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Genetic analysis of the chromosome of alkaliphilic *Bacillus halodurans* C-125

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Abstract Seventeen *Sse*8387I linking clones isolated from the chromosome of *Bacillus halodurans* C-125 for the purpose of constructing a physical map were sequenced and analyzed by comparison with the BSORF database and the nonredundant protein databank. The orientations of *Sse*8387I or *Asc*I linking clones serving to join adjacent fragments were determined by southern blot analysis using specific DNA probes. One-third of the open reading frames (ORFs) identified in the *Sse*8387I linking clones showed no significant similarity to any protein so far reported. The ORFs showing significant similarities to those of *Bacillus subtilis* were mapped in the chromosome of strain C-125, and the locations of the putative genes on the map were not well conserved between *B. halodurans* C-125 and *B. subtilis*.

Key words Alkaliphile · *Bacillus halodurans* C-125 · Genetic map · Genome analysis

Introduction

The facultatively alkaliphilic *Bacillus halodurans* C-125 (Takami and Horikoshi 1999), formerly called *Bacillus* sp. C-125, can grow well at pH 7–10.5 when sufficient sodium chloride (1%–2%) is present in the medium. 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 (Horikoshi 1996).

In 1997, whole genome analysis of *Bacillus subtilis*, which is closely related to strain C-125 except for the alkaliphilic phenotype, was 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 halodurans* C-125. An *Asc*I/*Sse*8387I physical map of its chromosome has been constructed (Takami et al. 1999a). In a previous study, 17 linking clones were isolated, serving to join adjacent *Asc*I or *Sse*8387I fragments in a chromosomal map, and the *Asc*I linking clones were sequenced (Takami et al. 1999a). The *oriC* region of the C-125 chromosome was identified, and an 18.5-kb DNA fragment containing the *oriC* region of the chromosome was obtained by inverse PCR (Triglia et al. 1988) and sequenced (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 of the C-125 chromosome (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* C-125 since the beginning of May 1998.

In this study, we sequenced *Sse*8387I linking clones and determined the orientations of all linking clones serving to join adjacent *Sse*8387I or *Asc*I fragments. Here we describe analysis of the sequences of the *Sse*8387I linking clones and partial genetic analysis of the chromosome of *Bacillus halodurans* C-125.

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Materials and methods

Sequencing and analysis of *Sse*8387I linking clones

Sequencing of *Sse*8387I linking clones was performed as described previously (Takami et al. 1999d). The sequences were analyzed for the locations of possible ORFs using the GeneWorks program (version 2.5.1N; 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 17 DNA fragments sequenced in this study were deposited at DDBJ with the accession numbers AB024548 for *Sse*8387I-a, AB0024549 for *Sse*8387I-b, AB024550 for *Sse*8387I-c, AB024551 for *Sse*8387I-d, AB024552 for *Sse*8387I-e, AB024553 for *Sse*8387I-f, AB024554 for *Sse*8387I-g, AB024555 for *Sse*8387I-h, AB024556 for *Sse*8387I-i, AB024557 for *Sse*8387I-j, AB0024558 for *Sse*8387I-k, AB024559 for *Sse*8387I-l, AB024560 for *Sse*8387I-m, AB024561 for *Sse*8387I-n, AB024562 for *Sse*8387I-o, AB024563 for *Sse*8387I-p, and AB024564 for *Sse*8387I-q.

Hybridization analysis

Agarose gels were blotted onto Hybond N^+ membranes using a vacuum blotting system (VacuGene XL; Pharmacia Biotech, Tokyo, Japan). Hybridization was performed using digoxigenin- (DIG-) labeled DNA probes in 40% (v/v) formamide hybridization solution at 42°C. Subsequent washing steps were performed according to the instructions provided with the DIG labeling and detection kit (Boehringer, Mannheim, Germany).

