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ORIGINAL PAPER

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A novel species of alkaliphilic *Bacillus* that produces an oxidatively stable alkaline serine protease

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Abstract A novel gram-positive, strictly aerobic, motile, sporulating, and facultatively alkaliphilic bacterium designated KSM-KP43 was isolated from a sample of soil. The results of 16S rRNA sequence analysis placed this bacterium in a cluster with *Bacillus halmapalus*. However, the level of the DNA–DNA hybridization of KSM-KP43 with *B. halmapalus* was less than 25%. Moreover, the G + C contents of the genomic DNA were 41.6 mol% for KSM-KP43 and 38.6 mol% for *B. halmapalus*. Because there were also differences in physiological properties and cellular fatty acid composition between the two organisms, we propose KSM-KP43 as a novel species of alkaliphilic *Bacillus*. This novel strain produces a new class of protease, an oxidatively stable serine protease that is suitable for use in bleach-based detergents. The enzyme contained 640 amino acid residues, including a possible ~200-amino-acid prepropeptide in the N-terminal and a unique stretch of ~160 amino acids in the C-terminal regions (434-amino-acid mature enzyme with a calculated molecular mass of 45,301 Da). The C-terminal half after the putative catalytic Ser255 and the contiguous C-terminal extension shared local similarity to internal segments of a membrane-associated serine protease of a marine microbial assemblage and the serine protease/ABC transporter precursors of the slime mold *Dictyostelium discoideum*, and to the C-terminal half of a cold-active alkaline serine protease of a psychrotrophic *Shewanella* strain.

Key words *Bacillus* · Oxidative stability · Subtilisin · Detergent enzyme · Phylogeny16S rRNA · DNA–DNA hybridization

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Introduction

Alkaliphilic *Bacillus* strains grow well in extremes of alkalinity higher than pH 9. The alkaliphilic *Bacillus* is a potentially rich source of useful enzymes (Ito et al. 1998; Horikoshi 1999). Among the isolated enzymes, highly alkaline serine proteases, a class of the subtilisin superfamily (Siezen and Leunissen 1997), are the enzymes most studied because of their industrial importance and are now mainly incorporated into modern heavy-duty detergent powders (Betzel et al. 1992; Van der Laan et al. 1992; Kobayashi et al. 1995).

To survive the extremes of high alkalinity and high chelator concentration in detergents, subtilisins have been improved with respect to thermostability, resistance to chelators, and pH- and temperature-activity profiles of the enzymes (Bryan 2000). However, because subtilisins all contain a Met residue located next to the catalytic Ser (Siezen and Leunissen 1997), they are readily inactivated by oxidants (Stauffer and Etson 1969). To improve their oxidative stability in bleach-based detergent formulations, the susceptible Met residue is replaced with nonoxidizable amino acids, but the mutation at this position reduces catalytic power on protein substrates (Estell et al. 1985; Bott et al. 1988). To prevent the loss of activity, several oxidatively stable serine proteases (OSPs) suitable for use in detergents have been isolated from alkaliphilic *Bacillus* strains (Saeki et al. 2000). Recently, we found that an isolate, KSM-KP43, which produces an OSP, is a novel species of *Bacillus*. We describe here the identification of the isolate, properties of the OSP, and its gene sequence.

Materials and methods

Bacterial strain and propagation

KSM-KP43 was originally isolated from a soil sample collected in Haga, Tochigi, Japan, and propagated at 30°C in

a medium (pH 9.0) composed of 0.5% glucose, 0.2% Polypepton S (Nippon Pharmaceutical, Osaka, Japan), 0.05% yeast extract (Difco, Detroit, MI, USA), 0.1% KH_2PO_4, and 0.26% Na_2CO_3 (separately autoclaved). *Bacillus halmapalus* DSM 8723^T was used as the reference strain. Alkaliphilic *Bacillus* strains D-6, Y, NCIB 12289, and SD521 (Saeki et al. 2000) and the enzymes produced were also used.

Purification of the enzyme and sequencing of its N-terminal region

The OSP designated KP-43 from KSM-KP43 was purified to homogeneity as described (Saeki et al. 2000) with a yield of 15%. The N-terminal amino acid sequence was determined in a protein sequencer (model 476A; Applied Biosystems, Foster City, CA, USA) that was connected to an on-line phenylthiohydantoin (PTH)-derivative analyzer.

Isolation of DNA and transformation

Preparation of genomic DNA and plasmid DNA and the methods of restriction digestion, ligation, and transformation were as described previously (Saeki et al. 2000). Sequencing was performed by the dideoxy chain termination method using a DNA sequencing kit (Dye Terminator Cycle Sequencing Ready Reaction; Perkin-Elmer, Norwalk, CT, USA) and an automated DNA sequencer (model 377; Applied Biosystems).

