A partial physical map for the chromosome of alkalophilic *Bacillus* sp. strain C-125

KATHARINE J. SUTHERLAND,[†] MICHIZANE HASHIMOTO, TOSHIAKI KUDO* and KOKI HORIKOSHI

The RIKEN Institute and JRDC, Hirosawa 2-1, Wako-shi, Saitama, 351-01, Japan

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***Bacillus* sp. strain C-125 has been chosen as a model alkalophilic bacterium to understand how adaptation to growth at high pH is achieved. To aid genetic analysis, we have started characterization of its genome. By using the two infrequently-cutting restriction endonucleases, *AscI* and *Sse8387I*, in conjunction with pulsed-field electrophoretic techniques, the size of the genome was found to be 3.7 Mb. Southern blot analysis of single, double and partial digests of *Bacillus* sp. strain C-125 DNA, using *AscI*-linking clones, gene probes and purified *Bacillus* sp. strain C-125 restriction fragments, allowed a putative chromosome map to be constructed.**

Introduction

How extremophiles are adapted to living in their extreme environments is one of the most interesting and challenging questions facing microbiologists today. Our group has a specific interest in alkalophilic bacteria and has been using a molecular biological approach to attempt to understand how they are adapted to living at high pH.

Detailed genetic and physical maps are available for the *Bacillus subtilis* 168 chromosome (Piggot & Hoch, 1989; Itaya & Tanaka, 1991). In addition, a physical map of the *Bacillus cereus* chromosome has been constructed (Kolsto *et al.*, 1990). To aid our studies on alkalophiles, we decided to construct a physical map for the alkalophilic *Bacillus* sp. strain C-125 chromosome. Alkalophilic *Bacillus* sp. strain C-125 was chosen as a model alkalophile since (i) it exhibits the standard alkalophilic properties of growing between pH 7.5 and 11, and of requiring Na⁺ for growth; (ii) it grows well on minimal medium, allowing cloning of genes by a selective marker and complementation approach; (iii) an efficient transformation system for cloning in *Bacillus* sp. strain C-125 is available; (iv) several genes have been cloned from this strain and thus can be used as markers on its chromosome map; and finally (v) we believe, on

the basis of GC content (Horikoshi, 1991) and gene sequence comparisons (Kang *et al.*, 1992), that this strain is closely related to *B. subtilis* 168 for which detailed physical and genetic maps are already available (Itaya & Tanaka, 1991; Piggot & Hoch, 1989).

The last few years have seen an escalation in the number of maps reported in the literature. Many different methods have been used to construct these physical maps. For example, two-dimensional pulsed-field gel electrophoresis (2D PFGE) was used to construct a physical map of the *Pseudomonas aeruginosa* PAO genome (Römling *et al.*, 1989; Römling & Tümmler, 1991). Other methods involving the probing of complete or partial digests with gene probes or linking clones (as described by Smith & Condemine, 1990) or with large overlapping restriction fragments have been used successfully (Taylor *et al.*, 1992; Yan & Taylor, 1991; Krause & Mawn, 1990; Chen *et al.*, 1991; Fonstein *et al.*, 1992); and an interesting gene-directed mutagenesis method whereby restriction sites were removed by point mutations was employed by Itaya & Tanaka (1991) to construct the physical map of the *B. subtilis* chromosome. Often a combination of two or more methods is required, as for example in the mapping of the *Chlamydia trachomatis* genome (Birkelund & Stephens, 1992).

This report describes evidence generated by using the two rare-cutting enzymes *AscI* and *Sse8387I* that suggests the size of the *Bacillus* sp. strain C-125 genome is approximately 3.7 Mb. The *AscI* fragments have been partially ordered using (i) *AscI* linking clones, (ii) Southern hybridization of single, double and partial

* Author for correspondence

[†] Present address: Department of Molecular Medicine, Collier Building, Royal Postgraduate School of Medicine, DuCane Road, London, W12 0NN, UK.

digests and (iii) cross-hybridization with purified fragments generated by restriction endonuclease digestion of the *Bacillus* sp. strain C-125 genome. A putative chromosome map is proposed. Hybridization probes derived from cloned genes from *Bacillus* sp. strain C-125 and *B. subtilis* 168 were used to locate the positions of these genes on the proposed *Bacillus* sp. strain C-125 chromosome map.

