Extremophiles (2000) 4:99–108

MINI-REVIEW

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Analysis of the genome of an alkaliphilic *Bacillus* strain from an industrial point of view

Received: July 11, 1999 / Accepted: December 27, 1999

Abstract *Bacillus* species and other microbes with pH optima for growth higher than pH 9 are defined as alkaliphiles. A large number of alkaliphilic *Bacillus* strains producing useful enzymes, have been isolated from various environments. Some of these enzymes, such as proteases and cellulases from alkaliphilic *Bacillus* strains, have been commercialized and have brought great advantages to industry and domestic life. To support further development of the enzyme industry, we initiated analysis of the genome of *Bacillus halodurans* C-125, which is 4.25 Mb in size, and constructed a physical and genetic map for comparison with the *Bacillus subtilis* chromosome. Systematic sequencing of the whole genome of *Bacillus halodurans* C-125 has been automated since the beginning of May 1998, and sequencing of 98% of the whole genome has been done so far. Through genome analysis, it became apparent that the genome organization of alkaliphilic *Bacillus halodurans* C-125 is totally different from that of *B. subtilis* orthologues.

Key words Alkaliphile · *Bacillus halodurans* · C-125 Physical mapping · Gene mapping · Genomic sequence · Industrial application · Enzyme production

Introduction

Generally, alkaliphilic *Bacillus* strains cannot grow or grow only poorly under neutral pH conditions, but grow well at pH higher than 9.5. Since 1969, we have isolated a great number of alkaliphilic *Bacillus* strains from various environments and have purified many alkaline enzymes (Horikoshi 1991). During the past two decades, our studies have focused on the enzymology, physiology, and molecular genetics of alkaliphilic microorganisms to elucidate their mechanisms of adaptation to alkaline environments (Horikoshi 1991). Industrial applications of these microbes have also been investigated, and some commercial enzymes from alkaliphilic *Bacillus* strains have brought great advantages to industry. Thus, it is clear that alkaliphilic *Bacillus* strains are quite important and interesting not only academically but also industrially. An alkaliphilic bacterium, strain C-125 (JCM9153), isolated in 1977, was identified as a member of the genus *Bacillus* and reported as a β-galactosidase (Ikura and Horikoshi 1979) and xylanase producer (Honda et al. 1985a). It is the strain most thoroughly characterized, physiologically biochemically, and genetically among those in our collection of alkaliphilic *Bacillus* isolates (Horikoshi 1991). Recently, this strain was reidentified as *Bacillus halodurans* based on the results of 16*S* rDNA sequence and DNA–DNA hybridization analyses (Takami and Horikoshi 1999). Whole-genome analysis of *B. subtilis*, which is taxonomically related to alkaliphilic *B. halodurans* strain C-125 (Fig. 1), except for the alkaliphilic phenotype, has been completed (Kunst et al. 1997). 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. From this research background, we initiated analysis of the genome of alkaliphilic *Bacillus* sp. C-125 for further development of the enzyme industry using alkaliphilic *Bacillus* strains. As the first step in genome analysis, we completed a physical map of the chromosome of strain C-125, which has a size of 4.25 Mb (Takami et al. 1999a), and the *oriC* region was identified (Takami et al. 1999b). At the same time, we reported analysis of the sequence of a 32-kb region containing a major ribosomal protein gene cluster, located in the 3S′/4A fragment in the physical map (Takami et al. 1999c). Also, a lambda phage library of the C-125 chromosome was constructed, and three independent DNA inserts (15–20 kb) were sequenced and analyzed to determine their genetic features (Takami et al. 1999d). We have been proceeding with systematic sequencing of the genome of alkaliphilic *Bacillus halodurans*

Communicated by C. Gerday

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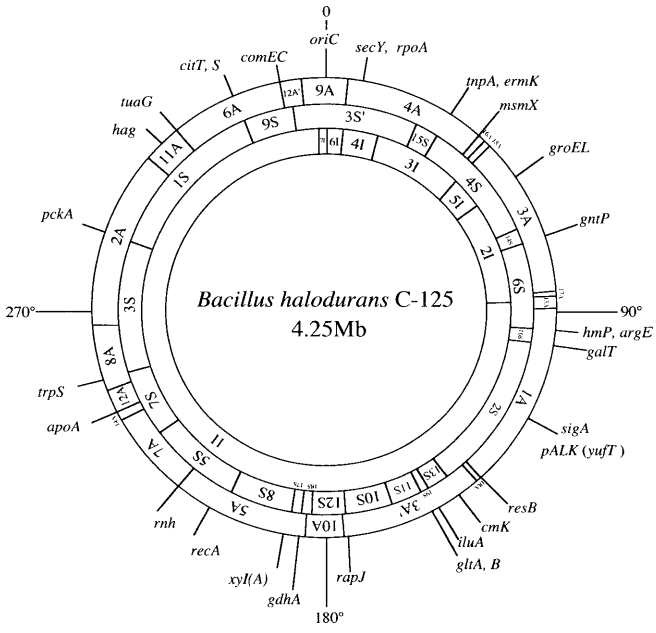

Fig. 1. *Asc*I/*Sse*83871/I-*Ceu*I physical map of the chromosome of *Bacillus halodurans* C-125. *Outer* and *inner circles* show the *Asc*I, *Sse*8387I, and I-*Ceu*I physical map, respectively. Locations of several genes are indicated on the map

C-125 since the beginning of May 1998. After completion of the sequencing of the C-125 genome, we will focus on the regulatory regions controlling the production of extracellular enzymes in this bacterium.