Results and discussion

Sequence analysis of *Sse*8387I linking clones

The determined sequence of each linking clone was searched for ORFs beginning with an ATG, GTG, or TTG start codon and comprising more than 100 codons. Sixty-one putative ORFs, including 18 partial ORFs (a1, b2, d1, 2, e4, g1, h1, 2, i2, j5, k4, l1, l2, m1, m5, o1, o4, and q7), were identified in the *Sse*8387I-linking clones, as shown in Table 1. Each ORF was identified on the basis of having a preceding Shine–Dalgarno (SD) sequence. The SD sequence was complementary to one found at the 3′-end (UCUUUCCUCCACUAG . . .) of the 16S rRNA of alkaliphilic *Bacillus halodurans* C-125 (Takami et al. 1997). One-third of the identified ORF products showed no significant similarity to any protein so far reported. The ORF b2 product showed 56% identity to NADP-specific glutamate dehydrogenase of *Prevotella ruminicola* (Wen and Morrison 1996). Two ORFs, q1 and q8, were found to encode products similar to a transposase (TnpA) produced by *Bordetella parapertussis* (Van der Zee et al. 1993) and transposase for insertion sequence element IS905 (Dodd et al. 1994), respectively. The transposase-like sequence from *Bordetella parapertussis* (Van der Zee et al. 1993) was also found in the insert of lambda clone no. 3 (Takami et al. 1999d). These findings suggest that this kind of transposable element may be involved in gene transposition in *Bacillus halodurans* C-125. Other ORF products showed significant similarity to those of *B. subtilis* with variation of 22%–79% (Table 1).

Determination of the orientation of *Sse*8387I or *Asc*I linking clones serving to join adjacent fragments

The orientations of linking clones serving to join adjacent *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. The sequences of the primers used to amplify specific probes and their positions in relation to each linking clone are described in Table 2. The primers were designed based on the position of the *Sse*8387I site in the linking clone. When the amplified DNA probe hybridized with several *Sse*8387I-generated fragments of the C-125 chromosome, the primer sequences were redesigned to correspond to a different position. It seems that a short repetitive sequence in the linking clone resulted in a complicated hybridization pattern, although the sequence has not been identified yet. As shown in Table 2, DNA probes specific for *Sse*8387I linking clones a–q hybridized with the complementary regions in two adjacent *Sse*8387I-generated fragments of the C-125 chromosome. From these results, the orientation of each linking clone was determined. The orientations of the *Asc*I linking clones previously sequenced (Takami et al. 1999b) were determined in the same manner (primer sequences not shown). The position of the *Sse*8387I fragment 16S (58 kb) on the physical map was revised from the previously assigned location between 1S and 3S (Takami et al. 1999b) to between 2S and 6S as a result of a series of detailed hybridization analyses (Fig. 1).

Mapping of putative genes in each linking clone in the chromosome of *Bacillus halodurans* C-125

The putative genes in the *Sse*8387I (Table 1) or *Asc*I linking clones (Takami et al. 1999b) were mapped on the *Sse*8387I linkage map of the *Bacillus halodurans* C-125 chromosome (Fig. 1). The map location, in degrees, of the *B. subtilis dnaA* locus was taken as zero degrees to position genetic loci of the C-125 chromosome listed in Table 1. 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 zero degrees. The *ydi* (*ydiH, ydiI, and ydiJ*), *yfi* (*yfiL, yfiM, and yfiN*), *ynd* (*yndF* and *yndE*), and *glt* (*gltA* and *gltB*) gene clusters and the *tuaG* and *hmp* genes were

