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Physical map of alkaliphilic *Bacillus firmus* OF4 and detection of a large endogenous plasmid

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Abstract Extremely alkaliphilic *Bacillus firmus* OF4 is among the best characterized of this group of alkaliphiles. Together with alkaliphilic *Bacillus* C-125 and numerous non-alkaliphilic *Bacillus* species whose chromosomes and gene organizations are currently being studied in detail, work on *B. firmus* OF4 offers the opportunity to discern whether there are features of chromosome and gene organization that are associated with alkaliphily. A physical map of the *B. firmus* OF4 is consistent with a circular chromosome of approximately 4Mb, with an extrachromosomal element of 110kb also detected. The previously identified cadmium-resistance locus and transposition functions in *B. firmus* OF4 were localized to the extrachromosomal element, whose genes exhibit a slightly different pattern of codon usage from chromosomal genes. No clustering of genes thus far identified with roles in alkaliphily has been found. Direct repeat sequences (DRS) were previously reported upstream of a gene encoding a Na^+/H^+ antiporter that has a role in pH homeostasis. In the current analyses, these sequences were found to be present in multiple copies on the chromosome, most of which are present in one 920-kb fragment. Such sequences might play a role in DNA rearrangements that allow amplification of important genes in this region.

Key words Alkaliphile · Physical map · Plasmid · Repeated sequence

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Introduction

The detailed information on chromosome and gene organization that has been provided by completion of the *Bacillus subtilis* genome sequencing project (Kunst et al. 1997) sets a new framework for comparative studies of levels of such organization that may specifically serve the lifestyles of various extremophiles. Although complete sequences for extremophile examples are already emerging, important interim information, especially with a completed sequence of a genus member as backdrop, may be obtained from comparative examination of detailed physical maps of extremophile and nonextremophile members of the genus. Such maps can be generated through approaches developed by Smith and colleagues (Smith and Condemine 1990) together with assignment of known markers. Among the nonextremophile *Bacillus* species, apart from *B. subtilis*, substantial information about chromosomal organization exists for *Bacillus cereus* (Kolstø et al. 1990; Carlson et al. 1992, 1996a; Carlson and Kolstø 1994) and *Bacillus thuringiensis* (Carlson and Kolstø 1993; Carlson et al. 1996a,b)

For the extremely alkaliphilic *Bacillus* species, only one, partial physical map has been reported to date, that for the 3.7-Mb chromosome of alkaliphilic *Bacillus* strain C-125 (Sutherland et al. 1993), a strain of *Bacillus lentus* (Aono 1995). An increasing number of gene loci that are implicated in alkaliphily are being identified in this species (Kudo et al. 1990; Aono et al. 1993; Hamamoto et al. 1994), but only one such locus was localized to a specific fragment on the initial map. Identification and localization of a larger number of genes that are critically involved in alkaliphily and comparative studies of several distinct alkaliphilic and nonalkaliphilic *Bacillus* species should allow assessment of whether there is a clustering of all or groups of genes related to alkaliphily, either on the chromosome or on extrachromosomal elements, and whether there are consistent differ-

ences in chromosomal organization of pH-inducible or housekeeping genes in alkaliphiles versus nonalkaliphilies. In the current study, we report the development of a physical map for alkaliphilic *Bacillus firmus* OF4 and the localization of dozens of genes on this map and on a large extrachromosomal element that was observed.

Materials and methods

DNA preparation

DNA from *B. firmus* OF4 (Guffanti el al. 1986) was used for all studies. This facultatively alkaliphilic strain was routinely grown at pH 10.5 in malate-containing medium. Bacterial DNA for pulsed field gel electrophoresis (PFGE) was prepared in agarose blocks (Smith et al. 1988; Kolstø et al. 1990). The cells were cast in agarose, and the blocks were incubated in 4 ml 10 mM EDTA–25 mM Tris (pH 8.0)–2% lysozyme–100 ml RNase (10 mg/ml) for 24 h at 50°C, followed by incubation in 2 ml ESP (0.5 M EDTA, pH 8.0, 1% sodium laurylsarcosin, 1 mg proteinase K) (Boehringer, Mannheim, Germany) per milliliter. The ESP was replaced after 24 h and the blocks kept at 4°C until use. Before digestion with restriction enzymes, the blocks were treated with 0.1 mM phenylmethyl-sulfonyl fluoride in TE (10 mM Tris-HCl (pH 7.5), 1 mM EDTA) twice for 30 min at room temperature. The agarose block was preincubated in the restriction enzyme solution at 4°C overnight to ensure sufficient diffusion of the enzyme, and then incubated at 37°C for *Asc*I, or 50°C for *Sfi*I, from 10 min to 7 h (Carlson and Kolstø 1993).

