borst P (1975) A freeze-squeeze method for recovering long DNA from agarose gel. Anal Biochem 66:213–220

Van der Zee A, Agterberg C, van Agterveld M, Peeters M, Mooi FR (1993) Characterization of IS1001, an insertion sequence element of *Bordetella parapertussis*. J Bacteriol 175:141–147