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Purification and Characterization of YfkN, a Trifunctional Nucleotide Phosphoesterase Secreted by *Bacillus Subtilis*

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YfkN isolated from the culture supernatant of *Bacillus subtilis* in the exponential phase of growth is a protein of 143.5 kDa that derives from a putative large precursor of 159.6 kDa processed at both the N- and C-terminal ends. Pulse-chase experiments indicated that the release occurs slowly with a half-time longer than 30 min, suggesting that the event is coupled with wall turnover. YfkN exhibits 2′,3′ cyclic nucleotide phosphodiesterase, 2′ (or 3′) nucleotidase and 5′ nucleotidase activities. *In vitro* the protein is reduced by subtilisin digestion to a shorter polypeptide (68 kDa), displaying phosphodiesterase activity but devoid of any 5′nucleotidase activity. This proteolytic processing led us to localize the potential active sites of the various nucleotidase activities. When bacteria were grown in low phosphate medium, the exocellular production of the enzyme was enhanced, suggesting that it plays a role in phosphate metabolism. Comparison with nucleotidase databases suggests that *yfkN* resulted from gene fusion.

Key words: 2′,3′ cyclic nucleotide phosphodiesterase, 2′ (or 3′) nucleotidase, 5′ nucleotidase, protein secretion, gene fusion.

Abbreviations: OD, optical density; PMSF, phenylmethanesulfonyl fluoride.

The complete sequencing of the genome of *Bacillus subtilis* (*1*) has made it possible to predict which of the 4106 gene products (*2*) are exported proteins. These have been estimated to number about 300 based on their amino acid termini, which contain a putative signal sequence (*3*, *4*). It has been shown that these proteins can be targeted to the membrane, the cell wall or the culture medium depending on additional signals. Two-dimensional (2D) gel protein electrophoresis is currently being used (*5*, *6*) to identify the proteins released by either *B. subtilis* wild-type or various mutant strains during each specific phase of growth and under specific environmental conditions. A recent analysis carried out with the *B. subtilis degU32*(Hy) mutant (*7*) led to the detection of YfkN in the culture supernatant. We found it tempting to characterize this protein under non denaturing conditions because it is the largest protein synthesized by *B. subtilis*. An exciting aspect of this enzyme stems from the fact that sequencing data (*8*) suggest that *yfkN*, with an extremely large open reading frame comprising 4386 nucleotides, seems to be the result of the fusion of two genes, one coding for a 2′,3′ cyclic nucleotide phosphodiesterase and the other for a 5′ nucleotidase. Examples of such gene fusions are scarce in microorganisms and a question arises as to whether the enzyme retains its size and remains in a multicatalytic state or rather undergoes specific proteolytic processing.

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MATERIALS AND METHODS

Substrates and Reagents—Purine and pyrimidine nucleotides, Protein A-Sepharose CL 4B, DEAE-Sepharose and subtilisin were from Sigma. [^{35}S]methionine was from Amersham.

Culture in Continuous Fermentor, Strain, and Medium—*Bacillus subtilis* QB112 [*degU32*(Hy)] strain (*9*) was used in this work. Aerobic cultures were run in a 1.5 liter fermentor (Bioflo 3000, New Brunswick Scientific). Cells were grown in minimal medium (*9*). The carbon source was 1.5% glucose at the beginning of growth. When the bacterial suspension reached an OD_{600} of 6, fresh minimal medium containing 1% glucose was added at a constant rate equal to that of the cell suspension outflow (660 ml h^{-1}). pH and temperature were controlled and maintained at values of 7.0 and 37°C, respectively. Solubilized O_2 was measured with a Clark electrode and 85% O_2 saturation was maintained by bubbling through the culture. The speed of agitation was 800 rpm. Under such conditions, the specific growth rate, μ, remained maximum and constant, equal to 0.69 h^{-1}. Growth yield (grams of biomass per mole of glucose) was approximately 78 g mol^{-1}.

Cells under conditions of phosphate limitation were grown in basal limitation medium prepared as described (*10*) containing 1.0% glucose and either 0.2 mM or 2 mM KH_2PO_4.