Amplification and sequencing of DNA

The complete KP-43 gene and its flanking regions were amplified with *Pwo* DNA polymerase by polymerase chain reaction (PCR) with the genomic DNA (100 ng) as template and appropriate primers (20 pmol each): 5′-AAATGGA TCCGCAGTATGCCTTTTAAGTC-3′ and 5′-TACAGG ATCCATATTAATTCTTCTACCC-3′. PCR experiments were done with 30 cycles of a 1-min denaturing step at 94°C, a 1-min annealing step at 50°C, and a 1-min extension step at 72°C. The amplified product of 2,110 bp was then sequenced directly.

16S rRNA (rDNA) fragments from KSM-KP43 and other bacilli (strains D6, Y, SD521, and NCIB12289) (Saeki et al. 2000) were analyzed using PCR direct sequencing, as described by Shima et al. (1994). Sequences were aligned using the Clustal multiple-alignment program (CLUSTAL X) (Thompson et al. 1994), and nucleotide substitution rates (K_{nuc} value) were calculated. Sites involving gaps were excluded from all analyses. A phylogenetic tree was inferred by the neighbor-joining method (Saitou and Nei 1987) in the CLUSTAL X program version 1.64b. The similarity values of the sequences were calculated using the GENETYX-MAC program version 9.0 (SDC Software Development, Tokyo, Japan).

DNA base composition and DNA–DNA hybridization

The KSM-KP43 genomic DNA was prepared according to the method of Marmur (1961). The G + C content of the DNA was determined by high performance liquid chromatography of the derived deoxyribonucleosides as described by Tamaoka and Komagata (1984). Levels of DNA–DNA relatedness with *Bacillus halmapalus* DSM 8723^T were determined by the method of Ezaki et al. (1989) using photobiotin-labeled DNA probes and microplates.

Other analytical methods

Protease activity was routinely assayed with Harmerstein casein (Merck, Rahway, NJ, USA) as substrate by color reaction with phenol reagent (Kanto, Tokyo, Japan). One unit of the activity was defined as the amount of enzyme that produced acid-soluble peptides equivalent to 1 μmol L-tyrosine/min. Protein was determined with bovine serum albumin (Bio-Rad, Richmond, CA, USA) as the protein standard. The oxidative stability was examined with Ala-Ala-Pro-Leu-pNA (Peptide Institute, Osaka, Japan) as substrate and H_2O_2 as oxidant. Molecular masses were estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the isoelectric point was determined in an electrofocusing system (Saeki et al. 2000).

Cellular fatty acids were extracted in *n*-hexane from 200 mg dry cells of KSM-KP43 and *B. halmapalus*. They were esterified by acid methanolysis, and the methyl esters were identified and quantified using a gas chromatograph (GC-17A; Shimadzu, Kyoto, Japan) and a gas chromatograph-mass spectrophotometer (Shimadzu GCMS-QP5050A), essentially as described previously (Koike et al. 1999).

Nucleotide accession numbers and deposition of organism

The nucleotide sequence of KP-43 has been submitted to the DDBJ, GenBank, and EMBL data banks with accession number AB051423. The accession numbers of 16S rRNA sequences of the OSP-producing bacilli determined in this study are reported in Fig. 6B. *Bacillus* sp. strain KSM-KP43 has been deposited as an international patent strain in the National Institute of Bioscience and Human Technology Agency of Japan with deposition number FERM BP-6532.

Results and discussion

Morphological and physiological characteristics

Taxonomic characteristics of the isolate KSM-KP43 are summarized in Table 1. KSM-KP43 is a gram-positive, strictly aerobic, motile, sporulating, rod-shaped (0.4–0.9 × 2.6–4.2 μm) organism with peritrichous flagella. Spores were ellipsoidal and located centrally to paracentrally, not

swelling the young sporangium. The isolate grew in nutrient broth at pH between 6.8 and 10 with an optimum around pH 9 and with up to 9% (w/v) NaCl at pH 7, indicating that it is a facultative alkaliphile. It grew on nutrient agar at pH 6.8. The range of temperature for growth was from 10° to 40°C, but not to 45°C, with an optimum around 30°C. KSM-KP43 was positive for utilization of D-glucose, D-mannose, fructose, maltose, sucrose, lactose, melibiose, D-sorbitol, D-mannitol, trehalose, and *N*-acetylglucosamine, hydrolysis of starch, gelatin, esculin, Tweens 20, 40, and 60, and enzyme tests of catalase and oxidase. It was negative for utilization of L-arabinose, D-xylose, galactose, inositol, glycerol, and D-raffinose, formation of indole, H_2S, and acetoin, utilization of citric acid, reduction of NO_3 to NO_2, and activities of β-glucuronidase, urease, lysine hydrolase, and arginine hydrolase.