Methods

Bacterial strains and media. Alkalophilic *Bacillus* sp. strain C-125 10596 (*Trp*⁻*Ura*⁻*Cm*^r) was isolated in our laboratory (Honda *et al.*, 1985; Kudo *et al.*, 1990). It was grown on Horikoshi-II medium (pH 10.3) containing 1% (w/v) soluble starch, 0.5% yeast extract, 0.5% polypeptone, 0.1% K₂HPO₄, 0.02% MgSO₄·7H₂O, and 1% (w/v) Na₂CO₃ (sterilized separately). Cloning of the *Bacillus* DNA was carried out in *Escherichia coli* MV1184 (*ara* Δ(*lac-proAB*) *rpsL* *thi* (φ80 *lacZ* ΔM15) Δ(*srl-recA*)306::Tn10(*ter*) F'[*traD* 36 *proAB lac*^r*lacZ* ΔM15]). *E. coli* was grown on 2 × yeast/tryptone medium (Sambrook *et al.*, 1989).

Plasmids, enzymes and chemicals. Restriction endonucleases *Asc*I, *Pac*I, and pNEB193 vector were from New England Biolabs. *Sse*8387I, other restriction endonucleases and T₄ DNA ligase were from Takara Shuzo Co. The DIG DNA labelling and detection kit was from Boehringer Mannheim. Hybond N⁺ membrane was from Amersham International. InCert agarose and λ DNA ladders were from FMC BioProducts.

Preparation of insert and digestion of chromosomal DNA. Cells of *Bacillus* sp. strain C-125 were harvested from a 10 ml stationary-phase culture. The cells were washed once in TE buffer (10 mM Tris, pH 8.0, 1 mM-EDTA) before being resuspended in 0.25 ml TE buffer. The following were then added to the cell suspension: 20 μl 10 mg ml⁻¹ RNAase A, 200 μl 0.1 mg ml⁻¹ lysozyme, and 1 ml 1.5% (w/v) InCert agarose solution (made in TE buffer). The solution was vortexed briefly before being poured into a single well of a 24-well Falcon tissue culture plate. The plate was placed at -20 °C for 2 min. The solidified gel was then cut into 8–10 pieces using a flamed spatula and these pieces were transferred to 2 ml of a 0.5 M-EDTA (pH 9.5), 1% *N*-lauroylsarcosine solution containing 2 mg proteinase K ml⁻¹. The plugs were incubated at 50 °C for 24 h. They were then washed 5 times with TE buffer (room temperature [RT] for 15 min), once with 0.1 mM-PMSF (RT for 1 h) and 3 times with TE buffer (RT for 15 min). Plugs prepared as above were stored indefinitely in TE buffer at 4 °C until required for digestion. For digestion, a portion of a DNA plug (25–50 μl in volume) was incubated twice in 200 μl of the appropriate 1 × digestion buffer (RT for 15 min) and then in 100 μl 1 × digestion buffer containing 5–20 U restriction endonuclease (at the appropriate temperature for 16 h). Partial digestion was achieved by lowering the concentration of enzyme. For loading, the DNA plug reaction mixture was incubated at 65 °C for 10 min and then loaded directly on to the gel prior to submerging the latter in the electrophoresis buffer.

Electrophoretic analysis. DNA fragments were resolved in a Pharmacia-LKB Pulsaphor apparatus using a hexagonal array of electrodes. The 1% (w/v) agarose gels were subjected to electrophoresis at 14 °C in 0.5 × TBE buffer [0.05 M-Tris/HCl, 0.05 M-boric acid, 2 mM-Na₂EDTA·2H₂O (pH 8.0)] at 160 V for 24 h with a pulse time of 5 to 55 s, varied according to the optimum size of separation desired.

Linking clone isolation. Approximately 2 μg genomic DNA from *Bacillus* sp. strain C-125 prepared by the conventional liquid isolation

method of Saito & Miura (1963) was digested with Enzyme A (*Hind*III or *Eco*RI). The digested DNA was circularized by ligation with T₄ ligase in a reaction volume of 100 μl (16 °C for 16 h). The circularized DNA was precipitated with ethanol and digested with *Asc*I. It was ligated to *Asc*I-bacterial alkaline phosphatase (BAP)-treated pNEB193 vector (16 °C for 16 h) and the ligation mix was then used to transform competent MV1184 cells. Putative linking clones were identified as recombinants which contained two *Asc*I sites and two Enzyme A sites, as the pNEB193 vector contains a *Hind*III and an *Eco*RI site.

Hybridization analysis. Gels were blotted using a vacuum blotter (Pharmacia LKB) on to Hybond N⁺. All blots were probed with digoxigenin-labelled probe in a 30% (w/v) formamide hybridization solution at 42 °C. Subsequent washing and detection steps were carried out according to instructions provided with the Boehringer Mannheim DIG-detection kit.

Uncloned DNA fragments used as probes were purified from low-melting point agarose by heating at 65 °C and extraction using phenol.