Purification of YfkN—Step1: Exocellular proteins were adsorbed on hydroxyapatite in batch. Solid hydroxyapatite was resuspended in culture supernatant (3 g per liter) and gently stirred for 20 min. When the stirring ceased, the hydrated hydroxyapatite was left to settle.

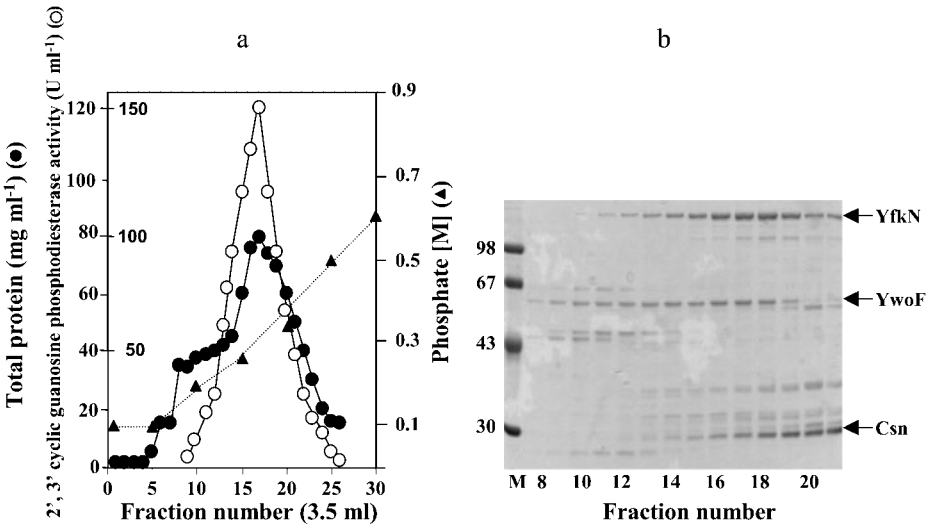

Fig. 1. **Identification of exocellular proteins displaying affinity to hydroxyapatite.** Solid hydroxyapatite (9 g) was resuspended in 3 liters of culture supernatant. (a) Column preparation and protein elution with a molarity gradient of sodium phosphate are described in Methods. Each fraction was assayed for 2′,3′ cyclic guanosine phosphodiesterase activity. (b) SDS gel electrophoresis pattern of the proteins eluted: 50 μl of each fraction was subjected to electrophoresis. The major proteins were identified by NH_2-terminal sequence analysis.

The supernatant was discarded and the pellet resuspended in a minimum volume of 0.1 M sodium phosphate, pH 7, and poured into a column. The hydroxyapatite was allowed to pack by gravity and was then washed with five volumes of the same buffer. Elution of the proteins was performed at room temperature with a molarity gradient (0.1 to 0.8 M) of sodium phosphate, pH 7. Fractions containing 2′,3′ cyclic nucleotide phosphodiesterase activity were pooled and dialysed overnight against 0.01 M sodium phosphate, pH 7.

Step 2: The dialysed protein solution was loaded onto a DEAE-Sepharose anion-exchange column (20 × 1 cm) pre-equilibrated with 0.01 M sodium phosphate, pH 7. The column was washed with the same buffer and proteins were eluted with a linear gradient of 0 to 1 M NaCl. YfkN activity was found in the fraction eluted at 0.3 M NaCl. Purified YfkN migrated as a single protein band on 10% (w/v) SDS-PAGE with an apparent molecular mass of 145 kDa.

Antibodies—Pure YfkN was used to raise antibodies against YfkN in rabbits.

Enzyme Assay of YfkN—YfkN activity was assayed in the presence of 2′,3′ cyclic nucleotide phosphates or 5′ nucleotide phosphates, in 0.1 M Tris-HCl, pH 7, at 37°C. Inorganic phosphate released by hydrolysis of various substrates was measured by the method of Ames and Dubin (*11*). One unit of enzyme activity was defined as the amount of enzyme that releases one nanomole of phosphate min^{-1} in the presence of 0.01 M substrate.