Electrophoretic analysis

The electrophoresis was run in either a Gene-line apparatus (Beckman, Palo Alto, CA, USA) using 0.25× modified TBE (25 mM Tris-borate buffer, 0.05 mM EDTA), or in a CHEF Mapper (BioRad, Hercules, CA, USA), using 0.5× modified TBE (50 mM Tris-borate buffer, 0.1 mM EDTA). In the Beckman apparatus, the electrophoresis was routinely run at 15°C, with a pulse time of 4 s for the first 10 min at 170 mA, after which the pulse was varied from 5 to 90 s at 150 mA for 18–24 h. In the CHEF Mapper, electrophoresis was run at 15°C at conditions for the separation of 50–1500 kb. Agarose gels (1%) were used in all experiments (SeaKem GTG, FMC Corp., Rockland, ME, USA). Size markers were *Saccharomyces cereviciae* chromosomes, for fragments larger than 500 kb, and phage lambda concatemers, both from New England Biolabs (Hertfordshire, England). The gels were stained with ethidium bromide and the DNA blotted onto MSI nylon filters in 20× SSC (1× SSC is 0.3 m NaCl and 0.05 M sodium citrate).

Mapping

The filters were hybridized with radioactive labeled gene probes (Table 1) as described previously (Kolstø et al. 1990). Routinely long exposures were used to detect hybridization to partial digested fragments. Extensive analysis of complete and partial digestion with *Asc*I or *Sfi*I has been performed, as well as a limited number of double digests, using both enzymes. In preliminary experiments *Not*I was used, but this enzyme produced too many fragments.

Results and discussion

Electrophoretic analysis

PFGE of digested DNA showed that the chromosome contained 8 *Asc*I fragments, ranging from 80 to 1140 kb, and 6 *Sfi*I fragments from 120 to 1300 kb (Table 2). A range of pulse times were used to obtain optimal separation of all the fragments. The sizes of the *Asc*I fragments were larger and the number of the *Asc*I fragments was smaller than the 17 *Asc*I fragments reported in the alkaliphilic *Bacillus* sp. strain C-125 (Sutherland et al. 1993). A similar high variation of the fragment pattern was observed in *Bacillus cereus* strains (Carlson et al. 1992, 1994). The presence of smaller fragments (less than 10 kb) in *B. firmus* cannot be excluded, although they would not contribute significantly to the overall chromosomal size of 4 Mb. The chromosomal size is somewhat larger than the 3.7-Mb chromosome of the alkaliphilic *Bacillus* sp. strain C-125 of 3.7 Mb (Sutherland et al. 1993), and somewhat smaller than the *B. subtilis* 168 chromosome of 4.2 Mb (Kunst et al. 1997). In other *Bacillus* species, the chromosomal size appears to vary considerably between strains (Carlson and Kolstø 1994).

In addition, an extrachromosomal band of 110 kb was noticed in runs of undigested as well as digested DNA (Fig. 1). This extrachromosomal element behaved as a linear DNA fragment rather than a circular plasmid that moves in a pulse-time-independent manner in PFGE (Beverley 1988). The extrachromosomal element may be a circular plasmid that has been linearized as seen in the gel runs or a phage DNA known to be present in several other *Bacillus* species. Although not shown, the element has been visualized in a circular form by electron microscopic examination of gently lysed preparations of *B. firmus* OF4 cells (A.A. Guffanti, E. Johnson, and T.A. Krulwich, unpublished data).

