## Alkalophilic *Bacillus* sp. Strain LG12 Has a Series of Serine Protease Genes

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Four tandem subtilisin-like protease genes were found on a 6,854-bp DNA fragment cloned from the alkalophilic *Bacillus* sp. strain LG12. The two downstream genes (*sprC* and *sprD*) appear to be transcribed independently, while the two upstream genes (*sprA* and *sprB*) seem to be part of the same transcript.

Bacteria isolated from alkalophilic and/or thermophilic environments may produce subtilisins with the characteristics needed in commercial applications (11, 14, 16, 18, 30, 33). In addition, these enzymes may provide sequence and biochemical information helpful in the design of new subtilisins (5, 8, 13, 17, 27, 34). To this end, we have cloned and sequenced a series of protease genes from *Bacillus* sp. strain LG1Z.

Cloning of the protease genes. An alkalophilic Bacillus sp. strain, LG12, was isolated from an alkaline creek bed and grown in Luria-Bertani medium with the pH adjusted to 8.5. This organism was found to produce at least two different alkaline proteases. Purified chromosomal DNA from Bacillus sp. strain LG12, digested with restriction enzymes (Boehringer Mannheim), was fractionated on 0.8% agarose gels and Southern blotted (29) on Nytran filters (Schleicher & Schuell). The nick-translated (24) subtilisin BPN' gene from Bacillus amyloliquefaciens (35) was used as a probe. Chromosomal DNA fragments of ca. 1 kilobase pair (kb) from a HindIII digest were cloned into pBR322 (2) and used to transform Escherichia coli MM294 (12), and colony hybridization was used to detect positive clones (9). The nucleotide sequence (25) of a 990-bp DNA insert from a positive clone was homologous to the 3' portion of the subtilisin BPN' gene. A ca. 6-kb BglII fragment was cloned and sequenced in order to obtain the remaining upstream sequences of the protease gene. Three additional subtilisin-like protease genes were discovered on this fragment.

The protease genes. The four open reading frames coding for subtilisin-like serine proteases have been designated *sprA*, *sprB*, *sprC*, and *sprD* (Fig. 1). The upstream portion of the *sprA* gene, coding for presumably the first 70 or so amino acids, is missing on the cloned *Bgl*II fragment. Another downstream open reading frame (ORF) may exist, but there is not enough sequence on the cloned fragment to determine if it codes for an additional protease.

The nucleotide sequence of the cloned region is shown in Fig. 2. Inverted repeat sequences, consistent with known rhoindependent terminators (22), were found immediately downstream of the *sprB*, *sprC*, and *sprD* genes. Additional inverted repeats were also found upstream of the *sprC*, *sprD*, and ORF genes and may have regulatory functions. Putative promoter sites, -10 and -35 regions (Fig. 2), were discovered upstream of the *sprC*, *sprD*, and ORF genes by comparison with promoter consensus sequences typical of *B. subtilis*  $\sigma^{A}$  recognition sequences (19).

Possible translational start sites are shown in Fig. 2 for the *sprB*, *sprC*, *sprD*, and ORF genes. The *sprC* coding region starts with GUG rather than AUG, which is not unusual for *Bacillus subtilis* genes (32). Two potential translational start sites were found for the *sprB* gene (Fig. 2); one 27 nucleotides downstream of the putative *sprA* termination codon with a recognizable ribosome binding site (RBS) (32), and the other nine nucleotides downstream without a discernible RBS (Fig. 2). Since there is such a small distance between the *sprA* and *sprB* 

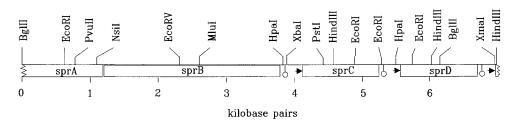


FIG. 1. Restriction endonuclease map of the DNA fragment carrying the four serine protease genes cloned from *Bacillus* sp. strain LG12. ○, putative terminator; ▶, promoter.

genes and there are not obvious transcriptional start or termination signals, it seems likely that they are part of the same transcript. If this is the case, then the two genes might be translationally coupled, avoiding the need for a good RBS (32).