Enzyme Assay of Peptides Resulting of Proteolytic Treatment of YfkN—A sample (50 μg) of pure YfkN was subjected to proteolysis by subtilisin in 100 μl of 0.1 M Tris-HCl, pH 7. The ratio of YfkN to subtilisin was 10. The reaction was allowed to proceed at 37°C for 5 min. PMSF (0.01 M final concentration) was added and the sample subjected in duplicate to SDS-PAGE. The gel was then soaked overnight in 100 ml of 0.1 M Tris-HCl, pH 7 in the presence of 1% Triton X100, at 4°C. The peptides released by proteolysis were localised by staining a part of the gel. Enzyme assay of the various peptides were carried out by incubating the gel slices in 200 μl of 0.1 M Tris-HCl, pH 7, containing 10 mM substrate. Aliquots of 50 μl were withdrawn at time intervals, and inorganic phosphate released was measured.

Mass Spectrometry Analysis—Proteins at concentrations higher than 1 pmole were mixed with an equal volume of 2,5-hydroxybenzoic acid solution in 50% acetonitrile/0.1% trifluoroacetic acid. The samples were analysed by a matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometer (Voyager-DE PRO PerSeptive Biosystems) (*12*). Protein mass was estimated using BSA as a calibration protein.

Amino Acid Sequencing—The NH_2 terminal sequence of exoproteins isolated from the culture supernatant was determined by the Edman degradation procedure (*13*) with a gas-phase sequencer (model 470 A, Applied Biosystems) equipped with an on line phenylthiohydantoin amino acid sequencer (model 120 A).

Pulse-Chase Experiment—At an OD_{600} of 2.5, a 1 ml aliquot of the culture suspension was pulse-labelled for 5 min with 0.5 mCi [^{35}S]methionine (1.8 10^7 Bq; 1,000 Ci/mmol) and chased at a final concentration of 4 mM. Samples (0.15 ml) were removed at intervals and centrifuged. Cell supernatants were diluted three fold with ice-cold TNE buffer (50 mM Tris-HCl, pH 8, containing 150 mM NaCl and 5 mM EDTA) and dialysed overnight at 4°C against the same buffer. Samples were diluted five fold in TNET (TNE buffer containing 1% v/v Triton X100). Antibodies against YfkN (50 μl) and 80 μl of 10% w/v ProteinA-Sepharose (Sigma) in TNET were then added. After overnight incubation at 4°C, the immunoprecipitates were recovered by centrifugation. The pellets were washed three times with 1 ml TNET and finally resuspended in electrophoresis sample buffer and analysed by SDS-PAGE. The bands were revealed by Phosphorimaging and quantified with Image-Quant software (Molecular Dynamics).

RESULTS

Purification of YfkN—We purified YfkN from the *degU32*(Hy) strain supernatant. The first step was the adsorption of the protein on hydroxyapatite. This method turned out to be a fast and reliable method for concen-

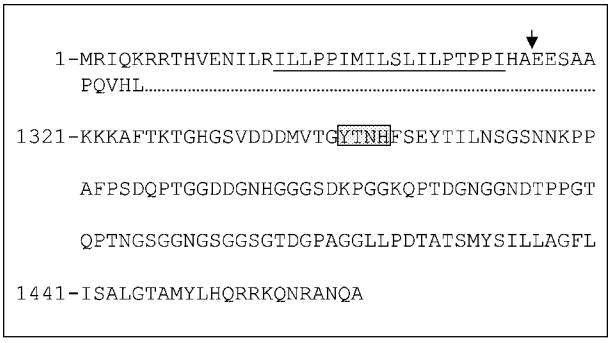

Fig. 2. **Processing of YfkN precursor form (1462 amino acids).** The arrow indicates the cleavage site of the N-terminal signal sequence. The location of the putative C-terminal end processing of the form released in the supernatant is indicated by a box.