Mapping

To construct a physical map of the *B. firmus* OF4 chromosome, total or partial digests with *Asc*I or *Sfi*I were separated by PFGE, blotted, and hybridized to the probes listed in Table 1. Gene probes were located to all the *Sfi*I and all the *Asc*I fragments except to the smallest one of 80 kb, and the sizes of the partial digested fragments were visible when long exposure times were used (see Fig. 1). The filters were reprobed three or four times (Figs. 1, 2). Double digests with *Asc*I and *Sfi*I were also used. For instance, the *msy*B probe hybridized to A4 (530 kb) and S6 (120 kb), and to a double-digest fragment slightly smaller than 120 kb, show-

Table 1. List of probes used to generate physical map of alkaliphilic *Bacillus firmus* OF4

Probe	Similarity	Source	Reference and/or accession #
arm19[a]	Unknown	Plasmid	M. Sturr, unpublished
arm32	Unknown	Plasmid	M. Sturr, unpublished
arm36	Unknown	Plasmid	M. Sturr, unpublished
arm43	Unknown	Plasmid	M. Sturr, unpublished
atp	*atp* operon fragments	PCR products	Ivey and Krulwich (1991)
C22H3L	*ftsH*	Pasmid	Ito et al. (1997a)
C24E1	*gerC*	Plasmid	Ito et al. (1997a)
cls	Cardiolipin synthase	Plasmid	B. Tropp, unpubished
katA	Catalase	Plasmid	Hicks (1995)
DRS	Direct repeat sequence in *nha*C region	Plasmid	Ito et al. (1997b)
*top*A	Toposiomerase	PCR product	Ivey et al. (1992a)
pBE22	*cad* operon	Plasmid	Ivey et al. (1992c)
pB159	Similarity to *msyB* of *E. coli*	Plasmid	M. Ito, unpublished, U64312
pBK3711	*fru*A, amidase	Plasmid	Ito et al. (1997a)
pCOX43	*cta* operon	Plasmid	Quirk et al. (1993)
pC23	Similarity to *dppA* of *E. coli*	Plasmid	Ito et al. (1997a)
pGNhaCH3	*nha*C	Plasmid	Ito et al. (1997b)
pJB14	ATP-binding protein	Plasmid	Ivey et al. (1992b)
pJB22	*cad* operon	Plasmid	Ivey et al. (1992c)
pM25	Similar to *E. hirae napA*	Plasmid	M. Ito, unpublished, U89914
pSASP	SASP (*sspA*)	Plasmid	Quirk (1993)
pSK2	2 ORFs in pSPT19, putative transporter	Plasmid	Ivey et al. (1992b)
p23SrRNA	23SrRNA	Plasmid	This study
pT22	Complements K^+ uptake defect	Plasmid	M. Ito, unpublished
pT26	*capBC*	Plasmid	Ito et al. (1997a)
pVM3[2]	Similar to *motAB* paralogue of *B. subtilis*	Plasmid	M. Ito, unpublished, U91841
pVM5	Hypothetical proteins	Plasmid	M. Ito, unpublished, U91842
pVM9	Succinate dehydrogenase (*sdh*)	Plasmid	M. Ito, unpublished, U91843
pVM10	Unknown	Plasmid	M. Ito, unpublished
pVM13	Unknown	Plasmid	M. Ito, unpublished
pVM15	Unknown	Plasmid	M. Ito, unpublished
URF	Putative hydrophobic protein	Plasmid	M. Sturr, unpublished

[a] arm, alkaliphile random map (random pieces of DNA cloned in pGEM 3Zf)
[b] VM, alkaliphile fragments that modestly complement a pH homeostasis mutant of *B.firmus* OF4