Generally, the codon usage for the four protease genes from *Bacillus* sp. strain LG12 (not shown), as in *B. subtilis* (26),

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	10	20	30	40	50	60	70	80	90	100
	$sprA \rightarrow \bullet$	•	•	•	•	•	•	•		100
1	AGATCTTTTGG	AAGAGATAGGAA	PACAGCAAGCCA	TCACATTTA	ATGATTTACC	CTTTGTAGGAG	CTTTGGCAA	CAAAGGAACA	AGTCGATCA	ATCCTC
	GAGTGGGATAA	CGTAATTTCCGTT	TATTACAACCA	GGAACTTCA	ATATGAGAAC	GATGATGCCAC	AGCTTATAC	TGGTGTGGAC	CGAGTTCGTA	AAAGATG
201		AAAATGAATGGCC								
301 401	GTTTGGTAACA	ATCTTGTCCAGA	ATGTCCTTGGAA	GTACCAACC'	I'GAA'I'GC'I'A'I'	GACAGGGATTC	TACCAATCA	CTTACGTCGA	AAATGTTCCC	CAATACG
501	TGATTCAAGTTC	CGGGCATGGAACA GGTTCCGGGGGCGG	CATGTTGCTGG	TACGGTTGG	AGGTACAGGA TTTCCCCCAT	GUAATGTUAGG	AGGGAAGTA	TGAAGGGGTT	JCCCCTGGAG	SAAAA'I"I'
	TACGAATTCTT	GGGGTGCAACGAG	CGATGCAGGTA	CCCACTTCG	AUCCUSAL	TIGACIAIGCC	TGACACAT	LAGCAGGAATA AAAAACTGTA	TCACCATCCC	SAATCAT ATTA ATT
		TGCTGGAAACTCT								
801		GCCGATTTCTCCT								
		GCGTTGACATTGI								
		TACATTCAGCGGA								
1101		ATACTCGAAGGCA	{ RBS }	atggaagga. sprB		GGGAAACTGGT	GCTGGCTAT	GITGATGCCT	ATGAAGCGGT	FTACGAA
1201	sprA-S	cop <u>AA</u> ACAATTCAT <u>AI</u>				TACTACTAT	GTGCGTTAT	ጥረጥጥሮልጥረርጥ	22000020000	ጥአሞአርሞ
		GGGAGAAGATACO								
1401		CAAAAAACCCACAG								
1501		AGAAGCAAGTCGA								
		AGTGAACAAAGTC								
		GACGGTACACATA								
1801 1901		ACACGGAAAATG1 GGGAGCGGCACCA								
		CAGGAATATGACA								
		GAGGAATCGTCGT								
2201	GTTGCGGCAGG	TGTCAAGGATGGC	ACATTGGCTGA	TTTCTCCTCT	CGTGGCACG	AAAGGGGTTGG.	AGGAACATT	CAGCATGGACO	GGATGGAAT	GGACTT
		CCAACCATAACTG								
2401		AACACCTCCCTTA								
		ACCAGATCAGGTC								
		GTGGATGCCTCTC TTGAAATAAACTA								
		GGAAGCTACGGGA								
		ATTGTATTGGTAG								
		AGGTACAAGGATC								
		TGAGAGATTATTI								
		CGCCAAAGCATTC AATTCCACCAAGA								
		GAGTCTTGGATTA								
		GAACACTTAAAAG								
									sprB-	
3601	ACCAAAGATCG	AGGCTACTTTCCA						ATTTTAATGTO	JTTTTTTAAT	T <u>GA</u> GGC
2701		AAACAGATTTTTA		>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>		·>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>		Cma C a mCCCmr		****