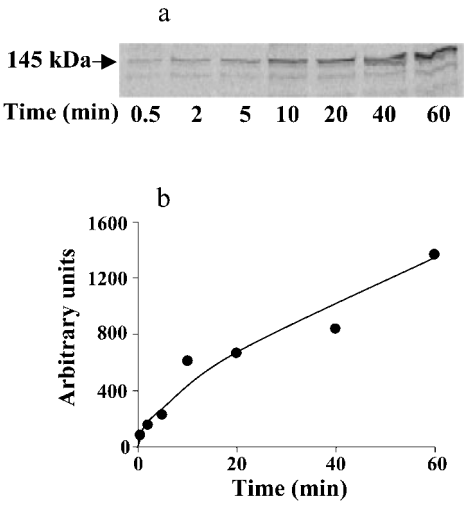

Fig. 3. **Kinetics of the release of YfkN in the culture supernatant.** At an OD_{600} of 2.5, 1 ml of the culture suspension was pulse-labelled for five min with 0.5 mCi [^{35}S]methionine and chased with a large excess of non-radioactive methionine (4 mM final concentration). Samples (0.15 ml) were removed at intervals and treated as described in Methods and analysed by SDS-PAGE (a). Kinetics of the appearance of labelled extracellular YfkN (b), counts are in arbitrary units.

trating YfkN under native conditions from the cell supernatant (Fig. 1). Few exocellular proteins had the ability to bind hydroxyapatite. The major proteins eluted were identified by NH_2 terminal sequence analysis. YfkN was unequivocally identified as the protein with an apparent molecular weight of 145 kDa eluted at 0.35 M phosphate. The second step was DEAE-Sepharose chromatography as described in Methods. The pure protein was eluted at 0.3 M NaCl.

Kinetics of YfkN Secretion—Mass spectrometry analysis of YfkN isolated from the supernatant revealed a molecular mass of 143.5 kDa. Analysis of the N-terminal amino acids indicated that the sequence is that expected from the cleavage of a signal sequence of 35 amino acids (4.095 kDa) according to the prediction of the SignalP algorithm (*14*).

Since a precursor with a molecular mass of 159.7 kDa can be deduced from the nucleotide sequence (*8*), we postulated that the extracellular form of YfkN undergoes a C-terminal processing either during its export or later in the supernatant. As shown in Fig. 2, the carboxyl domain that is removed corresponds to approximately 120 amino acids. The pulse-chase experiment in Fig. 3 shows that YfkN is released in its 143.5 kDa form and that its release into the culture supernatant is a slow process. This suggests that the C-terminal processing occurs during its export.

Enzyme Activities of YfkN—It has been suggested from the nucleotide sequence of *yfkN* (*8*) that YfkN is a bifunctional nucleotide phosphoesterase. We demonstrated experimentally (Table 1) that YfkN is a trifunctional nucleotide phosphoesterase. Indeed, the pure enzyme is able to catalyse the release of inorganic phosphate from 2′,3′ cyclic nucleotide phosphates. Such a chemical event involves both a 2′,3′ phosphodiesterase activity and a 2′ (or 3′) nucleotidase activity. In addition, YfkN also possesses a 5′ nucleotidase activity. However, the 2′,3′ cyclic phosphodiesterase activity is higher than that of the 5′ nucleotidase with various nucleotide phosphates used as substrates. Under the same conditions of reaction, the pure enzyme does not catalyse the release of inorganic phosphate from 3′,5′ cyclic nucleotide phosphates (results not shown).

In Vitro Proteolytic Processing of Isolated YfkN—We performed *in silico* research aimed at localizing the potential active sites of these nucleotidases in the YfkN sequence. Three sites sharing strong homology with common signatures of the nucleotidase family were identified (Fig. 4). Two of these sites, S1 (L45–M58) and S3 (L669–L682), showed homology with 2′ (or 3′) nucleotidase or 5′ nucleotidase active sites, and the third, S2 (Y134–N144), had a strong identity with the 2′,3′ cyclic nucleotide phosphodiesterase signature. In order to determine which

Table 1. **Nucleotide phosphoesterase activities of YfkN.** Reaction velocities were calculated from the initial rates of the release of inorganic phosphate. Each reaction mixture (200 μl), containing 10 mM substrate in 0.1 M Tris-HCl, pH 7, and 3.4 μg ml^{-1} YfkN, was incubated at 37°C. Aliquots of 50 μl were withdrawn at time intervals. Assay of inorganic phosphate was carried out according to Ames and Dubin (*11*).