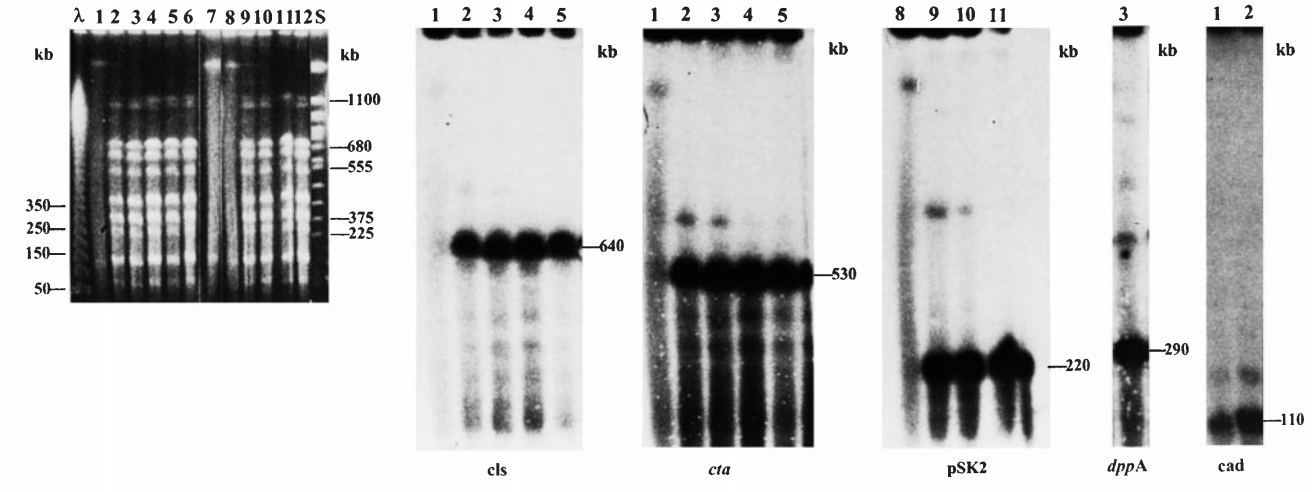

Fig. 1. *Asc*I fragments from *Bacillus firmus* OF4 DNA separated by PFGE. Running conditions: separation of 50- to 1500-kb fragments for 27 h (CHEF Mapper, BioRad). λ, lambda concatemers; *lanes 1–6*, DNA digested with increasing amounts (0.01, 0.1, 5, 10, and 20 U) of *Asc*I; *lanes 8–12*, as lanes 1–5; *lane 13*, *Saccharomyces cerevisiae* chromosomes. The gel was blotted onto a nylon membrane and hybridized with radioactive labeled probes as indicated. *Left panel*: the gel before blotting; the size of the markers is indicated

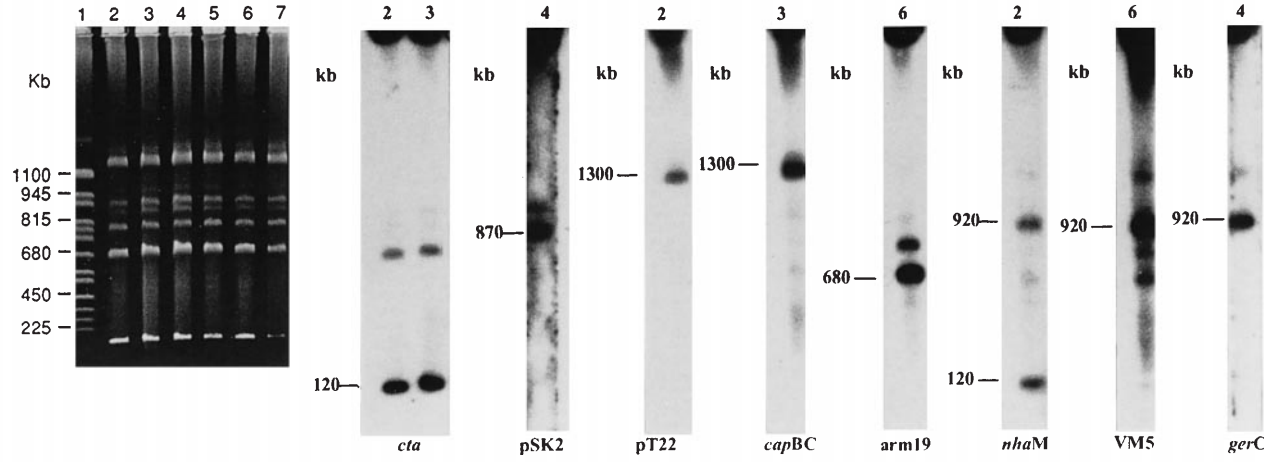

Fig. 2. *Sfi*I fragments from *B. firmus* OF4 DNA separated by PFGE. Running conditions: pulse time, 60 s for 20 h (Beckman). *Lane 1*, *S. cerevisiae* chromosomes; *lanes 2–7*, DNA digested with 20 U *Sfi*I. The gel was blotted onto a nylon membrane and hybridized with radioactive labeled probes as indicated. The size of the *S. cerevisiae* are indicated on the *left-hand* panel

Table 2. *Asc*I and *Sfi*I fragments of *Bacillus firmus* OF4

*Asc*I		*Sfi*I	
Fragment	Size (kb)	Fragment	Size (kb)
A1	1140	S1	1300
A2	700	S2	920
A3	640	S3	870
A4	530	S4	680
A5	400	S5	120
A6	290	S6	120
A7	220		
A8	80		
Total chromosome	4000		4010
Extrachromosomal DNA	120		120
Total genome	4120		4130