Isolation and taxonomic study of alkaliphilic *Bacillus* strains

In 1934, Vedder isolated aerobic, endospore-forming bacteria from human feces, and later from animal feces, that proved to be obligately alkaliphilic organisms; he named the organism *Bacillus alcalophilus* (Vedder 1934). On the other hand, the first report concerning an alkaline enzyme from an alkaliphilic *Bacillus* strain was published in 1971 (Horikoshi 1971a). Since this first report, we have isolated a great number of *Bacillus* strains from various environments and have purified many alkaline enzymes. The facultatively alkaliphilic *Bacillus* strains generally can grow well at pH 7.0–10.5 when sufficient sodium chloride (1%–2%) is present in the medium. Alkaliphilic *Bacillus* strains are usually classified in 11 groups based on 16*S* rDNA sequence data (Nielsen et al. 1995), and the major alkaliphilic *Bacillus* species are proposed to be the following: *B. pseudofirmus*, *B. agaradhaerens*, *B. clarkii, B. halodurans, B. clausii, B. cohnii, B. halmapalus, B. horikoshii, B. pseudoalcalophilus, and B. gibsonii*. Table 1 shows the growth patterns of some type strains of *Bacillus* species on Horikoshi II agar plates containing 2% sodium chloride adjusted to different pH conditions (Horikoshi 1996). Four type strains, *B. cohnii* DSM6307, *B. halodurans* DSM497, *B. clausii* DSM8716,

Table 1. Growth patterns of type strains of alkaliphilic *Bacillus* strains

Strain	Medium	
	Hokikoshi II (pH 7.5)	Horikoshi II (pH 9.5)
Bacillus alcalophilus DSM485	+	++
Bacillus cohnii DSM6307	++	++
Bacillus halodurans DSM497	++	++
Bacillus clausii DSM8716	++	++
Bacillus pseudofirmus DSM8715	++	++
Bacillus clarkii DSM8720	−	++

++, good growth; +, slight growth; −, no growth

and *B. pseudofirmus* DSM8715, could grow under both neutral (pH 7.5) and alkaline (pH 9.5) conditions. The type strain of *B. alcalophilus* DSM485 could grow slightly at pH 7.5 whereas it grew well at pH 9.5. The other type strain, *B. clarkii* DSM8720, which is an obligate alkaliphile, could not grow at pH 7.5. Thus, alkaliphilic *Bacillus* strains could be roughly classified in three groups on the basis of their growth patterns in neutral and alkaline media.

Alkaline enzymes from alkaliphilic *Bacillus* strains

It is well known that most alkaliphilic *Bacillus* strains produce various alkaline enzymes such as proteases, amylases, xylanases, cellulases, and pullulanases. Enzyme producers in our collection of alkaliphilic *Bacillus* isolates, which are classified into six taxonomic groups on the basis of 16*S* rDNA sequence data, are listed in Table 2.

Proteases

Horikoshi reported the production of an extracellular alkaline serine protease by alkaliphilic *Bacillus clausii* 221 (DSM2512; Table 2) in 1971 (Horikoshi 1971a). This strain, isolated from soil, was found to produce large amounts of alkaline protease that differed from enzymes of the subtilisin group. The optimum pH for activity of the purified enzyme was pH 11.5, with 75% of the activity retained at pH 13. The addition of 5 mM calcium ions resulted in a 70% increase in activity at the optimum temperature (60°C). Subsequently, two other *Bacillus* strains, AB42 and PB12, were reported, each of which also produced an alkaline protease (Aunstrup et al. 1972). These enzymes exhibited activity over a broad pH range, 9.0 to 12.0, and showed a temperature optimum of 60°C in the case of AB42 and 50°C in the case of PB12. Since the first report on the alkaline protease from alkaliphilic *Bacillus clausii* 221 was published, there have been extensive studies on alkaline proteases from other strains of alkaliphilic *Bacillus* such as YaB (Tsai et al. 1983, 1986), NKS-21 (Tsuchida et al. 1986), B-21 (Fujiwara and Yamamoto 1987a), and AH-101 (Takami et al. 1989). These alkaline proteases resemble each other in terms of physicochemical properties, amino acid composition, and NH_2-terminal sequence, and most of them show

Table 2. Alkaliphilic *Bacillus* strains that produce various useful enzymes

Species	Original no.	JCM no.	ATCC no.	DSM no.	Enzyme production
Bacillus halodurans	C-125	9153			β-Galactosidase, xylanase, protease, amylase, pectinase, thermostable protease (keratinase)
	AH-101[b]	9161			
	A-59	9148	21591	2153	Amylase
	C-3	9164			5′-Nucleotidase
	202-1	9151			Pullulanase
Bacillus pseudofirmus	124-1		21593	2517	Amylase
	A-40-2	9141	21592	2516	Amylase
	27-1	9144	21596	2520	Amylase
Bacillus clausii	Y-76	9138	21537	2515	Protease
	221	9139	21522	2512	Protease
	M-29		31084	2525	DNase
	O-4	9137	21536	2514	Protease
Bacillus sp., unknown group 1[a]	169	9147	21594	2518	Amylase
	N-4	9156	21833	2522	Cellulase
	135	9146	21595	2519	Amylase
Bacillus sp., unknown group 2[a]	13	9145	31006	2523	Amylase
	17-1	9142	31007	2524	Amylase
Bacillus sp., unknown group 3[a]	N-1	9140	21832	2521	Cellulase

[a] Nielsen et al. (1995)
[b] Takami et al. (1999e)

optimum activity in the pH range 10–12, as do most bacterial alkaline proteases.