Substrate	Specific activity (U mg^{-1})	Ratio of specific activities (2′,3′ cyclic phosphodiesterase/5′ nucleotidase)
Cyclic 2′,3′ AMP	3,900 ± 300	4.2
5′ AMP	960 ± 60	
Cyclic 2′,3′ CMP	2,300 ± 200	3.8
5′ CMP	600 ± 40	
Cyclic 2′,3′ GMP	1,600 ± 120	1.3
5′ GMP	1200 ± 100	
Cyclic 2′,3′ UMP	3,500 ± 300	5.8
5′ UMP	600 ± 40	

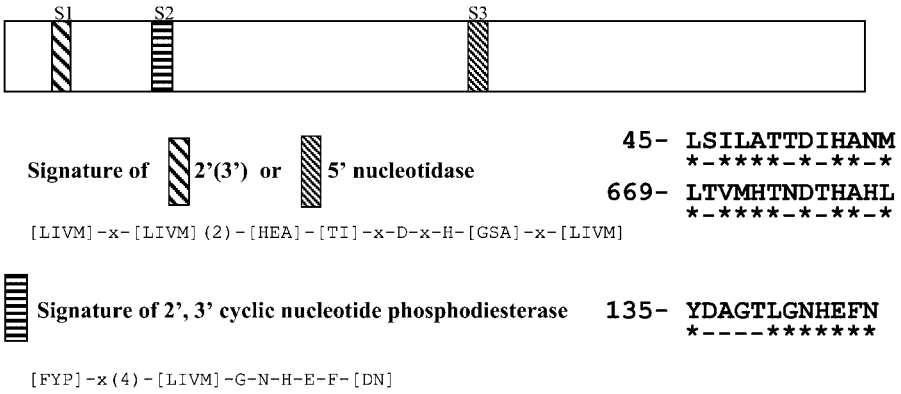

Fig. 4. **Signatures of nucleotidase activities.** Signatures of nucleotidase activities were identified from the PROSITE databank (http://www.expasy.org/prosite/). The sites S1 (L45–M58) and S3 (L669–L682) correspond to 2′ (or 3′) and 5′ nucleotidase activities and the site S2 (Y134–N144) corresponds to the 2′,3′ cyclic nucleotide phosphodiesterase. The consensus motives are indicated and the corresponding sequences of YfkN detailed. Stars indicated the residues corresponding to the consensus.

specific nucleotide phosphate activity is associated with S1 and S3, we attempted to dissociate them by proteolytic treatment of the protein. Treatment with various concentrations of subtilisin resulted in the release of two polypeptides displaying resistance to proteolysis (Fig. 5) whose accumulation made sequencing their N-terminal amino acids possible. The 68 kDa polypeptide is characterized by the following N-terminal sequence: EESAAPQV, whereas the 47 kDa polypeptide NH_2 terminal sequence is ENPEAK. From these data it was possible to localize the sites of proteolytic attack generating the two polypeptides (Fig. 5). Subsequent incubation of the gel slices with various phosphonucleotide substrates, as described in Methods, indicated that the 68 kDa fragment is able to catalyse the release of phosphate from 2′,3′ cyclic nucleotide phosphates, but not from 5′ nucleotide phosphates. The 47 kDa polypeptide did not modify the various substrates. These findings, therefore, allow us to ascribe a 5′ nucleotidase signature to the S3 internal sequence, and a 2′ (or 3′) nucleotidase signature to S1.