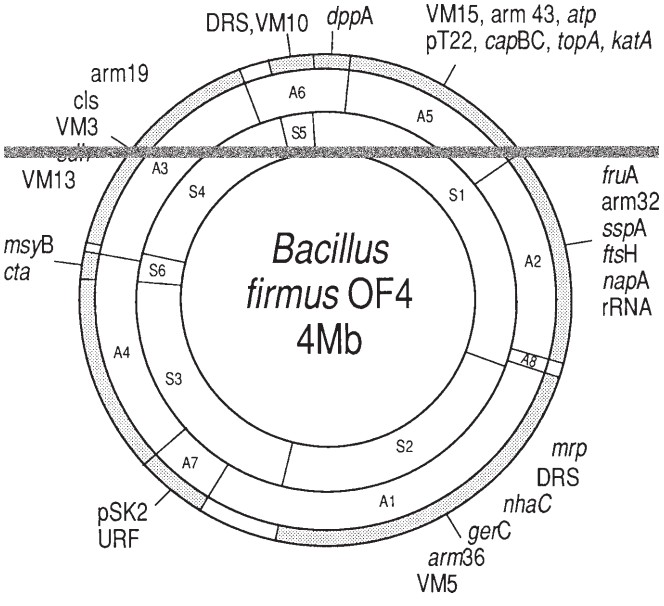

Fig. 3. Physical map of the *B. firmus* OF4 chromosome. *Inner circle*, *Sfi*I fragments; outer *circle*, *Asc*I fragments

ing overlap between the A4 and the S6 fragments (Fig. 3). The VM10 probe hybridized to a similar-sized *Sfi*I fragment, S5 (120 kb), while it hybridized to A6 (290 kb) and to a 290-kb double-digest fragment, showing that the S5 fragment overlapped completely with A6. The probe *dpp*A hybridized to A6 and S1 (1300 kb), and to a double-digest fragment smaller than A6, showing an overlap between S1 and A6. All the probes that hybridized to A2 and A5 hybridized to S1, indicating that the A2 and A5 fragments completely overlapped with S1. This was confirmed by double digests in which the A2 and A5 retained their sizes. Likewise the S2 (920 kb) completely overlapped with the A1 (1140-kb) fragment, and A7 (220 kb) with the S3 (870-kb) fragment.

The physical map (Fig. 3) shows that the genes involved in growth on nonfermentative carbon sources, such as the *atp* (F_1F_0-ATPase) (Ivey and Krulwich 1991) and *cta* (cytochrome oxidase) (Quirk et al. 1993; Gilmour and Krulwich 1997) operons are nowhere near each other on the chromosome. The two antiporter-encoding genes that are involved in alkaliphily in this or other alkaliphilic *Bacillus* species, *nhaC* and *mrp* (Hamamoto et al. 1994; Ito et al. 1997b; Krulwich et al. in press), map to the same fragment but there is no evidence that they are close together within that fragment. A comparison of the localization of the genes common in *B. subtilis* and the *B. firmus* OF4 chromosome indicates a different gene organization of the nine genes analyzed (Fig. 4). This may not be so surprising, taking into consideration the random organization of orthologous

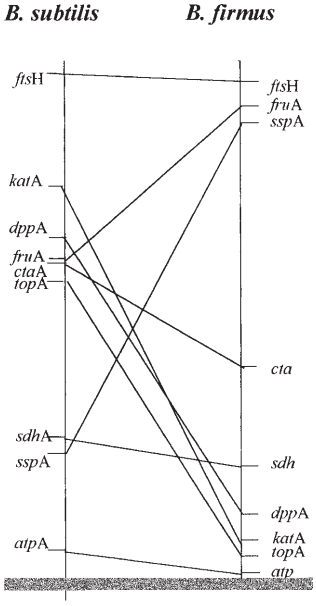

Fig. 4. Comparison of gene localization on *B. firmus* OF4 and *Bacillus subtilis* 168 chromosomes. The *B. subtilis* chromosome is linearized, with *dna*A as the first gene on the top (Kunst et al. 1997). only the genes equivalent to the mapped *B. firmus* OF4 genes are included. The *B. firmus* map is linearized with the 700-kb *Asc*I fragment on the top to obtain maximum similarity between the maps