Table 1. Open reading frame (ORF) analysis of *Sse*8387I linking clones

Clone	ORF	SD/initiation codon	n.t.	a.a.	Gene	Characteristics
a	1(P*)	----------TTG	37–594	186		Unknown
b	1(W)	AAAAAGGGGGaccgtctaGTG	79–1179	366		Unknown
	2(P*)	----------ATG	2235–1339	298	*gdhA*	Similar to *Prevotella ruminicola*, NADP-specific glutamate dehydrogenase (EC 1.4.1.4) (444 aa, 56.0% identity)
c	1(W)	AGAAatAGGAGTGAgagagATG	1192–2196	334		Unknown
	2(W)	GaAAAGAGGaAtaatgATG	2273–3643	456	*gntP*	Similar to *B. subtilis* gluconate permease (197 aa, 29.0% identity)
	3(W)	AGAAAGGAtGgTGAgtgaccgTTG	3847–4173	108	*yulD*	Function unknown; similar to *B. subtilis* YulD (104 aa, 37.0% identity)
d	1(P)	----------	2–1276	424	*yuiF*	Function unknown; similar to *B. subtilis* YuiF (442 aa, 44.9% identity)
	2(P)	GAAGGAgGGAcagcttTTG	1885–2526	214	*rapJ*	Similar to *B. subtilis* response regulator aspartate phosphatase (373 aa, 36.0% identity)
e	1(W)	AAAGGAGGcgtggacgcgaaggaGTG	151–711	186	*sleB*	Similar to *B. subtilis* spore cortex-lytic enzyme (305 aa, 62.4% identity)
	2(W)	GGAGGTGAgcgattGTG	731–2074	447	*ypeB*	Function unknown; similar to *B. subtilis* YpeB (450 aa, 42.2& identity)
	3(W)	AGGGAAGGAtggcgcgATG	2174–2851	225	*ypfA*	Function unknown; similar to *B. subtilis* YpfA (217 aa, 25.5% identity)
	4(P)	AGGtgtAGGaaTGAagaagATG	2986–3480	165	*cmk*	Similar to *B. subtilis* cytidylate kinase (224 aa, 57.4% identity)
f	1(W)	GGAtGGAtATG	721–1203	160		Unknown
	2(W)	GGGGGTActtgaaTTG	1255–2889	544		Unknown
	3(W)	AAAGGGGGAgttaaatATG	3438–3869	143		Unknown
	4(W)	AGAAAGGAttcttataATG	5496–4180	438		Unknown
	5(W)	GGAGGAAGctcccttATG	5777–6931	385		Unknown
g	1(P)	AGaAGGTGgAagagtcATG	324–1673	450	*galT*	Similar to *B. subtilis* galactose-1-phosphate uridyltransferase (513 aa, 60.9% identity)
h	1(P)	----------	2–682	226	*gltA*	Similar to *B. subtilis* glutamate synthase (large subunit; 1520 aa, 62.4% identity)
	2(P)	GGAGTGAgtgctATG	777–1379	201	*gltB*	Similar to *B. subtilis* glutamate synthase (small subunit; 493 aa, 70.6% identity)
i	1(W)	GGGAGGacATG	286–1398	370	*ilvA*	Similar to *B. subtilis* threonine dehydratase (422 aa, 66.5% identity)
	2(P)	AGgGGAGgaTGAacATG	1600–2007	136	*cheV*	Similar to *B. subtilis* modulation of CheA activity in response to attractants (303 aa, 54.6% identity)
j	1(W)	AGAAAacGAGGggaagtcATG	86–631	181	*ydeG*	Similar to *B. subtilis* metabolite transport (430 aa, 70.7% identity)
	2(W)	----------ATG	633–1418	261	*ydeG*	Similar to *B. subtilis* metabolite transport (430 aa, 65.5% identity)
	3(W)	AGGAGccaatgagATG	1872–1528	114		Unknown
	4(W)	AGaaAGGTGAttacagTTG	2165–3154	329	*qoxA*	Similar to *B. subtilis* cytochrome aa3 quinol oxidase (subunit I; 321 aa, 59.2% identity)
	5(P)	AGaGAGGgttttaccgTTG	3188–4150	320	*qoxB*	Similar to *B. subtilis* cytochrome aa3 quinol oxidase (subunit I; 649 aa, 78.8% identity)
k	1(W)	AGAGGGGatTTG	459–1247	262		Unknown
	2(W)	AGGAGtGTGAagtttaATG	1247–2158	303		Unknown
	3(W)	AGGGGattactcATG	2155–2532	125		Unknown
	4(P)	GGGAGGgagacgtATG	2529–2837	103		Unknown