The enzyme from *Bacillus* sp. Ya-B has been characterized as an alkaline elastase because it displays strong elastolytic activity. This enzyme was found to be most active toward elastin at pH 11. Takami et al. isolated a new extremely thermostable alkaline protease from alkaliphilic *Bacillus* sp. no. AH-101 (JCM9161; Table 2) (Takami et al. 1989) that is closely related to *Bacillus halodurans* as determined on the basis of 16*S* rDNA sequence data (Takami et al. 1999e). This enzyme was found to be most active toward casein at pH 12–13 and was stable for 10 min at 60°C in the pH range 5–12. The temperature optimum for activity was about 80°C in the presence of 5 mM calcium ions. The AH-101 alkaline protease showed higher hydrolyzing activity with insoluble fibrous natural proteins as the substrate, such as elastin or keratin, in comparison with subtilisin-type enzymes and proteinase K (Takami et al. 1990, 1992a). This enzyme easily digested human hair and nail in alkaline buffer (pH 11–13) containing 1% thioglycolic acid (Takami et al. 1992b). Kobayashi et al. (1995) have isolated and purified M-protease showing optimum activity at pH 10.6 from alkaliphilic *Bacillus* KSM-K16, and this enzyme was found to be suitable for use in detergents.

The main industrial application of alkaliphilic enzymes is detergents. Detergent enzymes account for approximately 30% of total worldwide enzyme production, and these alkaliphilic or alkalitolerant proteins represent a good example of successful commercial products. Not all these proteins are produced by alkaliphilic bacteria. However, several alkaline proteases produced by alkaliphilic *Bacillus* strains are commercially available from several companies. On the other hand, alkaline proteases have been used in the hide-dehairing process where dehairing is carried out under conditions between pH 8 and 10. In addition, an alkaline protease from *Bacillus* sp. B21 (Fujiwara and Yamamoto 1987a) has been found to be useful to recover silver by decomposing the gelatinous coating on X-ray films (Fujiwara and Yamamoto 1987b; Fujiwara et al. 1991; Ishikawa et al. 1993).

Starch-degrading enzymes

In 1971, Horikoshi reported that an alkaline amylase was produced in Horikoshi II medium by the alkaliphilic *Bacillus pseudofirmus* no. A-40-2 (JCM 9141; Table 2) (Horikoshi 1971b). Alkaline amylases have been classified into four types according to their pH activity curves. The type I curve has only one peak at pH 10.5; the type II curve has two peaks, at pH 4.0–4.5 and pH 9.0–10.0; the type III curve has three peaks, at pH 4.5, 7.0, and 9.5–10.0; the type IV curve has one peak at pH 4.0 with a shoulder at pH 10.0. Type III amylase (*Bacillus* sp. no. 38-2 enzyme) and type IV amylase (*Bacillus* sp. no. 17-1 and no. 13 enzymes) have high cyclomaltodextrin glucanotransferase activity, acting to convert starch to cyclodextrins (Horikoshi 1971b). The amylase from *Bacillus pseudofirmus* no. A-40-2 (JCM9141; Table 2) is most active at pH 10.0–10.5 and retains 50% of its activity between pH 9.0 and 11.5. The enzyme is a type of saccharifying α-amylase, as 70% of the starch substrate is hydrolyzed to glucose, maltose, and maltotriose. Subsequently, Boyer and Ingle reported an alkaline amylase in strain NRRL B-3881 that showed optimum activity at pH 9.2 (Boyer and Ingle 1972; Boyer et al. 1973). This enzyme produces maltose, maltotriose, and a small amount of glucose and maltotetraose from starch, all of which have a β-configuration. Both these amylases are more stable in the presence of EDTA than the corresponding enzymes from *Bacillus amyloliquefaciens* or *B. subtilis*.

Bacillus sp. no. 38-2 and no. 17-1 (JCM 9142; Table 2) were selected as the best producers of CGTases from among approximately 1000 strains (Nakamura et al. 1975; Nakamura and Horikoshi 1976a–c). *Bacillus* sp. no. 38-2 produces a mixture of three enzymes: an acid CGTase, a neutral CGTase, and an alkaline CGTase, which show optimal activity at pH 4.6, 7.0, and 8.5, respectively. Since strain no. 38-2 was isolated, many other CGTase-producing alkaliphilic *Bacillus* strains have been reported. Nomoto et al. isolated the CGTase-producing alkaliphilic *Bacillus* sp. no. HA3-3-2 from soil in Taipei, Taiwan (Nomoto et al. 1984). Abelyan et al. developed a method for isolation of CGTase using cyclodextrin polymers and their derivatives (Abelyan et al. 1994a,b). A psychrophilic alkaliphilic *Bacillus* sp. no. 3-22 has been isolated from deep-sea mud. This strain grows at 4°C and its CGTase produces predominately β-cyclodextrin (CD) with minor amounts of α- and γ-CDs (Georganta et al. 1993). Several attempts to produce β-CD on an industrial scale have been made by Corn Products International Co. and The Teijin Ltd. in Japan. These companies have produced β-CD using *B. macerans* CGTase but encountered the following serious problems in the production process: (1) the CGTase from *B. macerans* is not suitable for industrial use because it does not have sufficient thermostability; (2) the yield of CD from starch was not high, usually only 20%–30% on an industrial scale; and (3) toxic organic solvents such as trichloroethylene, bromobenzene, and toluene had to be used to precipitate CD because of the low conversion rate. A CGTase produced by alkaliphilic *Bacillus* sp. no. 38-2 overcame all these problems, and a successful process for mass production of crystalline CD at low cost without the need for organic solvents has been established. The yield of CD using this enzyme ranges from 85% to 90% with amylose as the substrate and from 70% to 80% with potato starch as the substrate on a laboratory scale. This simple method has paved the way for the use of CD in large quantities in foodstuffs, chemicals, and pharmaceuticals.