Table 1. Phenotypic properties of KSM-KP43

Reaction of strain		
Property	KSM-KP43	*Bacillus halmapalus*[a]
Form	Rod	Rod
Mobility	+	+
Flagella	Peritrichous	Peritrichous
Spore	+ (central to paracentral)	+ (central)
Gram stain	Positive	Positive
Catalase	+	
Oxidase	+	
NO_3 to NO_2	–	–
Substrate utilized:		
D-Glucose	+	
D-Mannose	+	+
Fructose	+	
Galactose	–	–
Maltose	+	
Sucrose	+	
Lactose	–	–
L-Arabinose	–	–
D-Xylose	–	–
Inositol	–	–
D-Sorbitol	+	–
D-Mannitol	+	
Glycerol	–	–
Melibiose	+	–
D-Raffinose	–	–
Rhamnose	+	–
Trehalose	–	
Growth at 10°C	+	+
Growth at 20°C	+	+
Growth at 40°C	+	+
Growth at 45°C	–	–
Growth at pH 7	+	+
Growth at pH 8	+	+
Growth at pH 9	+	+
Growth at pH 10	+	+
Growth in 5% NaCl	+	–
Growth in 7% NaCl	+	–
Growth in 9% NaCl	+	–
Hydrolysis of starch	+	+
Hydrolysis of gelatin	+	+
Hydrolysis of escculin	+	
Hydrolysis of pullulan		+
Hydrolysis of Tweens 20, 40, and 60	+	–
β-Glucuronidase	–	–
Arginine hydrolase	–	
Lysine hydrolase	–	
Urease	–	
Utilization of citrate	–	
H_2S production	–	
Indole production	–	
Acetoin production	–	

[a]Results of Nielsen et al. (1995)

16S rRNA (rDNA) sequence and phylogeny

A number of new taxa have been proposed for alkaliphilic *Bacillus* strains (Spanka and Fritze 1993; Nielsen et al. 1995; Agnew et al. 1995; Fritze 1996; Yumoto et al. 1998; Switzer Blum et al. 1998). To further characterize KSM-KP43, we constructed a phylogenetic tree based on comparison of the 16S rDNA gene sequence of the isolate and those of eight type (standard) strains of *Bacillus* spp. (Fig. 1). The sequence of alkaliphilic KSM-KP43 had the closest match (98.8% homology) with that from alkaliphilic *B. halmapalus*

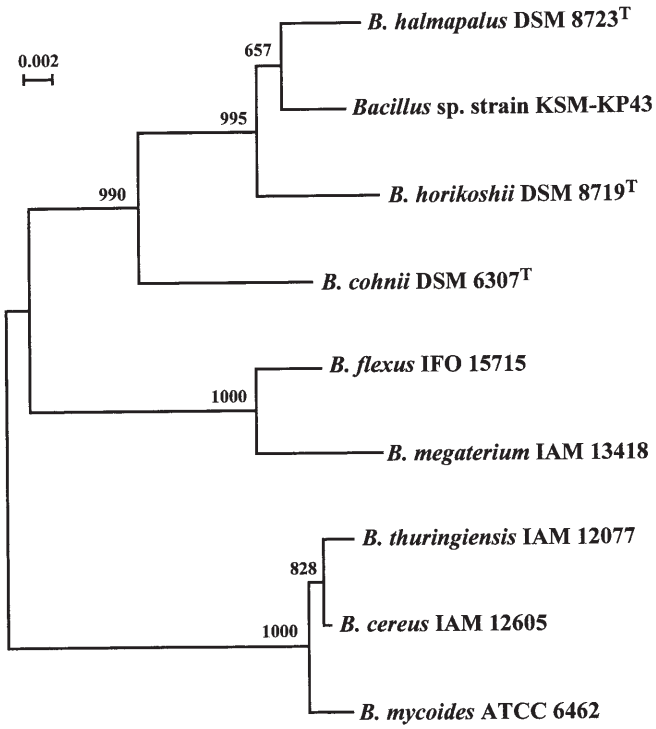

Fig. 1. Phylogenetic tree of KSM-KP43 associated with other members of the genus *Bacillus*. The 16S rDNA sequence of KSM-KP43 was determined and compared with those of related *Bacillus* spp. The *numbers* at internal nodes are bootstrap values above 500 derived from 1,000 samples in which the group to the *right* of the node was monophyletic. Bootstrap probability values less than 50% were omitted from the figure. Sequences incorporated in the figure are under the following accession numbers: *Bacillus cohnii* DSM 6307T, X76437; *Bacillus mycoides* ATCC 6462, AB021192; *Bacillus cereus* IAM 12605, D16266; *Bacillus thuringiensis* IAM 12077, D16281; *Bacillus flexus* IFO 15715, AB021185; *Bacillus horikoshii* DSM 8719T, AB043865; *Bacillus halmapalus* DSM 8723T, X76447; and *Bacillus megaterium* IFO 13418, D16273. *Bar* 0.02 K_{nuc} unit