Results and Discussion

Selection of endonucleases and determination of chromosome size.

Intact chromosomal DNA of *Bacillus* sp. C-125 was prepared in agarose using a method developed by combining and modifying those of Itaya & Tanaka (1991) and of Römmling *et al.* (1989), used for *B. subtilis* and *Pseudomonas aeruginosa*, respectively.

Restriction endonucleases which recognise an 8 bp sequence were tested for their ability to digest the chromosome. *Pac*I enzyme with the recognition sequence 5'-TTAATTA-3' generated over 50 fragments of less than 50 kb. This result is perhaps not surprising, since in addition to the likelihood that the *Pac*I recognition site occurs frequently in regions upstream and downstream of coding sequences, the *Bacillus* sp. C-125 chromosome (GC content 43.7%) (Horikoshi, 1991) has a slight bias for A and T.

In contrast to results obtained with *B. subtilis*, the chromosome of which contains over 70 *Not*I sites, *Not*I does not digest the *Bacillus* sp. C-125 chromosome. It is likely, however, that *Not*I sites are present on the *Bacillus* sp. C-125 chromosome but in a modified form, since when *Bacillus* sp. C-125 DNA is cloned into *E. coli*, *Not*I digestion occurs (data not shown).

*Sfi*I enzyme (with the recognition sequence 5'-GGCCNNNNNGGCC-3') generated approximately 20 fragments ranging in size from 20 kb to 700 kb. However, the restriction pattern was uneven, and smearing made it hard to identify fragments and their sizes accurately. Smearing may have been a result of the increased reaction temperature (50 °C) required for *Sfi*I digestion stimulating or enhancing activity of nucleases, and the unevenness of the pattern may have been due to the degenerate nature of the *Sfi*I restriction site, some sites being more readily digested than others.

The two remaining enzymes, *Asc*I and *Sse*8387I,

Table 1. Sizes of restriction fragments generated by *AscI* and *Sse8387I* cleavage of the *Bacillus* sp. strain C-125 genome

Band number	Size (kb) of fragments generated with:	
	<i>AscI</i>	<i>Sse8387I</i>
1	570	530
2	530	510
3	440	430
4	420	390
5	390	330
6	330	265
7	250	220
8	170	195
9	150	165
10	125	155
11	120	125
12	80	105
13	45	70
14	35	60
15	25	55
16	20	48
17	3.5	40
18		18
Total	3703.5	3711

generated 17 and 18 resolvable fragments, respectively. The sizes of these fragments were determined by comparison with size standards on a series of PFGE gels (Fig. 1, Table 1). The mean total genome size of *Bacillus* sp. C-125, obtained by totalling the sizes of the *AscI* or *Sse8387I* fragments, is estimated to be 3707 kb. The *B. subtilis* and *B. cereus* genomes have been estimated to be 4.2 and 5.7 Mb in size, respectively (Itaya & Tanaka, 1991; Kolsto *et al.*, 1990). Thus, it appears that even among *Bacillus* species large size differences in the chromosome can be accommodated.

Constructing an *AscI* physical map

As the *AscI* enzyme gave consistently better digestion than the *Sse8387I* enzyme, we decided to construct a physical map of *AscI* sites on the *Bacillus* sp. C-125 chromosome.

Initially, *AscI* linker clones of *Bacillus* sp. strain C-125 DNA were used in an attempt to position *AscI* sites on the map. A linker clone contains DNA which overlaps two adjacent restriction fragments and can be used as a hybridization probe to establish the identity of these two contiguous fragments (Smith & Condemine, 1990).

Linking clone libraries of approximately 100 clones each were constructed using *HindIII* or *EcoRI* as Enzyme A. Plasmid DNA was isolated from individual clones

Fig. 1. PFGE of restriction enzyme digests of total DNA from *Bacillus* sp. strain C-125. (a) Optimal separation conditions for 4–150 kb fragments [1.0% (w/v) agarose, 24 h, 14 °C, 16 V cm⁻¹, 8 s pulse time]. Lane 1, *Sse8387I*; lane 2, lambda standards; lane 3, *AscI*; lane 4, *AscI* plus *Sse8387I*. (b) Optimal separation conditions for 200–500 kb fragments [1.0% (w/v) agarose, 24 h, 14 °C, 6 V cm⁻¹, 40 s pulse time]. Lane 1, lambda standards; lane 2, *AscI*; lane 3, *AscI* plus *Sse8387I*; lane 4, *Sse8387I*. Sizes of the standards are shown on the left.