Physiological Function of YfkN—The multifunctional activity of YfkN strongly suggests that this enzyme plays a role in the cellular reprocessing of nucleotide phosphates present in the culture medium. *B. subtilis* produces extracellular RNases that release 3′ or 5′ phosphonucleotides from RNA hydrolysis (*15*). Therefore, the nucleotidase activities of YfkN should facilitate the

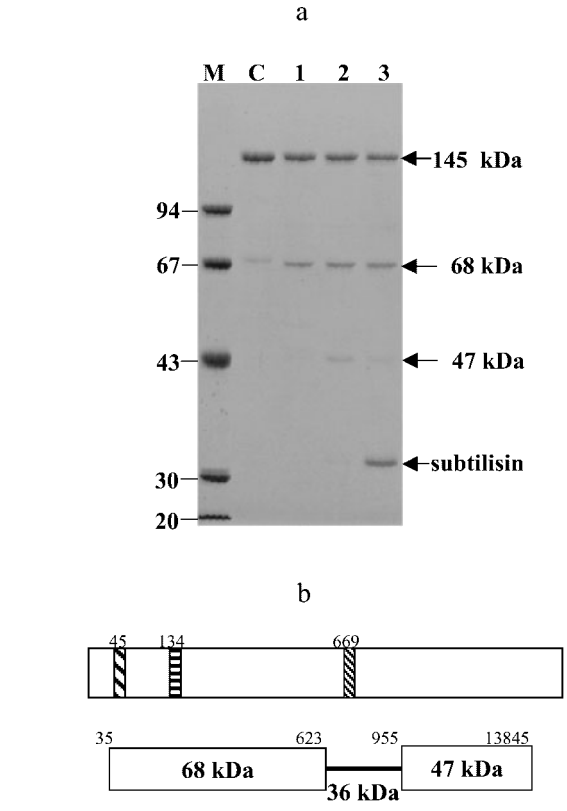

Fig. 5. **Processing of isolated YfkN by subtilisin.** Samples (2.5 μg) of pure YfkN were subjected to proteolysis with increasing concentrations of subtilisin in 25 μl of 0.1 M sodium phosphate buffer, pH 7. The reaction proceeded at 37°C for 5 min. Phenyl methyl sulfonyl fluoride (0.01 M final concentration) was then added and the samples were subjected to SDS-PAGE analysis (a). Lane C: control; lanes 1, 2, and 3: the ratios of YfkN to subtilisin were 100, 10 and 1, respectively. The schematic localisation (b) of the 68 and 47 kDa fragments, in reference to the non processed protein (upper part), is based on the N-terminal sequences of these fragments.

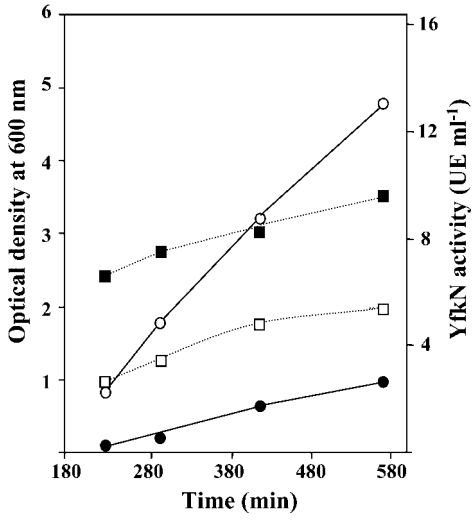

Fig. 6. **Production of extracellular YfkN by *B. subtilis degU32*(Hy) growing in medium containing low phosphate (0.2 mM) or in medium containing 2 mM phosphate.** Culture suspension (0.5 ml) at an OD_{600} of 3.0 was diluted in 30 ml fresh medium at 2 mM or 0.2 mM final phosphate concentration. Samples were withdrawn at intervals and centrifuged. Supernatants were dialysed against 0.1 M Tris-HCl, pH 7, and assayed for 2′,3′ cyclic guanosine phosphodiesterase activity. OD_{600} of cultures grown in medium containing 2 mM (open circles) or 0.2 mM (solid circles) phosphate and the YfkN activity of the samples withdrawn from these cultures (open squares) or (solid squares).