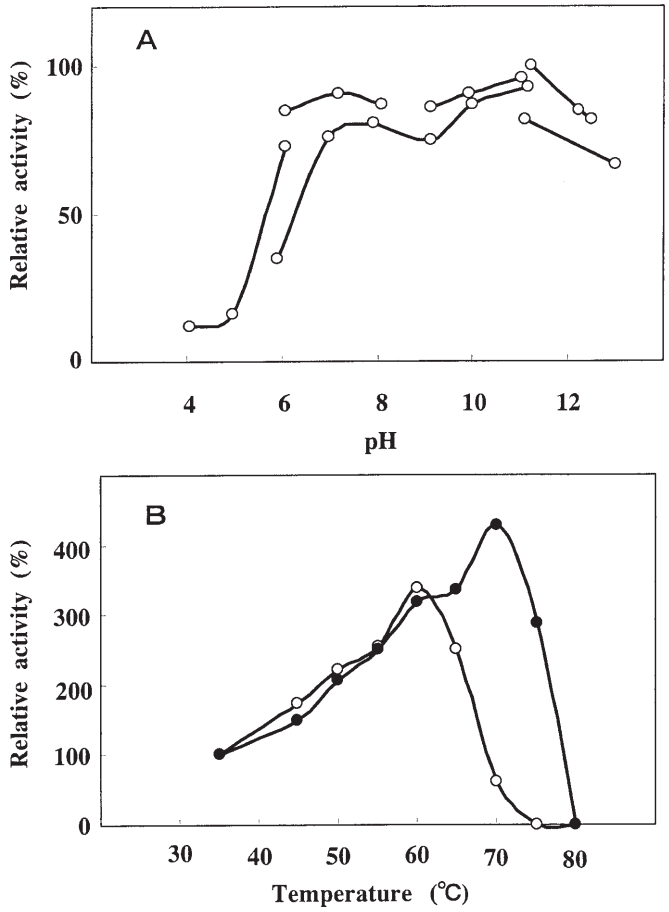

Fig. 2A,B. Effects of pH and temperature on the activity of KP-43. **A** Effect of pH on activity. Acetate buffer (pH 4–6), sodium phosphate buffer (pH 6–8), Tris-HCl buffer (pH 7–9), glycine-NaOH buffer (pH 8–11), borate buffer (pH 9–11), and KCl-NaOH buffer (pH 12–13) were used. Assays were done at 35°C for 10 min in the indicated buffers at 20 mM with casein as substrate. The values are shown as percentages of the maximum specific activity of KP-43 observed at pH 11 in glycine-NaOH buffer, which is taken as 100%. **B** Effect of temperature on activity. The reactions were done at the indicated temperatures for 5 min and at pH 10 in 50 mM glycine-NaOH buffer in the absence (*open circles*) or presence (*solid circles*) of 5 mM $CaCl_2$. The values are shown as percentages of the activity observed at 35°C in the absence of $CaCl_2$, which is taken as 100%

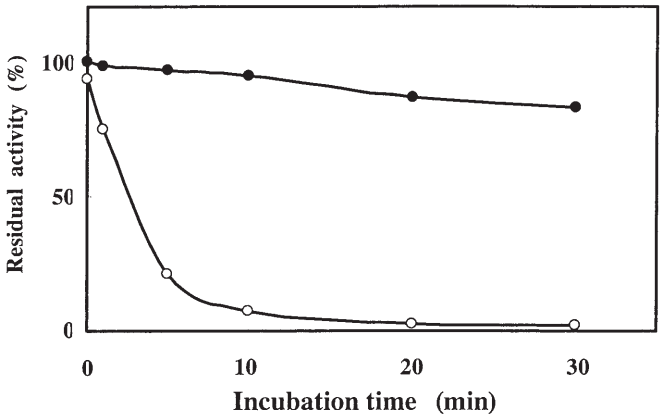

Fig. 3. Rates of oxidative inactivation by H_2O_2 of KP-43. The enzyme was incubated in the presence of 50 mM H_2O_2 at 30°C and at pH 10 in 5.0 ml of 20 mM Britton–Robinson buffer. Samples (0.5 ml) were withdrawn at appropriate time intervals and mixed with 50 µl of catalase solution (20 mU/ml) to remove residual H_2O_2. The residual activities of KP-43 (*solid circles*), including a highly alkaline enzyme, M-protease (Kobayashi et al. 1995), as reference (*open circles*), were measured by incubation at 30°C for 10 min with 5 mM Ala-Ala-Pro-Leu-pNA as the substrate

Table 2. Cellular fatty acid compositions of KSM-KP43 and *B. halmapalus*

Fatty acid	KSM-KP43	*B. halmapalus*
C14:0	0.4[a]	0.3
iso-C15:0	11.2	9.9
anteiso-C15:0	5.7	7.0
C15:0	0.7	0.4
C16:1 (*cis*-5)	3.0	3.0
iso-C16:0	3.2	4.2
C16:1 (*trans*-5 + *trans*-6)	7.2	1.9
C16:1 (*trans*-9)	0.3	0.3
C16:0	21.8	12.0
C17:l (*cis*-5)	9.0	6.1
C17:1 (*trans*-5)	14.8	4.9
iso-C17:0	2.0	6.0
anteiso-C17:0	7.5	7.9
C17:0	1.1	1.4
2-Hydroxy-C16:0	0.2	0.7
iso-C18:0	0.1	0.2
C18:2	1.1	6.9
C18:1 (*cis*-9)	6.4	14.8
C18:1 (*trans*-9)	0.4	0.8
C18:0	2.2	4.3
anteiso-C19:0	0.5	0.7
C20:0	0.1	0.3