Pullulanases

Nakamura et al. discovered that alkaliphilic *Bacillus halodurans* 202-1 (JCM 9151; Table 2) produces an extracellular pullulanase in Horikoshi II medium (Nakamura et al. 1975). This enzyme has a pH optimum at 8.5–9.0 and is stable for 24 h at pH 6.5–11.0 at 4°C. The enzyme is most active at 55°C, and is stable up to 50°C for 15 min in the absence of substrate. Subsequently, a novel alkaline pullulanase was isolated from alkaliphilic *Bacillus* sp. KSM-1876 (Ara et al. 1992; Igarashi et al. 1992). This enzyme shows optimal activity at around pH 10.0–10.5, which is the highest pH optimum known for pullulanase activity. This enzyme is a good candidate for use as a dishwasher detergent additive, especially for removal of starch from dishes in the presence of detergents. Several other alkaliphilic *Bacillus* strains producing highly alkaline pullulanases have been reported (Kim et al. 1993; Lin et al. 1994; Lee et al. 1994).

Xylanases

Xylanases have been found widely in bacteria and fungi. From the industrial point of view, alkaline xylanases are of interest because the enzyme can readily hydrolyze xylan, which is soluble under alkaline conditions. The first report of xylanase from alkaliphilic *Bacillus* was published in 1973 (Horikoshi and Atsukawa 1973). The purified enzyme from alkaliphilic *Bacillus* sp. no. C-59-2 showed a broad pH optimum, from pH 6.0 to 8.0. In the culture broth of *Bacillus halodurans* C-125 (Tables 2, 3) two xylanases were found, xylanase A and xylanase N, with molecular weights of 43 000 and 16 000, respectively (Honda et al. 1985a). Xylanase N is most active at pH 6–7 whereas xylanase A shows high activity in the pH range 6–10 and even retains some activity at pH 12. The xylanase A gene has been cloned and expressed in *E. coli* and sequenced (Honda et al. 1985b). Four thermophilic alkaliphilic *Bacillus* strains [W1 (JCM2888), W2 (JCM2889), W3, and W4] have been shown to produce xylanases (Okazaki et al. 1984). The optimum pH for activity is 6.0 in the case of W1 and W3 and between 6 and 7 in the case of W2 and W4. All these enzymes are stable between pH 4.5 and 10.5 for 1 h at 45°C. The optimum temperature for activity is 65°C in the case of W1 and W3 and 70°C in the case of W2 and W4. The degree of hydrolysis of xylan was found to be about 70% after 24-h incubation.

Since the finding that xylanases are useful for enzymatic debleaching, there have been extensive studies on thermostable alkaline xylanases from other strains of alkaliphilic *Bacillus* such as *Bacillus* sp. KCIM 59 (Dey et al. 1992), *Bacillus stearothermophilus* T-6 (Khasin et al. 1993), 41M-1 (Nakamura et al. 1993a), and TAR-1 (Nakamura et al. 1994). Recently, enzymatic debleaching using thermostable alkaline xylanases has become popular in the pulpmilling industry and has been studied extensively. Xylanase A from *Bacillus halodurans* C-125 has been investigated for food processing because it is active over a broad pH range from 6 to 10. Currently, most of the paper companies in the world are using thermostable xylanases for debleaching in pulpmilling. In Japan, Oji Paper Company has been using a thermostable xylanase developed by their own company for this purpose.

Cellulases

Two alkaliphilic *Bacillus* strains, no. N-4 and no. 1139, producing extracellular carboxymethylcellulases (CMCases), have been isolated (Horikoshi et al. 1984; Fukumori et al. 1985). *Bacillus* sp. no. N-4 (JCM 9156) produces multi-CMCases that are active over a broad pH range from 5 to 10. Two alkaline CMCases (E1 and E2) showing optimal activity at pH 10.0 were partially purified from a crude enzyme preparation. The enzyme E2 is stable up to 80°C and E1 up to 60°C. No differences between the two enzymes have been observed so far in terms of the types of products formed. Sashihara et al. have cloned the cellulase

genes of *Bacillus* sp. no. N-4 (JCM 9156; Table 2) in *E. coli* HB101 using the plasmid vector pBR322 (Sashihara et al. 1984). Another CMCase produced by *Bacillus* sp. no. 1139 is most active at pH 9.0 and still retains some activity at pH 10.5. The enzyme is stable over the pH range 6–11 (at 4°C for 24 h and at temperatures up to 40°C for 10 min), and it hydrolyzes cellotriose or cellotetraose but not cellobiose. The cellulase gene of strain no. 1139 has been cloned and sequenced (Fukumori et al. 1986). The evolutionary relationship between the cellulases of these two strains has been studied (Fukumori et al. 1986, 1987a,b, 1989). The amino acid sequence of the cellulase from *B. subtilis* (BSC) and that of the N-4 cellulase show significant homology in most parts except for the C-terminal portions. Nakamura et al. constructed many chimeric cellulases using the BSC and N-4 cellulase genes in an effort to understand the alkaliphily of the N-4 cellulase (Nakamura et al. 1991). The pH activity profiles of the chimeric enzymes in the alkaline range were found to depend on the sequence of the C-terminal region. Hitomi et al. have reported very productive research on the factors that determine pH activity profiles (Hitomi et al. 1994). They found substrate-dependent changes in the pH activity profile of an alkaline cellulase from an alkaliphilic *Bacillus* strain. The optimum pH of the BSC is about 6 for all cellulose-derived oligosaccharides examined. On the other hand, the optimum pH of the alkaline cellulase from strain NK-1 varies depending on the substrate; i.e., a sharp optimum was observed at pH 6 with G4 and G5OH, and a broad optimum of pH 6–10.5 was observed with G5, G6, and G6OH.