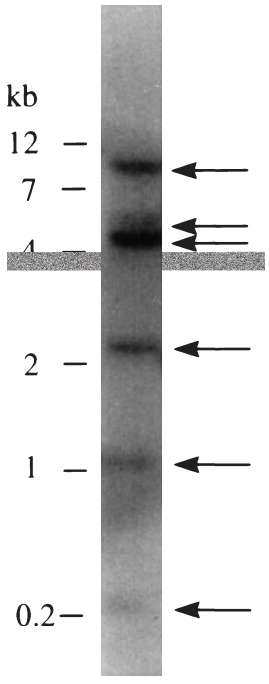

Fig. 5. Southern analysis of *B. firmus* OF4 chromosomal DNA using a region of the direct repeat sequences as probe. Chromosomal DNA (10 μg) from *B. firmus* OF4 was digested with *Sph*I, electrophoresed through a 0.6% agarose gel, and blotted to nitrocellulose. For the probe, amplified DNA corresponding to the most downstream DRS (DRS2) previously reported in the *nhaC* gene region (Ito et al. 1997b) was prepared by PCR and labeled with a random priming kit (New England Biolabs, Beverly, MA, USA). The *numbers at the left* indicate the positions of standard molecular weight markers. The bands that hybridized with the probe are indicated by *arrows*

genes in other bacteria that are not very closely related (Kolstø 1997).

Analysis of codon usage of genes associated with chromosome versus genes associated with the extrachromosomal element

It was notable that transposition-related genes and a cadmium-resistance locus that had earlier been identified (Ivey et al. 1992c) were localized to an extrachromosomal element that is likely to be a large endogenous plasmid. An analysis of the codon usage of the genes that can now be assigned to that element and the more extensive catalog of sequenced genes which are localized to the chromosome indicates that there are small but signficant differences in codon usage (Table 3). For example, TAG is not used in any of the translational stop codons thus far proposed in the extrachromosomal element, whereas it represents 28% of the stop codons of the chromosomal genes. TGA has been found, conversely, to be used significantly more frequently in the extrachromosomal element than in the chromosome, and this codon is also at least occasionally read through as tryptophan (Ivey et al. 1992a). Similar significant differences in use of codons are seen in AGC versus TCC for serine and CGC for arginine. The differences in codon usage suggest a separate origin for the extrachromosomal element, perhaps by horizontal transfer from other soil organisms. Most importantly, none of the genes that thus far is implicated as having a role in the energetics of alkaliphily or pH homeostasis was localized to the extrachromosomal element.

Southern analysis of direct repeat sequence distribution

Novel direct repeat sequences (DRS) had been found earlier just upstream of the Na^+/H^+ antiporter-encoding *nhaC* gene (Ito et al. 1997b). The two DRS had 95.7% nucleotide sequence identity over a 138-bp overlap. In the physical mapping, a DRS probe hybridized, as expected, to the fragment that also hybridized to an *nhaC* probe, and also to the S120–A290 fragment (see Fig. 2, probe *nha*M). As shown in Fig. 5, a DRS probe hybridized to at least six independent loci in a standard southern analysis of *Sph*I-digested chromosomal DNA. Because only two of the fragments in the physical map were identified as having these sequences, there is an apparent nonrandom distribution in the chromosome of the DRS. Such sequences could play a role in regulation or in chromosomal rearrangements that promote amplifications which might be useful as a response to environmental stresses. It is notable in this context that Amano and Shishido (1995) noted DRS in the regions of several *B. subtilis* strains that flank the tetracycline-resistance determinant. Recent work in our laboratory has shown that this determinant encodes a multifunctional antiporter that has important physiological roles in Na^+ resistance and pH homeostasis in the moderately alkaline range in addition to providing defense against low levels of the antibiotic