[a] Expressed as percent of the total fatty acids

(Nielsen et al. 1995). *Bacillus cohnii* (Spanka and Fritze 1993) and *Bacillus horikoshii* (Nielsen et al. 1995) showed 96.2% and 98.0% homology, respectively, to that of KSM-KP43, but they were on a different branch.

G + C content and DNA–DNA hybridization

The G + C content of the KSM-KP43 genomic DNA was 41.6 mol%, whereas that of *B. halmapalus* was 36.8 mol% by the method of Tamaoka and Komagata (1984). Stackebrandt and Goebel (1994) have emphasized the importance of DNA–DNA relatedness in the species definition, even if almost identical 16S rRNA sequences are obtained. It was found that the DNA–DNA hybridization of KSM-KP43 with *B. halmapalus* revealed a low association (less than 25%).

Cellular fatty acids

To verify the taxonomic difference between KSM-KP43 and *B. halmapalus*, their cellular fatty acid compositions were analyzed (Table 2). Although both strains possessed isomers

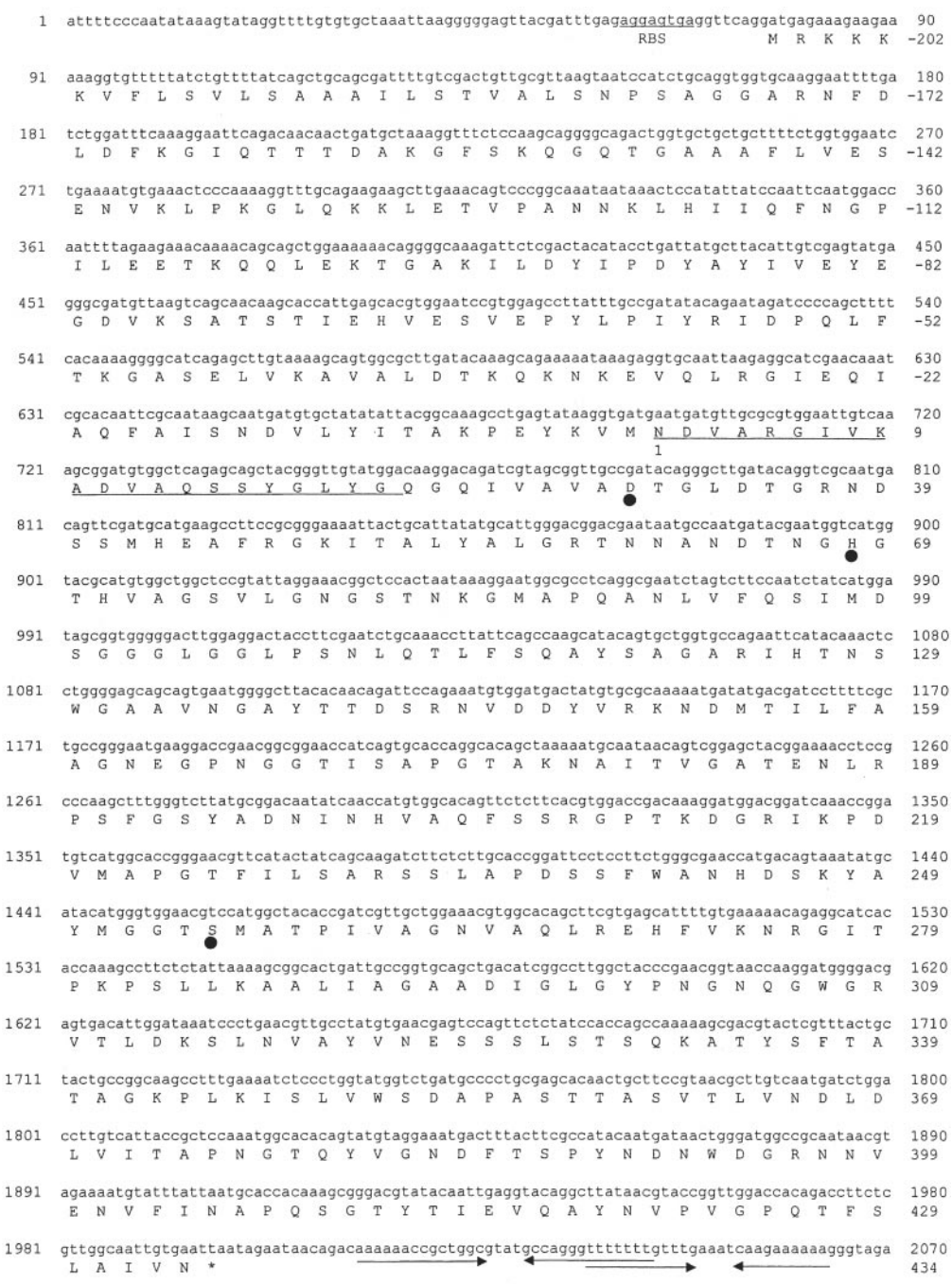

Fig. 4. Nucleotide and deduced amino acid sequences of the KP-43 precursor. The deduced amino acid sequence of the gene product is indicated by the *single-letter codes* under the nucleotide sequence determined. A putative ribosome-binding site is *underlined*. The opening reading frame (ORF) extends from Met (–206) to Asn434. The *underlined* amino acid sequence (Asn1–Gly21) refers to the N-terminal sequence of KP-43 exoproduced by *Bacillus* sp. strain KSM-KP43. *Convergent arrows* show inverted repeats downstream from the stop codon TAA of the ORF. *Solid circles* under the deduced amino acid sequence indicate the residues that form the catalytic triad

of C15 at similar levels, the compositions of isomers with C16, C17, and C18 were clearly different qualitatively and quantitatively.

B. halmapalus is unable to grow in the presence of 5% NaCl, at pH 9–10, on lactose, melibiose, and rhamnose, or to hydrolyze Tweens, 20, 40, and 60 (see Table 1; Nielsen et al. 1995). Moreover, the DNA–DNA relatedness is low, and the G + C content and components and concentration of cellular fatty acids differ. Therefore, KSM-KP43 is not closely related to any of the species of *Bacillus* reported to date and is categorized as a new species of alkaliphilic *Bacillus*.

Physicochemical and catalytic properties of KP-43

The purified KP-43 had a molecular mass of ~43 kDa and a specific activity of approximately 120 units/mg protein at pH 10.5 in 50 mM borate buffer. The pI value of the enzyme was pH 9.7–9.9. The N-terminal amino acid sequence determined was Asn-Asp-Val-Ala-Arg-Gly-Ile-Val-Lys-Ala-Asp-Val-Ala-Gln-Ser-Ser-Tyr-Gly-Leu-Tyr-Gly. In the absence of calcium ions, caseinolytic activity of KP-43 at 35°C was observed at a pH range of 4–13, with an optimum pH at 11–12 in glycine-NaOH buffer (Fig. 2A). KP-43 was stable at pH 6–12 but unstable below pH 5 and above pH 13