Saito and Ito (personal communication) of Kao Corp., Japan, mixed our alkaline cellulases with their laundry detergents and studied the washing effect by washing cotton underwear. The best results were obtained using an alkaline cellulase produced by alkaliphilic *Bacillus* sp. N-1 (JCM 9140; Table 2). However, the yield of enzyme was not sufficient for industrial purposes. Through collaboration with us, Ito et al. isolated an alkaliphilic *Bacillus* sp. no. KSM-635 from soil and succeeded in producing an alkaline cellulase as a laundry detergent additive on an industrial scale (Ito et al. 1989). Members of the genus *Bacillus* usually require CMC for the production of cellulases (Horikoshi et al. 1984; Fukumori et al. 1985; Au and Chan 1987), but the cellulase of no. KSM-635 was found to be produced almost constitutively, in terms of quantity, during growth on various carbohydrates, and neither the structure nor the composition of the carbon source used affected the production pattern. Ito et al. improved the productivity of the KSM-635 cellulase through genetic engineering, use of the single-cell isolation method, optimization of culture conditions, and so on. Ultimately, they succeeded in producing 20–25 g of the enzyme per liter of culture broth using a variant of strain KSM-635, and this is one of the highest yields so far reported (Ito et al. 1991; Ito 1997). Besides KSM-635 cellulase, the cellulases from *Bacillus* sp. KSM-19 and KSM-520 are not inhibited by metal ions or various components of laundry products such as surfactants, chelating agents, and proteinases.

Analysis of the genome of facultatively alkaliphilic *Bacillus halodurans* C-125

The facultatively alkaliphilic *Bacillus halodurans* C-125 (Takami and Horikoshi 1999), which is the strain most thoroughly characterized physiologically, biochemically, and genetically, produces at least five kinds of enzymes of industrial interest, and the pH and temperature profiles of the major extracellular enzymes are shown in Table 3. The enzymes produced by *Bacillus halodurans* C-125 show comparatively high optimum temperatures for activity (50°–70°C) and high pH optima (Table 3). The amount of each enzyme produced varies depending on the culture conditions, particularly with different combinations of nitrogen and carbon sources. Thus, alkaliphilic *Bacillus* strains have the potential to produce different kinds of enzymes, which are expressed under specific culture conditions, and it should be possible to control extracellular enzyme production for each purpose on determining all the information in the genome of an alkaliphilic *Bacillus* strain.

Detailed genetic and physical maps are available for the *B. subtilis* 168 chromosome (Itaya and Tanaka 1991; Biaudet et al. 1996) and physical maps of the chromosomes of *B. cereus* (Kolsto et al. 1990), *B. thuringiensis* (Carlson and Kolsto 1993; Carlson et al. 1996), and *B. firmus* OF4 (Gronstand et al. 1998) have been constructed. In addition, a partial physical map of the chromosome of alkaliphilic *Bacillus* sp. C-125 has been proposed (Southerland et al. 1993). These physical maps will be helpful for comparative studies, including exploration of the substantial differences in genome structure among bacilli.

Construction of a physical map of the genome

As the first step in genome analysis, we attempted to construct a physical map of *Asc*I and *Sse*8387I sites on the *Bacillus halodurans* C-125 chromosome, because enzymatic digestion with *Asc*I or *Sse*8387I resulted in 20 resolvable fragments (Takami et al. 1999a). A linking clone contains DNA that overlaps two adjacent restriction fragments and can be used as a hybridization probe to establish the identity of these two contiguous fragments (Smith and Condemine 1990). Isolation of *Asc*I- and *Sse*8387I-linking clones was attempted to join adjacent fragments generated by *Asc*I or *Sse*8387I digestion in mapping the chromosome.

Table 3. Properties of extracellular enzymes produced by *Bacillus halodurans* C-125

Enzyme	Optimum pH	Optimum temperature (°C)
Protease[a]	10	60
Amylase[a]	10	60
Pectinase[a]	9	50
Xylanase A[b]	6–10	70
Xylanase N[b]	6–7	70

[a] Optimum pH was measured at 40°C; optimum temperature was measured at pH 10
[b] Honda et al. (1985a,b)