Table 1. *Continued*

Clone	ORF	SD/initiation codon	n.t.	a.a.	Gene	Characteristics
1	1(P)	----------	2–991	329	*ykvW*	Function unknown; similar to *B. subtilis* YkvW (637 aa, 57.1% identity)
	2(P)	AGGGGGgagaaagcATG	1207–2184	326	*yraO*	Function unknown; similar to *B. subtilis* YraO (438 aa, 38.0% identity)
m	1(P)	----------	2–343	113		Unknown
	2(W)	AAAGGAaGGAagccaTTG	358–828	156	*ywpB*	Similar to *B. subtilis* hydroxymyristoyl-(acyl carrier protein) dehydratase (132 aa, 35.0% identity)
	3(W)	AGGgAGGagcgATG	825–2036	403		Unknown
	4(W)	AAAGGAtGGAttgttgcATG	3374–2571	267		Unknown
	5(P*)	----------GTG	3978–3397	193		Unknown
n	1(W)	AGGAGaGcgggccATG	62–1678	538	*citS*	Similar to *B. subtilis* two-component sensor histidine kinase (542 aa, 36.1% identity)
	2(W)	AAAGGAGGgtTGAgtatcATG	1684–2376	230	*citT*	Similar to *B. subtilis* two-component response regulator (226 aa, 44.2% identity)
	3(W)	AAAGaAGGcgatacgATG	2482–3513	343		Unknown
	4(W)	AGAAgAaGAGGctcagccaaataagcacgggacgATG	3907–3539	122	*yoaI*	Function unknown; similar to *B. subtilis* 4-hydroxyphenylacetate-3-hydroxylase (375 aa, 53.8% identity)
o	1(P)	----------	1–462	153	*pakA*	Similar to *B. subtilis* phosphoenolpyruvate corboxykinase (527 aa, 65.4% identity)
	2(W)	AGAGGTGAgttaaaATG	1116–793	107	*yvaP*	Function unknown; similar to *B. subtilis* YvaP (236 aa, 67.0% identity)
	3(W)	AGGAGGaatacaatcatATG	1264–1653	129	*yfiD*	Function unknown; similar to *B. subtilis* YfiD (134 aa, 50.8% identity)
	4(P)	AAAGActGGAGGcacttATG	1677–2288	204	*yfiE*	Function unknown; similar to *B. subtilis* YfiE (285 aa, 45.6% identity)
p	1(W)	AGaAGGcgatttttTTG	312–1172	286		Unknown
	2(W)	AAGGAGGAgagaacATG	2084–3016	310	*yfiL*	Function unknown; similar to *B. subtilis* YfiL (311 aa, 51.6% identity)
	3(W)	AAAGGAGGggcagaATG	3029–4300	423	*yfiM*	Function unknown; similar to *B. subtilis* YfiM (396 aa, 22.5% identity)
	4(W)	AAAGGGGGgaagctcaATG	4297–5403	368	*yfiN*	Function unknown; similar to *B. subtilis* YfiN (385 aa, 22.1% identity)
	5(W)	GGAGtGaaTGAtATG	5886–5446	146	*yhdE*	Function unknown; similar to *B. subtilis* YhdE (146 aa, 57.2% identity)
	6(W)	AAAGtGAGGcgatccatccATG	6216–7451	411	*hmp*	Similar to *B. subtilis* flavohemoglobin (399 aa, 50.5% identity)
	7(P*)	----------ATG	7513–8376	287	*argE*	Similar to *B. subtilis* acetylornithine deacetylase (436 aa, 34.8% identity)
q	1(W)	AAgAGaAGGgtcatcatGTG	27–650	207	*tnpA*	Similar to *Bordetella parape*, transposase TnpA (406 aa, 26.3% identity)
	2(W)	AGGaaAGGgaTGAtcgctATG	727–1581	284	*ermK*	Similar to *B. subtilis* rRNA methylase (287 aa, 66.9% identity)
	3(W)	AAAGGgtgtgtaaataaATG	1597–2499	300	*ycbJ*	Function unknown; similar to *B. subtilis* YcbJ (209 aa, 47.8% identity)
	4(W)	AGAAAGGAGcatctgagcaATG	3311–2619	230	*yhcG*	Function unknown; similar to *B. subtilis* YhcG (232 aa, 50.9% identity)
	5(W)	AGttGAGGTGtAatcaATG	3688–3308	126	*yhcF*	Function unknown; similar to *B. subtilis* YhcF (GntR family; 121 aa, 45.1% identity)
	6(W)	AGGAGGaataattATG	4516–3692	274	*yhcE*	Function unknown; similar to *B. subtilis* YhcE (253 aa, 24.0% identity)
	7(W)	AGGcaAGGgatcggtcaaaaatctATG	5166–5792	208		Unknown
	8(W)	GAGAGGatGTG	6806–6084	240		Similar to transponsase for insertion sequence element IS905 (391 aa, 35.8% identity)

P, partial ORF; W, whole ORF; P*, The SD-like sequence was not found because the 5′-flanking region was truncated