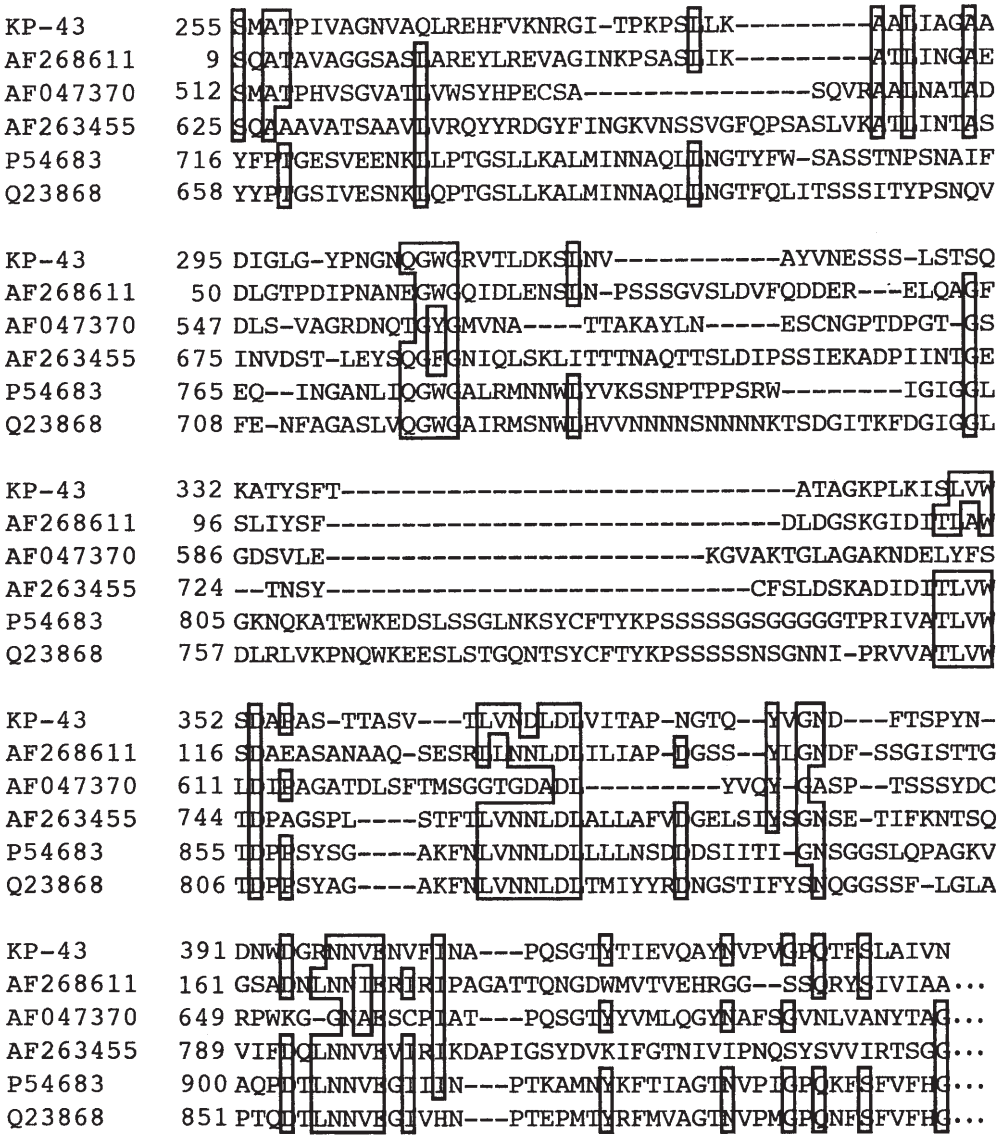

Fig. 5. Multiple sequence alignment of KP-43 with some related proteins. Segments that showed local homology were aligned first automatically and then manually. The conserved amino acids are *boxed*. Sequence sources (*left column*) are a membrane-associated serine protease of a marine microbial assemblage (AF268611), an alkaline serine protease of a *Shewanella* strain (AF047370), and three serine protease/ABC transporter precursors of *Dictyostelium discoideum*, i.e., TagA (AF263455), TagB (P54683), and TagC (Q23868)

after a 24-h incubation at 25°C. The optimal temperature for activity at pH 10 was 60°C in the absence and 70°C in the presence of 5 mM $CaCl_2$ (Fig. 2B). The enzyme was stable up to 55°C in the absence and to 65°C in the presence of 5 mM $CaCl_2$ after a 30-min incubation at pH 10. Phenylmethylsulfonyl fluoride and diisopropyl fluorophosphate (1 mM each) inhibited KP-43 activity almost completely, a result that is consistent with the classification of KP-43 as a serine protease.

The striking feature of KP-43 is its strong resistance to excess H_2O_2 (Fig. 3), as has been reported for OSPs E-1, LP-Ya, NP-1, and SD-521 (Saeki et al. 2000). *o*-Phenanthroline, ethylenediaminetetraacetic acid (EDTA), and ethyleneglycoltetraacetic acid (EGTA) (5 mM each) did not inhibit the enzyme activity at all. Therefore, KP-43 is an effective additive to bleach-based detergent formulations.

Nucleotide and deduced amino acid sequences of KP-43

Figure 4 shows the nucleotide and deduced amino acid sequences of KP-43 that contain a single open reading frame (ORF; nucleotides 77–1,996), a putative ribosome-binding sequence, and two possible long inverted repeats. The ORF encodes 640 amino acids. The calculated molecular mass of the mature enzyme (434 amino acids) was 45,301 Da. The KP-43 precursor had a possible 206-amino-acid prepropeptide, as suggested previously for other OSPs (Saeki et al. 2000). KP-43 exhibited limited homology with known typical subtilisins and related high-alkaline proteases with 24%–26% identity. Nevertheless, the catalytic triads of KP-43 are integrally conserved as Asp30, His68, and Ser255. Oxidative inactivation of subtilisins is caused by oxidation of the conserved Met222 (in subtilisin BPN'