Table 4. DNA probes used for the construction of the physical map

Probe	Size	Characteristics	Source or reference
*Asc*I-A	1.1	C-125 *Asc*I-linking clone	Takami et al. (1999a)
*Asc*I-B	1.5	C-125 *Asc*I-linking clone	Takami et al. (1999a)
*Asc*I-C	1.6	C-125 *Asc*I-linking clone	Takami et al. (1999a)
*Asc*I-D	1.7	C-125 *Asc*I-linking clone	Takami et al. (1999a)
*Asc*I-E	2.5	C-125 *Asc*I-linking clone	Takami et al. (1999a)
*Asc*I-F	3.6	C-125 *Asc*I-linking clone	Takami et al. (1999a)
*Asc*I-G	2.4	C-125 *Asc*I-linking clone	Takami et al. (1999a)
*Asc*I-H	6.9	C-125 *Asc*I-linking clone	Takami et al. (1999a)
*Asc*I-I	3.9	C-125 *Asc*I-linking clone	Takami et al. (1999a)
*Asc*I-J	0.6	C-125 *Asc*I-linking clone	Takami et al. (1999a)
*Asc*I-K	0.6	C-125 *Asc*I-linking clone	Takami et al. (1999a)
*Asc*I-L	6.5	C-125 *Asc*I-linking clone	Takami et al. (1999a)
*Asc*I-M	5.7	C-125 *Asc*I-linking clone	Takami et al. (1999a)
*Asc*I-N	4.2	C-125 *Asc*I-linking clone	Takami et al. (1999a)
*Asc*I-O	2.8	C-125 *Asc*I-linking clone	Takami et al. (1999a)
*Asc*I-P	2.2	C-125 *Asc*I-linking clone	Takami et al. (1999a)
*Asc*I-Q	3.5	C-125 *Asc*I-linking clone	Takami et al. (1999a)
*Sse*8387I-a	0.5	C-125 *Sse*8387I-linking clone	Takami et al. (1999a)
*Sse*8387I-b	2.2	C-125 *Sse*8387I-linking clone	Takami et al. (1999a)
*Sse*8387I-c	4.5	C-125 *Sse*8387I-linking clone	Takami et al. (1999a)
*Sse*8387I-d	2.4	C-125 *Sse*8387I-linking clone	Takami et al. (1999a)
*Sse*8387I-e	3.2	C-125 *Sse*8387I-linking clone	Takami et al. (1999a)
*Sse*8387I-f	6.3	C-125 *Sse*8387I-linking clone	Takami et al. (1999a)
*Sse*8387I-g	1.5	C-125 *Sse*8387I-linking clone	Takami et al. (1999a)
*Sse*8387I-h	1.3	C-125 *Sse*8387I-linking clone	Takami et al. (1999a)
*Sse*8387I-i	2.0	C-125 *Sse*8387I-linking clone	Takami et al. (1999a)
*Sse*8387I-j	4.0	C-125 *Sse*8387I-linking clone	Takami et al. (1999a)
*Sse*8387I-k	2.8	C-125 *Sse*8387I-linking clone	Takami et al. (1999a)
*Sse*8387I-l	2.2	C-125 *Sse*8387I-linking clone	Takami et al. (1999a)
*Sse*8387I-m	4.0	C-125 *Sse*8387I-linking clone	Takami et al. (1999a)
*Sse*8387I-n	4.0	C-125 *Sse*8387I-linking clone	Takami et al. (1999a)
*Sse*8387I-o	2.1	C-125 *Sse*8387I-linking clone	Takami et al. (1999a)
*Sse*8387I-p	8.0	C-125 *Sse*8387I-linking clone	Takami et al. (1999a)
*Sse*8387I-q	6.0	C-125 *Sse*8387I-linking clone	Takami et al. (1999a)
C-125 *gyrB*	1.9	DNA gyrase subunit B	Takami et al. (1999b)
C-125 *rpoA*	1.0	RNA polymerase α-subunit	Takami et al. (1999c)
C-125 *sigA*	1.1	RNA polymerase major sigma factor (σ^A)	Nakasone et al. (1998)
C-125 *secY*	2.0	Preprotein translocase subunit	Kang et al. (1992)
C-125 *hag*	2.7	Flagellin	Sakamoto et al. (1992)
C-125 pALK	2.4	Alkaline resistance	Kudo et al. (1990)
C-125 *xyl* (A)	2.6	Alkaline xylanase	Honda et al. (1985a,b)
C-125 *groEL*	1.8	Heat-shock protein (chaperonin)	Xu et al. (1996)
B. subtilis trpS	1.0	*B. subtilis* tryptophanyl-tRNA synthetase	Chow and Wong (1988)

Linking clone libraries were constructed using *Hin*dIII or *Eco*RI or *Eco*RV as the R enzyme. Plasmid DNA was isolated from individual clones and screened for the presence of two *Asc*I or *Sse*8387I sites and two R enzyme sites. Seventeen possible *Asc*I-linking clones (A–Q) or *Sse*8387I-linking clones (a–q) were identified and screened for authenticity by Southern blot analysis of R enzyme-digested *Bacillus halodurans* C-125 chromosomal DNA. All *Asc*I- and *Sse*8387I-linking clones listed in Table 4 were used as hybridization probes in Southern blot analyses of *Asc*I and *Sse*8387I digests of C-125 chromosomal DNA in PFGE gels. In most cases, the linking clones for a particular enzyme hybridized to two different fragments obtained after digestion with the enzyme. Each *Asc*I or *Sse*8387I contiguous fragment was joined by means of the hybridization and cross-hybridization patterns obtained on analysis of chromosomal DNA digested with *Sse*8387I or *Asc*I when hybridized to *Sse*8387I- or *Asc*I-linking clones.