3701	CALIGITAACA	AACAGAIIIIIA	AIAGI 166661	AGGAGAGAAA	-35	LICAGCIICII	IIIIGAIII	-10	.CATGIGAAA	MATCCA
3801	CACCAATTCCA	TTCTATAGGATAA	TCAAGGAAACT	ATTACAGATI		GAAACTTGGT	GTCTATTA	GCATATAATO	CACCCTATTT	TTTCTC
					·>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>		<<<<<<		{ RBS	
3901		FATGAATTTCTCG $prC \rightarrow$	TGACTGATGGT	AAACTTTCGI	TAATAGTTC	PTAATTAGTTA(	GAATTATTA	AAAATCCTTAI	TCCAAGGAG	TGAATC
4001		DIC → IGGATGAAAGGTT	TTTTCCCTCATA	СТТАТАССА	TATTGGCTT	TCTCGTTAGCT	PCGGAACTG	TTCAGGCAGA	ассатсааат	TCCGAAT
		GAAAGAATACTTA								
		CAGTACATGGATG								
4301	CGTCGAAATGA	IGGCTACTGCTCA	AACAGTTCCTT	GGGGCATCCC	TCATATCAA	AGCTGACAAAG	CGCATGCTG	CTGGAGTGACI	IGGAAGCGGT	GTGAAG
		GGATACAGGAATT								
		GGAACACACGTAG CTTCAGGTAGTGG								
		TTCAACTGCTTTA								
		ATGGGTTATCCTG								
									>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>	
4901		PAATGGCACCAGG	TGTCAACATTT	TGAGCACAAC	TCCTGGCAA			JTACATCCATO	GCAGCTCCT	CATGTT
	< <<<<<<				>>>>>>>					
5001	GCAGGAGC'I'GC'	IGCATTAATCAAA	IGUGAAATACCC	AAGCATGACA sprC-9		ATTCGTGAACG	ICIGAAAAA			ATCCTT erm
5101	TCTTCTACGGA	AAAGGTGTCATCA	ATGTGGAAAGT			ATCCCCCCCTC	TAAATAAGA			
		<<<< >>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>								
5201		GTGTTTTTTTATT			TTTAGCACT	ATGACTTATT'	TTTGAAGTT	ATTACCATGAT	IGCACCATTG	GGAAAT
						>>>> -35 <-		-10		
5301	TCGGGTGTAGA	ATCTCCGGATTAG			TCCCAATAA	CAATTCCTATA	rtgttatgc'	TAGTATTATAG	эттаастааа	TAATTT
5401	CATTATTACA	{ RBS AAAAAAGAAGGAG	} :CCTC & & TT & C &	$sprD \rightarrow$		<u>ለ አ አ አ ም አ ምም</u> ር አ ርሳ	~ <u>പന്നത്ത്</u> നം	20000070077	៶ຓຬຬຓຬຓຓຉຓ	൙൱൬൬൙൬
		rctgatgcaagtg								
		PTGGTGGAACGGT								
5701	CCTCTAGTGGA	GTATGTGGAAGAA	AACGTTGAAAT	GCAAACAACC	GCTCAAACA	<b>STTCCATATGG</b>	AGTACCTCA	TATCAAAGCGG	JATGTAGCAC	ATGCAC
		GGATCTGGTGTGA								
		ATGCTTTGACGGA								
		ICTTTATGCAGTA AATATGAGTCTGG								
		AATATGAGTCIGG GTGGTTCCGTGCT								
		CTTCTCAAGTGTA								
		ATGGCTTCTCCTC								
							<i>sprD</i> -S			>>> >
6501		CCAATTTGGGATC		ATGGCCATGG						
c c c c c c		Term <<<<<<		000000000	-35					
		ITGTGTGGGGGCGG CGTTTCGCAAAAA								
0,01	ICHICICIAIA	RE {				STRUCCAIGI				101001
6801	AGTTCTGAGAA	AGCACTCCCGGGA			CAACAAGCTT					