and screened for the presence of two *AscI* sites and two Enzyme A sites. A total of fourteen possible linker clones were identified and screened for authenticity by Southern blot analysis of Enzyme A-digested *Bacillus* sp. strain C-125 DNA (Fig. 2). Only seven of the fourteen clones (Table 2) proved to be genuine linker clones binding as expected to one Enzyme A fragment, and to two *AscI* fragments. Table 3 lists the results of using these genuine linker clones for hybridization against *AscI*-, *Sse8387I*-

Fig. 2. Check of linker clone authenticity using Southern blot analysis. In these examples, digoxigenin-labelled clones pLINKH4, pLINKH10 and pLINKH6 were used to probe blots (c) containing *Hind*III (H) digests of *Bacillus* sp. strain C-125 DNA. [The *Eco*RI (E) tracks are superfluous and should be ignored]. The clone insert DNA (b) and regions of the chromosome from which these originated (a) are represented schematically as shaded boxes (not to scale). Sizes indicated on the right refer to the hybridized *Hind*III fragments. Lambda-*Hind*III/ ϕ X174-*Hae*III markers were hybridized to themselves. A, *Asc*I.

Table 2. Probes and sources

Probe	Size (kb)	Gene product/phenotype	Source or reference
C-125 <i>uncH</i> ,A,G	2.2	F ₁ ATPase α , β & γ subunits	Kobayashi
C-125 <i>hag</i>	2.7	Flagellin	Sakamoto <i>et al.</i> (1992)
C-125 pALK	0.5	Alkaline resistance	Kudo <i>et al.</i> (1990)
C-125 23S <i>rrn</i>	3.0	23S rRNA	Sutherland
C-125 <i>secY</i>	2.0	Secretion of proteins	Kang <i>et al.</i> (1992)
C-125 <i>xyl</i> (A)	2.6	Xylanase (alkaline)	Honda <i>et al.</i> (1985)
C-125 <i>xyl</i> (N)	0.9	Xylanase (neutral)	Kobayashi
<i>B. subtilis trpS</i>	0.9	Tryptophanyl tRNA synthase	Chow & Wong (1988)
<i>B. subtilis spoII</i> G	0.8	Stage II sporulation	Stagier <i>et al.</i> (1984)
<i>B. subtilis</i> pNEXT39	3.4	<i>B. Subtilis</i> <i>NotI</i> -linking clone	Itaya & Tanaka (1991)
<i>B. subtilis</i> pNEXT41	2.9	<i>B. Subtilis</i> <i>NotI</i> -linking clone	Itaya & Tanaka (1991)
C-125 pLINK H1	2.9	C-125 <i>Asc</i> I-linking clone	This study
C-125 pLINK H4	4.0	C-125 <i>Asc</i> I-linking clone	This study
C-125 pLINK H5	1.5	C-125 <i>Asc</i> I-linking clone	This study
C-125 pLINK H10	1.4	C-125 <i>Asc</i> I-linking clone	This study
C-125 pLINK H20	3.7	C-125 <i>Asc</i> I-linking clone	This study
C-125 pLINK H23	0.7	C-125 <i>Asc</i> I-linking clone	This study
C-125 pLINK E6A	1.5	C-125 <i>Asc</i> I-linking clone	This study
16A fragment	20	C-125 <i>Asc</i> I fragment (Table 1)	This study

Table 3. Hybridization patterns of pLINK clones to restriction endonuclease fragments of *Bacillus* sp. strain C-125 DNA

Fragments generated with either *Asc*I or *Sse*8387I are identified by their band numbers (Table 1). The *Asc*I-plus-*Sse*8387I fragments and fragments generated as a result of partial *Asc*I or *Sse*8387I digestion are identified by their sizes in kb.

Probe	Hybridization with fragment (kb)		
	<i>Asc</i> I	<i>Sse</i> 8387I	<i>Asc</i> I-plus- <i>Sse</i> 8387I
pLINK H1	17A 13A	6S	17A 13A
pLINK H4	3A 10A	12S	100 5
pLINK H5	11A 6A	1S	11A 200
pLINK H10	14A 7A	7S	14A 45
pLINK H20	5A 10A	17S	18 30
pLINK H23	9A 12A	3S	9A 60
pLINK E6A	6A 12A	9S	145 20

and *Asc*I plus *Sse*8387I-digested *Bacillus* sp. strain C-125 (as illustrated by the example in Fig. 3). Two of the original fourteen clones hybridized to several fragments (even at increased hybridization and washing stringency) and were assumed to contain repetitive DNA sequences from the *Bacillus* sp. strain C-125 chromosome. The remaining five clones bound to two Enzyme A fragments. Since a genuine linker clone should bind to only one Enzyme A fragment, it was concluded that these five clones, which appear to be the result of the cloning of two noncontiguous *Asc*I-Enzyme A fragments were artefactual linker clones (e.g. pLinkH6, Fig. 2).