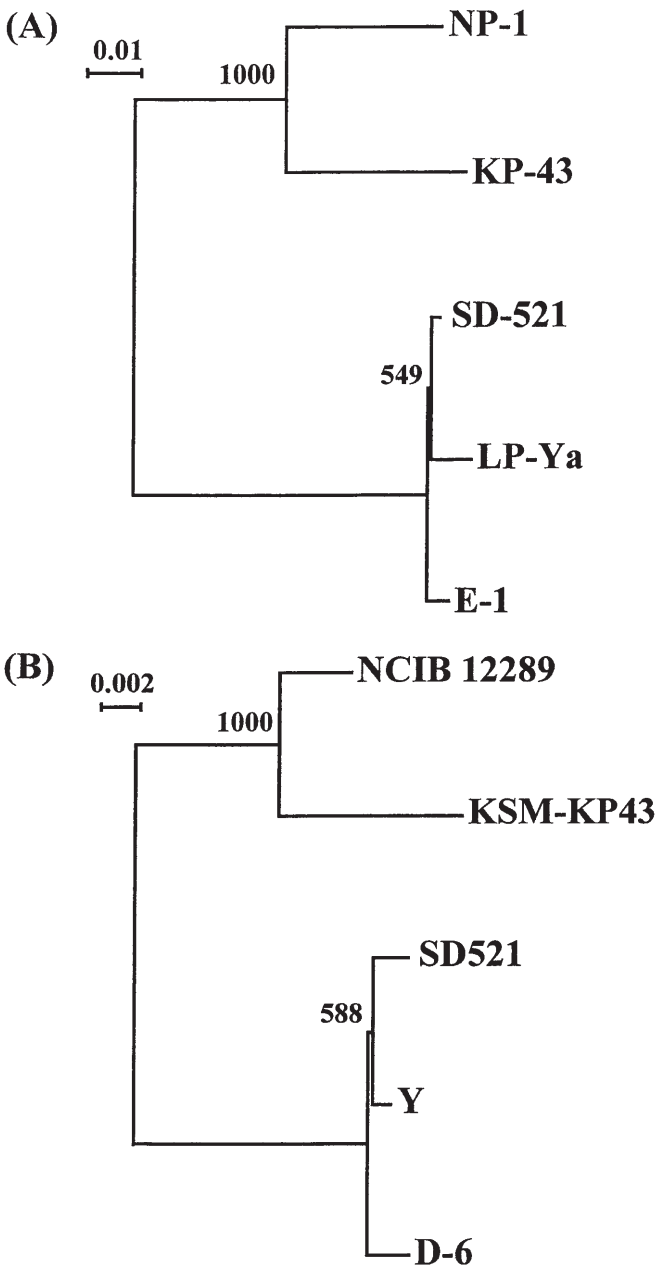

Fig. 6A,B. Phylogenetic trees of oxidatively stable serine proteases (OSPs) and the OSP-producing *Bacillus* strains. **A** Phylogenetic tree of the OSPs. The tree was inferred from the amino acid sequence alignment of the conserved regions around active sites (Saeki et al. 2000). Conserved sequence segments around the catalytic triads (Asp30, His68, and Ser255 in KP-43 numbering) of the OSPs were aligned manually with each other. Sources of sequences aligned: KP-43, AB051423 (this study); E-1, AB046402; LP-Ya, AB046404; SD-521, AB046405; NP-1, AB046406 (Saeki et al. 2000). *Bar* K_{nuc} unit. **B** Phylogenetic tree of the OSP-producing bacilli. The tree was inferred from 16S rRNA sequences. Sources of sequences aligned: KSM-KP43 (KP-43 producer), AB055093; D-6 (E-1 producer), AB055094; Y (LP-Ya producer), AB055095; SD521 (SD-521 producer), AB055096; NCIB 12289 (NP-1 producer), AB055097. *Bar* K_{nuc} unit

numbering) to its sulfoxide derivative (Stauffer and Etson 1969). Surprisingly, the oxidant-susceptible Met residue, as in the cases of subtilisins, occurs at position 256 just after the conserved Ser255. The mechanism of the oxidative stability of KP-43 has not yet been clarified.

The mature KP-43 included a unique C-terminal extension of ~160 residues, as has been identified in other OSPs (Saeki et al. 2000). The C-terminal half after the putative catalytic Ser255 and the contiguous C-terminal extension shared local similarity to internal segments of a membrane-associated subtilisin-like serine protease of a marine microbial assemblage and the serine protease/ABC transporter precursors TagA, TagB, and TagC of the slime mold *Dictyostelium discoideum*, and to the C-terminal half of a cold-active alkaline serine protease of a psychrotrophic gram-negative *Shewanella* strain (Fig. 5). This result raises the possibility that the conserved C-terminal regions of the OSPs and the *Shewanella* enzyme are involved somehow in membrane transport machinery or, by inference, suggests that they, including the ABC transporters, evolved from a common ancestral protein.

Phylogeny of OSPs and organisms

The phylogenetic tree showed that KP-43 and other OSPs are evolutionarily close, but they can be placed unequivocally into two separate clusters (Fig. 6A). One is the group of KP-43 and NP-1, and another is the group of E-1, LP-Ya, and SD-521. The latter group is further divided into two subgroups. The evolutionary distance of the OSP-producing bacilli was also computed, and a phylogenetic tree was constructed (Fig. 6B). The analyses of 16S rRNA sequences place strains D-6 (E-1 producer), Y (LP-Ya producer), and SD521 (SD-521 producer) in *Bacillus cohnii*, NCIB 12289 (NP-1 producer) in *B. halmapalus*, and KSM-KP43 (KP-43 producer) in a novel species of alkaliphilic *Bacillus*. It is interesting that the difference in the amino acid sequence of the OSPs closely reflects the evolutionary distances of the respective OSP-producing *Bacillus* spp. We have already crystallized KP-43 (Nonaka et al. 2001), and its tertiary structure would help further understanding of the physiological role of the C-terminal extension and oxidative stability of OSPs.

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