FIG. 2. Nucleotide sequence of the 6,854-bp DNA fragment containing the four *spr* genes (shown schematically in Fig. 1). The putative start and stop codons for the genes are underlined, and potential RBSs are labelled. Putative -10 and -35 promoter regions are indicated. Inverted repeats (> <) that could function as rho-independent terminators are labelled (Term). The beginning of another possible ORF is shown.

		-60	-40	
sprA			DLLEEIGIQQAITFNDLE	FVGALATKEQV
sprB		<u>EG</u> EDTVSSVVQEKVWSVLKDATTTAQIIITF		
sprC		EASNPNASAKKEYLVGFAKGNKA		
sprD		DASANGKPLMKEYLIGLKTGP		
BPN'	MRGKKVWISLLFALALIFTMAFGSTSS	AOAAGKSNGEKKYIVGFKQTMST	MSAAKKKDVISEKGGKVQKQFKYVI	DAASATLNEKAV
	-20 +1	20 •	40	
sprA		GVDRVRKDESFRKMNGGMPVSGNGIGIVIND		
sprB		GVNKVRTDKEMTKGNGGMPISGNGIGVVVND WGIPHIKADKAHAAGVTGSGVKVAILD		
sprC sprD		GIPHIKADKAHAAGVIGSGVKVAILD GVPHIKADVAHAQNVTGSGVKVAVLD		
BPN'		GVSQIKAPALHSQGYTGSGVKVAVLD		
511			101DADADDD	
	60 • 80	100	120	140
sprA		EGVAPGENLIGYGSGAVVAMLDTLGGFD		
sprB		EGAAPGADLIGYGSGGALFILDGIGGFD		
sprC		LGVAYNADLYAVKVLSASGSGTLSGIAQGIE		
sprD		LGVSYDVDLYAVKVLSAGGSGTLAGIAQGIE		
BPN'	TNPFQDNNSHGTHVAGTVAALINNSIGV.	LGVAPSASLYAVKVLGADGSGQYSWIINGIE	WAIANNMDVINMSLGGPSGSAA	LKAAVDK
	160	180	200	
sprA		KAPWVITVAAGSVEGDLADFSSRGVQGNGGS		TPLSALSAOKD
sprB		KAPWVISVAAGVKDGTLADFSSRGTKGVGGT		
sprC		RYSSVIAVGAVSSNNTRASFSSVGSE		
sprD		RYDSVIAVGAVDSNNNRASFSSVGSQ		
BPN'	AVASGVVVVAAAGNEGTS.GSSSTVGYPO	KYPSVIAVGAVDSSNQRASFSSVGPE	LDVMAPGVSIQST	LPGNK.
	220•	240 260		
sprA		Z40 Z60 ALMLEVNPTLDALEIKEILEGTAIPMEGYAI		
sprB		ALMLEADPTLSPDOVKEIIOHTATNMPGYEA		MNOTEVSNUTT
sprC		ALIKAKYPSMTNVQIRERLKNTATNLGDP		
sprD		ALLLAQNPNLTNVQVRERLRDTATNLGS.A		
BPN'	YGAYNGTSMASPHVAGAA	ALILSKHPNWTNTQVRSSLENTTTKLGDS	FYYGKGLINVQAAAQ	
-				
sprB		ILLARVDGKGMMEATGNPINLVLTAPDGTEYS IPAEAAIOHGVSERLFDGYKNGSFKPDAKLTR		
sprB		TKGELAYSLVOSLGLOEEALAHKGDIKVMYK		
sprB sprB	DLKPKIEATFHGNEEITRGDFAVAFTRYF		DEPAARVOOR LEURGIAÕRVOUT	AVAIVE SV TQGRY
Shrp	DERIVICAL FROMEET INODE AVAF INTE	TAAT T TA		
0	· · · · · · · · · · · · · · · · · · ·		1	

FIG. 3. Computer-generated (pileup program, Genetics Computer Group) amino acid sequence alignment of the Spr proteins, with the subtilisin BPN' sequence added for comparison. Numbering is based on the BPN' sequence (35). Putative signal sequences at the beginning of the Spr proteases are underlined. The amino acid sequence in the C-terminal extension of SprB having homology to the S-layer-like repeat sequences in the cellulosome from *C. thermocellum* (7) is also underlined. The catalytic aspartate, histidine, and serine residues are indicated ( $\cdot$ ).

favors codons with A or T in the third position (an exception is TCC). This reflects the overall G+C content of 44%, which is consistent with a number of *Bacillus* species (23). Unlike in *B. subtilis* and *Escherichia coli*, there does not seem to be a strong preference against ATA as an isoleucine codon. Like the other members of the subtilisin family (4), all four of these genes use TCN as the codon for the active-site serine.

Finding a series of four subtilisin-like protease genes was surprising since in *B. subtilis* the protease genes map to very different loci on the chromosome (21). Tandem gene duplications can arise by homologous recombination of repeated sequences (15), and chromosomal amplifications of antibiotic genes can be induced in *B. subtilis* by increasing the selective pressure (1, 37). However, no obvious repeated sequences, indicative of previous recombination events, were found in the sequenced region.