Table 3. Codon usage for *Bacillus firmus* OF4 genomic and plasmid genes

A											
Gly	GGG	0.20	Arg	AGG	0.10	Trp	TGG	1.0	Arg	CGG	0.07
Gly	GGA	0.28	Arg	AGA	0.26	End	TGA	0.15	Arg	CGA	0.12
Gly	GGT	0.34	Ser	AGT	0.19	Cys	TGT	0.65	Arg	CGT	0.34
Gly	GGC	0.18	Ser	AGC	0.15	Cys	TGC	0.35	Arg	CGC	0.12
Glu	GAG	0.38	Lys	AAG	0.37	End	TAG	0.28	Gln	CAG	0.31
Glu	GAA	0.62	Lys	AAA	0.63	End	TAA	0.57	Gln	CAA	0.69
Asp	GAT	0.77	Asn	AAT	0.62	Tyr	TAT	0.69	His	CAT	0.67
Asp	GAC	0.23	Asn	AAC	0.38	Tyr	TAC	0.31	His	CAC	0.33
Val	GTG	0.20	Met	ATG	1.0	Leu	TTG	0.10	Leu	CTG	0.09
Val	GTA	0.31	Ile	ATA	0.14	Leu	TTA	0.36	Leu	CTA	0.11
Val	GTT	0.36	Ile	ATT	0.57	Phe	TTT	0.68	Leu	CTT	0.26
Val	GTC	0.14	Ile	ATC	0.29	Phe	TTC	0.32	Leu	CTC	0.08
Ala	GCG	0.16	Thr	ACG	0.22	Ser	TCG	0.09	Pro	CCG	0.24
Ala	GCA	0.38	Thr	ACA	0.45	Ser	TCA	0.26	Pro	CCA	0.32
Ala	GCT	0.35	Thr	ACT	0.21	Ser	TCT	0.25	Pro	CCT	0.38
Ala	GCC	0.12	Thr	ACC	0.12	Ser	TCC	0.06	Pro	CCC	0.06
B											
Gly	GGG	0.11	Arg	AGG	0.14	Trp	TGG	1.0	Arg	CGG	0.07
Gly	GGA	0.44	Arg	AGA	0.31	End	TGA	0.33	Arg	CGA	0.21
Gly	GGT	0.32	Ser	AGT	0.18	Cys	TGT	0.83	Arg	CGT	0.23
Gly	GGC	0.12	Ser	AGC	0.07	Cys	TGC	0.17	Arg	CGC	0.04
Glu	GAG	0.27	Lys	AAG	0.30	End	TAG	0.00	Gln	CAG	0.29
Glu	GAA	0.73	Lys	AAA	0.70	End	TAA	0.67	Gln	CAA	0.71
Asp	GAT	0.83	Asn	AAT	0.73	Tyr	TAT	0.81	His	CAT	0.86
Asp	GAC	0.17	Asn	AAC	0.27	Tyr	TAC	0.19	His	CAC	0.14
Val	GTG	0.13	Met	ATG	1.0	Leu	TTG	0.15	Leu	CTG	0.03
Val	GTA	0.31	Ile	TAT	0.21	Leu	TTA	0.39	Leu	CTA	0.16
Val	GTT	0.39	Ile	ATT	0.57	Phe	TTT	0.75	Leu	CTT	0.23
Val	GTC	0.18	Ile	ATC	0.23	Phe	TTC	0.25	Leu	CTC	0.03
Ala	GCG	0.10	Thr	ACG	0.15	Ser	TCG	0.05	Pro	CCG	0.11
Ala	GCA	0.40	Thr	ACA	0.32	Ser	TCA	0.26	Pro	CCA	0.30
Ala	GCT	0.41	Thr	ACT	0.34	Ser	TCT	0.32	Pro	CCT	0.49
Ala	GCC	0.10	Thr	ACC	0.19	Ser	TCC	0.12	Pro	CCC	0.10

Sections A and B represent the codon usage by genes putatively coded for by genomic and plasmid DNA, respectively. In each section of the table, amino acids are represented by their three-letter code, followed by the relevant codon and the frequency of that codon. The table was generated using the Codon Frequency program of the GCG Software package. Sequence data for section A were from Genbank accession numbers L02548, L02551, M60117, M79460, M94110, M96341, U18744, U39410, U60883, U61168, U61539, U61844, U64312, U64313, U64314, U64514, U64515, U89914, U91841, U91842, U91843, X59424, and Z14112. Sequence data for section B were from Genbank accession numbers M90749, M90750, U33209, and Z17326

(Cheng et al. 1996). It will be of interest to determine the effects of introducing and deleting DRS from *B. firmus* OF4 on the adaptability of this versatile extremophile to extremely alkaline conditions at various concentrations of Na^+.

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