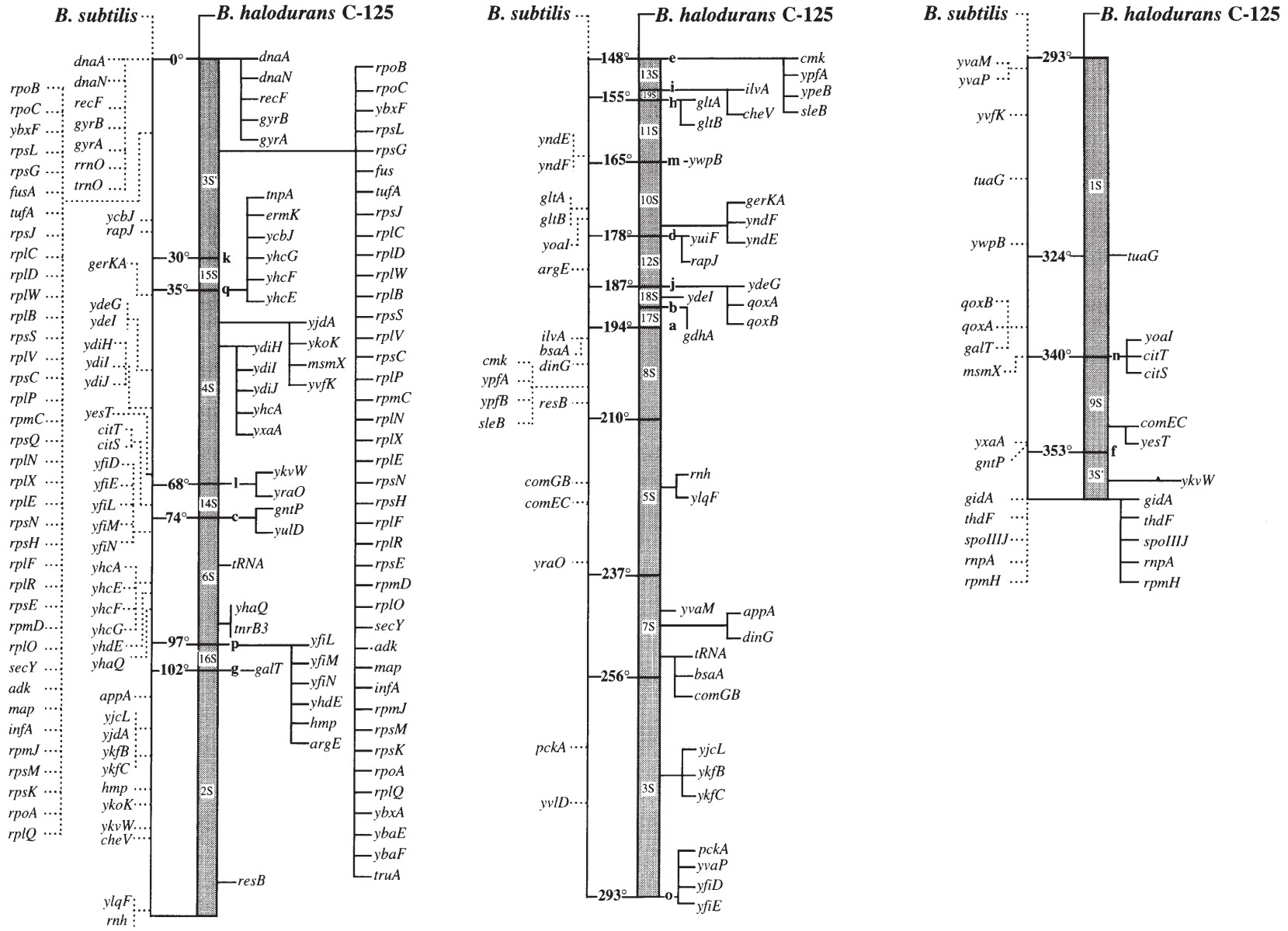

Fig. 1. Genetic analysis of the chromosome of alkaliphilic *Bacillus halodurans* C-125 and comparison with the genetic map of *B. subtilis*. Each gene from alkaliphilic *B. halodurans* C-125 was mapped on the linkage map of *Sse*8387I fragments (S). The scale of 360°, beginning with zero at the *dnaA* 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 our previous reports (Takami et al. 1999a–c). The *dashed lines* and *solid lines* represent the *B. subtilis* chromosome and the *B. halodurans* C-125 chromosome, respectively

Table 2. *Sse*8387I-linking clones and primer sequences for DNA probes used in hybridization experiments