**Protease expression.** For protease production, the *sprC* and *sprD* genes were cloned in the replicating vector pBN2, a hybrid plasmid of pUC18 (36) and pUB110 (10), as ca. 1.7-kb *XbaI-HpaI* and ca. 2.5-kb *HindIII-XmaI* fragments, respectively (Fig. 1). Both the genes were expressed in *B. subtilis* BG2036 ( $\Delta npr \ \Delta apr$ ) utilizing their own intrinsic transcriptional and translational signals. Protease activity was detected by the formation of clearing zones around colonies on skim milk plates and assayed by using the synthetic substrate *N*-succinyl-L-Ala-L-Pro-L-Phe-*p*-nitroanilide (6) (data not shown).

**Protease sequence features.** The predicted protein sequences of SprB, SprC, and SprD have typical signal sequences indicative of secreted proteins (20), although the precise signal peptidase cleavage sites have yet to be located (Fig. 3). All four proteases appear to have propeptides (20), with the promature junction in SprC and SprD most likely identical to that of subtilisin BPN' (35). The SprA and SprB junctions are also likely to be near this region (Fig. 3), but as yet the N-terminal sequences of the mature proteases have not been determined.

The propeptides of SprB, SprC, and SprD are not as highly charged as other subtilisins (17 charged residues for SprD versus 25 for BPN'). It has been suggested that propeptides with a large net negative charge are indicative of subtilisins from alkalophilic *Bacillus* species (31), and although the propeptides of SprB, SprC, and SprD do have predicted net negative charges, the values are not very large (the most negative is -4).

The predicted SprC and SprD mature proteases are closely related to subtilisin BPN' (Table 1). However, the SprA and SprB proteases are not as closely related to BPN' and also are not as similar (<40% identity) to other subtilisins, including the minor extracellular proteases, Epr, Bpr, and Vpr, from *B. subtilis* (21). SprB has a long C-terminal extension of over 350 amino acids (Fig. 3) like Epr, Bpr, and Vpr, but there does not appear to be any homology among the sequences of the Cterminal extensions. The function of C-terminal extensions is

TABLE 1. Percent identities among mature protease sequences

Dustana	% Identity with protease					
Protease	BPN'	SprA	SprB	SprC		
SprA	32					
SprB	40	69				
SprC	65	34	36			
SprD	65	32	37	77		

unknown, but in the case of Vpr the extension may function as a membrane anchor (28). SprB may also be associated with the cell surface, since there is some sequence homology (ca. 35% identity in a 43-amino-acid sequence) in the C-terminal extension (Fig. 3) to that of the S-layer-like repeat sequences in the cellulosome from *Clostridium thermocellum* (7).

Besides the catalytic triad (D32, H64, and S221) and oxyanion-binding residue (N155), SprA, SprB, SprC, and SprD have additional amino acids reported to be highly conserved in subtilisins (G23, G34, H39, G65, T66, G70, G83, S125, G127, G146, G154, G219, T220, and P225) (27). The side chains of the calcium binding site, Q2, D41, and N77, are conserved in SprC and SprD. Consistent with the BPN' structure (27), SprD would be expected to form the salt bridges K136-D140 and R170-E195, and both SprC and SprD have the side chains necessary for a E197-R247 salt bridge.

There are one cysteine in the signal sequence of SprB, one cysteine in the mature sequence of SprC, and no cysteines in SprA or SprD. Thus, as in other bacterial subtilisins (27), there are no internal disulfide bridges in these proteases.

A number of subtilisins from thermophilic and/or alkalophilic bacteria have a deletion of four amino acids relative to BPN' at the P1 binding site in the region of amino acid 160 (31). However, SprC, SprD, and the alkaline protease from the thermophilic species *Bacillus smithii* (18) do not have this deletion; in fact, SprD has an insertion of one amino acid at this site (Fig. 3). Deletion of these four amino acids has been found to lower the catalytic efficiency of BPN' (3). The insertions in SprA and SprB, relative to BPN' (Fig. 3), are consistent with the variable regions noted for other subtilisins (27).

Of the amino acid substitutions known to increase the alkaline activity (change of Y to F at position 104 [Y104F]) or stability (M50F, I107V, and K213R) of BPN' (5, 34), only F50 is present in SprC and SprD (Fig. 3). Twenty-nine residues have been found to be unique in subtilisins isolated from alkalophilic bacteria compared with proteases from mesophiles (30). Of these residues, only six are conserved in both SprC and SprD (A15, G25, P55, A108, Q109, and R170).

**Nucleotide sequence accession number.** The GenBank accession number for the DNA sequence identified in this study is U39230.

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