We were unable to isolate all the necessary linking clones required to determine the positions of every *Asc*I site on the chromosome. Certain *Asc*I sites may be unclonable due to *Bacillus* gene toxicity in *E. coli*. Alternatively, they may be contained on very long *Hind*III or *Eco*RI fragments that are beyond the limit of plasmid cloning. The latter problem could have been overcome by using alternative enzymes as Enzyme A. However, this would have involved the screening of several hundred more clones, a very time-consuming and expensive exercise.

To obtain more information and to add detail to the map, clones of *Bacillus* sp. strain C-125 and *B. subtilis* DNA, and the 20 kb *Asc*I fragment (Table 2) were used as hybridization probes. The results are listed in Table 4.

Of the *B. subtilis* clones used, pNEXT39, *Spo*IIIG and

Fig. 3. Southern blot analysis of *Bacillus* sp. strain C-125 genomic DNA. In this example digoxigenin-labelled pLINKH23 was used to probe a blot containing *Sse*8387I (S), *Asc*I plus *Sse*8387I (A-S), and *Asc*I (A) digests of *Bacillus* sp. strain C-125 DNA. Sizes on the right indicate the migration distance for annealed concatemers of lambda DNA. Positions and identities (as described in Table 1) of observed bands are highlighted on the left. The 230 kb *Asc*I fragment is due to partial digestion of the linked 9A and 12A fragments.

pNEXT41 confirmed results already obtained with the *Bacillus* sp. strain C-125 clones pALK, pLINKH10 and *xyl*(A), respectively. Only *B. subtilis trpS*, important for linking the 8A fragment to the 14A fragment, generated novel data.

An *Asc*I physical map of the *Bacillus* sp. strain C-125 chromosome

The data listed in Tables 3 and 4 have allowed us to arrange the majority of the *Asc*I fragments into two regions (Fig. 4). It was not been possible to link all the *Asc*I fragments but, if circularity of the *Bacillus* sp. strain C-125 chromosome is assumed there are only two possible arrangements for the regions shown in Fig. 4, namely (i, ii, iii, iv) or (i, iv, ii, iii). The binding of the *SecY* probe to a 460 kb partial *Asc*I fragment, however, rules out arrangement (i, iv, ii, iii). We therefore propose that arrangement (i, ii, iii, iv) (Fig. 5), the only circular

Fig. 4. Partial linkage map of *Bacillus* sp. strain C-125 *AscI* (A) fragments and corresponding *Sse8387I* (S) fragments. (i) and (ii) show two sets of linked fragments. (iii) and (iv) show *AscI* fragments that remain unlinked. Fragment numbers correspond to their allotted band numbers as given in Table 1. The 4S and 12S fragments are represented by dotted lines as their actual positions in relation to the corresponding *AscI* fragments are unknown. The correct orientations of the 13A and 17A doublet, the *rrn* and *xyl*(N) on 3A or 6S, the *rrn* and *secY* on 4A or 3S, and the pALK and pNEXT39 on 1A or 2S are unknown.

Table 4. Hybridization patterns of probes to restriction endonuclease fragments of *Bacillus* sp. strain C-125 DNA

Fragments generated with either *AscI* or *Sse8387I* are identified by their band numbers (Table 1). The *AscI* plus *Sse8387I* fragments and fragments generated as a result of partial *AscI* or *Sse8387I* digestion are identified by their sizes in kb.

Probe	Hybridization with fragment				
	<i>AscI</i>	<i>Sse8387I</i>	<i>AscI</i> -plus- <i>Sse8387I</i>	Partial digests of	
				<i>AscI</i>	<i>Sse8387I</i>
<i>uncH,A,G</i>	6A	1S	200	—	—
<i>hag</i>	11A	1S	11A	—	—
pALK	1A	2S	470	—	580
23S <i>rrn</i>	3A	6S	—	—	—
	15A	4S	—	—	—
	4A	3S	—	—	—
	9A	—	—	—	—
<i>secY</i>	4A	3S	230	460	—
	—	—	—	580	—
<i>xyl</i> (A)	5A	17S	—	640	245
<i>xyl</i> (N)	3A	6S	150	—	—
<i>trpS</i>	8A	7S	140	—	—
<i>spoIIG</i>	7A	7S	45	—	—
pNEXT39	1A	2S	470	—	—
pNEXT41	7A	5S	150	—	—
16A fragment	16A	4S	16A	50	—