Clones (fragment no.)	Length (site position)	Probe length	Primer 1 sequence (position)	Primer 2 sequence (position)	Fragment hybridized
a (8S/17S)	594 (283)	515	5′-tgggattgcgggttaccca-3′, (37–56)	5′-tccgaaacgcgctcagaca-3′, (532–552)	8S
b (17S/18S)	2242 (2186)	1026	5′-ctatgaggttggaggaca-3′, (806–1348)	5′-ggtcgtctagtgtttgca-3′, (1330–1348)	18S
c (6S/14S)	4817 (1231)	426	5′-gctgatctaacgaagacc-3′, (1623–1641)	5′-tcgaaatcgtccgctcaa-3′, (2032–2049)	6S
d (10S/12S)	2528 (419)	761	5′-gatcgcagcaatggtatt-3′, (1254–1272)	5′-gtcatgtatggtaagctg-3′, (1997–2015)	12S
e (2S/13S)	3482 (469)	491	5′-tatcgctcttgacagctc-3′, (1328–1346)	5′-atgcagctcgttgatagc-3′, (1801–1819)	2S
f (3S′/9S)	6932 (4589)	496	5′-gtcgattggcatcagatg-3′, (3624–3642)	5′-gagcggaaaaatgcctgt-3′, (4102–4120)	3S
g (2S/16S)	1674 (836)	390	5′-acatccgattcctgcaga-3′, (72–90)	5′-ccggctctagttcatcta-3′, (444–462)	16S
h (11S/19S)	1379 (1363)	500	5′-aactttggtgctggtatg-3′, (322–340)	5′-gaggtgtttcccgcttat-3′, (804–822)	19S
i (13S/19S)	2007 (994)	485	5′-aagcagacgatccgcttc-3′, (38–49)	5′-ctacgaacgtttcccctg-3′, (538–523)	13S
j (12S/18S)	4150 (514)	303	5′-gatgggagatgtagctga-3′, (631–649)	5′-tggtgttgcatatggacg-3′, (952–934)	18S
k (3S′/15S)	2838 (1785)	464	5′-acacgatggattgtgtcg-3′, (2187–2205)	5′-cgtatacggcccgttata-3′, (2633–2651)	3S′
l (4S/14S)	2184 (1990)	416	5′-gcttcaattcacgccttg-3′, (1130–1148)	5′-atccgtccaagtgagcg-3′, (1529–1546)	4S
m (10S/11S)	3990 (861)	302	5′-atgtcggcttgtaatcggtg-3′, (1286–1306)	5′-gatatgcttctccatcggttt-3′, (1607–1588)	10S
n (1S/9S)	3962 (2551)	495	5′-acgcatcgcgggtttgg-3′, (1540–1557)	5′-catacgcgtctaaccgtt-3′, (2053–2035)	9S
o (1S/3S)	2290 (1531)	528	5′-tagatgttgccttgctcg-3′, (1558–1576)	5′-tccaatcccatgatgaaga-3′, (2085–2066)	1S
p (6S/16S)	8387 (7425)	762	5′-gaagacgaggactcattt-3′, (6079–6097)	5′-atatgagtatgctcatgtc-3′, (6822–6841)	6S
q (4S/15S)	6851 (5052)	595	5′-gattaaggtatagaacggt-3′, (3773–3792)	5′-tatcctaatcggcaggtc-3′, (4352–4370)	15S

mapped at positions comparatively similar to those of the analogous *B. subtilis* loci. However, the positions of other putative genes differed from those of the *B. subtilis* orthologs, suggesting that genome organization is not conserved between *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).

Bacteria may be divided into two subgroups in terms of genome stability; one group consists of stable species whereas members of the other group show a higher level of heterogeneity within the species (Fonstein and Haselkon 1995). *Bacillus halodurans* may belong to the latter group although the genome organization of other strains of this species including the type strain (DSM197) is still not known. Homologous recombination between the *rrn* loci seems to be one of the major causes of such heterogeneity. In fact, it is known that the organization of the *rrn* gene operon in clostridial chromosomes varies considerably, and the *rrn* operon organization of *Clostridium acetobutylicum* is more similar to that of *B. subtilis* than to other clostridia (Cornillot et al. 1997). In contrast to clostridia, *B. subtilis* 168 has a generally stable structure, as shown by the examination of many derivatives, in agreement with the high genetic stability of this species (Itaya 1993).

In this study, it became apparent that the genome organization of alkaliphilic *Bacillus halodurans* C-125 is totally different from that of *B. subtilis* orthologs. In a previous study, we found that the region around 200°–250° on the *B. halodurans* C-125 map was different from that of *B. subtilis* and may correspond to the region around 100°–140° on the *B. subtilis* genetic map (Takami et al. 1999b). In addition, it became apparent that strain C-125 has at least 8 *rrn* operons in the chromosome (unpublished data) and transposon-like sequences, although the repetitive sequence has not been identified. Thus, it seems that these factors may be related to the genetic arrangement in the C-125 chromosome. The systematic sequencing of the whole genome of alkaliphilic *Bacillus halodurans* C-125 will be finished soon, and we will search for repetitive sequences in the chromosome.

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