The DNA probes listed in Table 4 were used for mapping of genetic loci on the physical map. In addition, the ORFs identified and annotated in the linking clones were also used for mapping. The assigned positions of the genes on the physical map of *Bacillus halodurans* C-125 were compared with those on the genetic and physical maps of *B. subtilis* (Anagnostopoulos et al. 1993; Itaya and Tanaka 1991; Kunst et al. 1997). DNA probes for *secY* (Kang et al. 1992) and *rpoA* from strain C-125 hybridized to the same fragments 4A and 3S′. The genetic loci on the physical map of strain C-125 were within approximately 12° of their positions on the *B. subtilis* physical map (see Fig. 1). It was also found that probes for *groEL* (Xu et al. 1996) and *hag* (Sakamoto et al. 1992) hybridized to 3A and 4S and to 11A and 1S, respectively. Their positions on the map were also close to those of *B. subtilis* (55° for *groEL* and 310° for *hag*), as shown in Fig. 1. On the other hand, it appeared that genes such as *trpS*, *appA*, *rnh*, and *recA* have divergent map posi-

tions, being located at 103° to 150° (Fig. 1) on the *B. subtilis* genetic map and approximately 200° to 250° on the map of strain C-125. The map positions of three genes [*sigA*, *pALK* (*yuft*), *resB*] located at 100°–140° also differ from the positions 206°–277° on the *B. subtilis* map. Thus it appears that the region around 200° to 250° on the C-125 map may correspond to the region around 100° to 140° on the genetic map of *B. subtilis*.

Genetic map of the chromosome of alkaliphilic *Bacillus halodurans* C-125 and comparison with that of *B. subtilis*

All *Asc*I- and *Sse*8387I-linking clones isolated from the chromosome of *Bacillus halodurans* C-125 for the purpose of constructing a physical map were analyzed by comparison with the BSORF database (http://bacillus.tokyo-center.genome.ad.jp:8008/). The orientations of linking clones serving to join adjacent *Asc*I or *Sse*8387I fragments were determined by Southern blot analysis using specific probes, each of which should hybridize with only one of the two adjacent *Sse*8387I fragments.

A genetic map of the chromosome of alkaliphilic *B. halodurans* C-125 is shown in Fig. 2. The map location, in degrees, of the *B. subtilis dnaA* locus was taken as 0° to position the genetic loci of the C-125 chromosome. The 360° scale brings the *B. halodurans* C-125 map into conformity with the *B. subtilis* map. Thus, the linkage map of *Sse*8387I fragments was arranged to place the *dnaA* locus at the position of 0°. The putative genes in the *Asc*I- or *Sse*8387I-linking clones (Takami et al. 1999a) were mapped on the *Sse*8387I linkage map of the *Bacillus halodurans* C-125 chromosome (Fig. 2). The *ydi* (*ydiH*, *ydiI*, *ydiJ*), *yfi* (*yfiL*, *yfiM*, *yfiL*), *ynd* (*yndF*, *yndE*), and *glt* (*gltA*, *gltB*) gene clusters and the *tuaG* and *hmp* genes were mapped at positions similar to those of the analogous *B. subtilis* loci. However, the positions of other putative genes differed completely from those of the *B. subtilis* orthologues, suggesting that genome organization is not conserved between

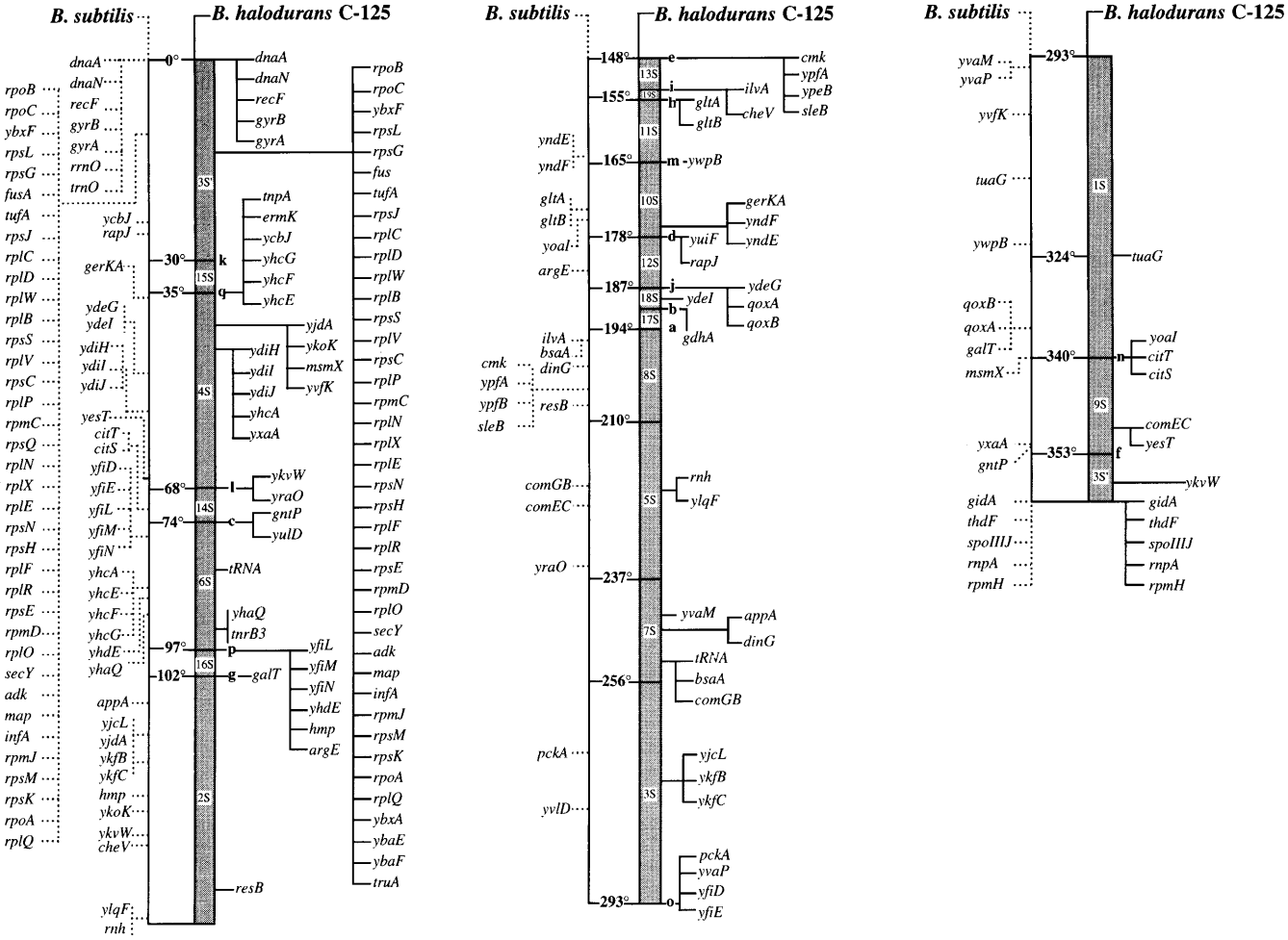

Fig. 2. Genetic map of the chromosome of alkaliphilic *Bacillus halodurans* C-125 and comparison with that of *B. subtilis*. The scale of 360°, beginning with 0° at the *dnaA* locus, is based on the *B. subtilis* map (Anagnostopoulos et al. 1993; Biaudet et al. 1996; Kunst et al. 1997). The genetic symbols used in this figure are defined in Table 1 and in previous reports (Takami et al. 1999b–d). Each gene from alkaliphilic *B. halodurans* C-125 was mapped on the linkage map of *Sse*8387I fragments (S). *Dashed lines* and *solid lines* represent the *B. subtilis* chromosome and the *B. halodurans* C-125 chromosome, respectively

B. subtilis and *B. halodurans* C-125, although 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). This kind of phenomenon was also observed in the case of the *B. cereus* chromosome. Okstad et al. reported that the genome organization is not conserved between *B. cereus* and *B. subtilis* (Okstad et al. 1999).

Shotgun sequencing of the whole genome of *B. halodurans* C-125

A 20-μg aliquot of chromosomal DNA was sonicated for 5–25 s with a Bioruptor UCD-200TM (Tosho Denki, Yokohama, Japan). The sonicated DNA fragments were blunt-ended using a DNA blunting kit (Takara Shuzo, Kyoto, Japan) 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 pUC18, which had been previously treated with BAP, and introduced into competent XL1-Blue cells by the standard method (Sambrook et al. 1989). We usually could obtain transformants with a frequency of $5–6 \times 10^5$/μg DNA. Inserts in the plasmids carried by these transformants were amplified by the colony PCR method.

The DNA fragment inserted in pUC18 was amplified by PCR using M13–20 and reverse primers. PCR fragments treated with exonuclease I and shrimp alkaline phosphatase (Amersham 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 Terminator Cycle Sequencing Kit (Perkin Elmer, Norwalk, CT, USA). We have already sequenced 57000 shotgun clones, with statistical coverage of the sequenced region having reached ninefold at this stage, and these clones sequenced seem to be almost saturated (Fig. 3).

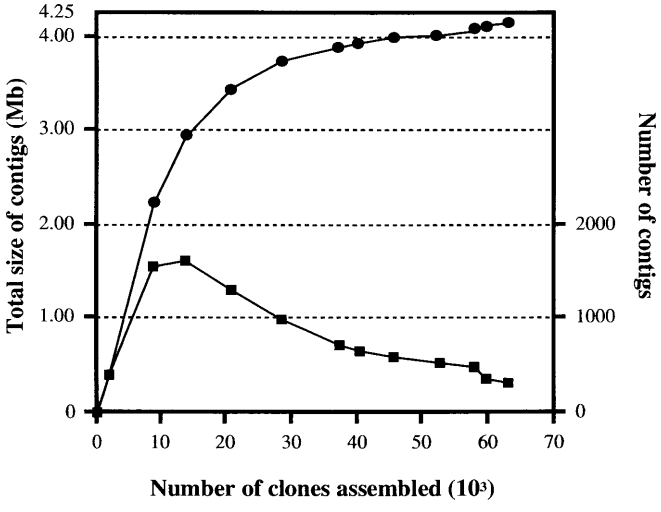

Fig. 3. Summary of the assembly of sequences of shotgun clones from the whole genome of alkaliphilic *Bacillus halodurans* C-125. *Circles*, total size of constructed contigs; *squares*, number of constructed contigs

DNA sequences determined by means of the ABI sequencer were assembled into contigs using Phrap (http://bozeman.mbt.washington.edu/phrap.docs/phrap.html) with the default parameters and without quality scores. At a statistical coverage of ninefold, the assembly using Phrap yielded 260 contigs (Fig. 3); the average contig length was 16 kb. The total length was 4.15 Mb, corresponding to 97.5% of the whole genome of *Bacillus halodurans* C-125 (4.25 Mb) (Fig. 3). To complete the sequencing of the entire genome, we are trying to fill the gaps using the sequences of both ends of large inserts in libraries such as lambda and cosmid clone libraries and by means of PCR with primers designed based on the internal sequence in each contig. The systematic sequencing of the whole genome of alkaliphilic *Bacillus halodurans* C-125 will be finished soon, and we will search the chromosome for the sequences of all extracellular enzymes and the regulatory regions controlling enzyme production in this bacterium.

Acknowledgments We express special thanks to Dr. K. Nakasone, Mr. Y. Takaki, and all members of the genome sequencing team for their great effort. We also thank Dr. H. Kobayashi for his dedication to enzymatic work.

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