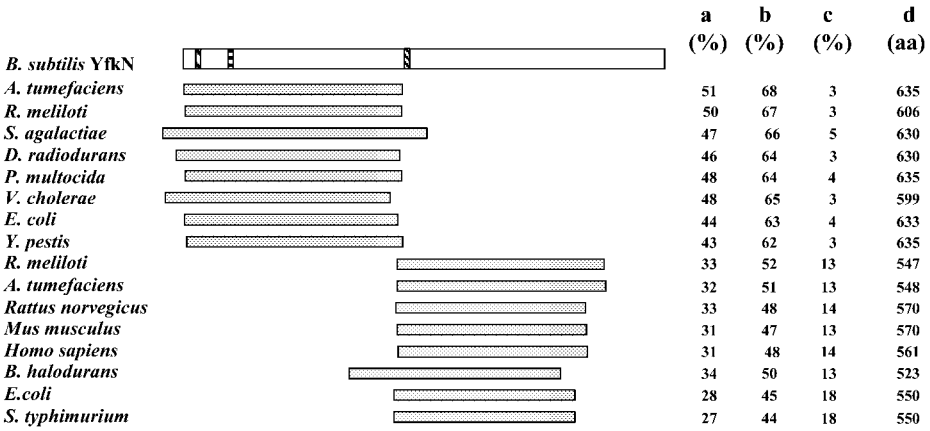

Fig. 7. **Sequence comparison of *B. subtilis* YfkN with proteins in general databanks.** Search for homology was done using the BLAST 2 program for proteins in the non redundant protein database at the Swiss Institute of Bioinformatics (http://www.ch.embnet.org). The percentages of identity (a), positives (b), gaps (c), and the number of residues taken into account in the percentages (d) are indicated. Additional close homologs are omitted from the figure. The names of the homolog proteins and their number of amino acid residues are as following: *Bacillus subtilis* (YfkN, 1462); *Agrobacter tumefaciens* (CpdB,662); *Rhizobium meliloti* (CpdB,657); *Streptococcus agalactiae* (GBS1921, 800); *Deniococcus radiodurans* (DR1736, 678); *Pasteurella multocida* (CpdB, 656); *Vibrio cholerae* (VC2562,681); *Escherichia coli* (CpdB, 647); *Yersinia pestis* (CpdB, 656); *R. meliloti* (RO2802, 628); *A. tumefaciens* (AGR_L_213p, 632); *Rattus norvegicus* (NT5E, 576); *Mus musculatus* (NT5E, 574); *Homo sapiens* (NT5E, 574); *Bacillus halodurans* (BH0026, 641); *E. coli* (UshA, 550); *Salmonella typhimurium* (UshA, 550).

reprocessing of nucleotide phosphate components under conditions of shortage. Such an hypothesis was substantiated by the increase in YfkN production when the bacteria were grown in phosphate restricted medium (Fig. 6).

Does yfkN Result from Gene Fusion or Duplication?—As stressed previously (*8*), *yfkN* could result from the duplication of an ancestral gene or from the fusion of two genes coding for a bifunctional 2′,3′ cyclic nucleotide phosphodiesterase, 2′ (or 3′) phosphodiesterase and 5′ nucleotidase, respectively. Database search using the BLAST 2 program showed that the N-terminal part of *yfkN* coding for the 68 kDa fragment (amino acids 36 to 660) displays a high degree of identity (around 50%) with genes coding for bifunctional enzymes, 2′,3′ cyclic nucleotide 2′ (or 3′) phosphodiesterases found in various Gram positive or Gram negative bacteria (Fig. 7). Among them, the *cpdB* genes of Enterobacteriaceae (*Escherichia coli* and *Salmonella typhimurium*) (*16–18*), *Haemophilus influenzae*, *Vibrio alginolyticus* and *Yersinia enterocolitica* (*19–21*) have been characterized.

The C-terminal part of *yfkN* shows similarity to structural genes coding for 5′ nucleotidases of eukaryote and prokaryote origins (Fig. 7). Based on the protein sequences listed in the databases, *Rhizobium meliloti* and *Agrobacterum tumefaciens* synthesize the three activities on two different proteins (CpdB and RO2802, CpdB and AGR_L_213p, respectively) to ensure the three catalytic activities of YfkN. On the other hand, the BLAST 2 program showed that the two halves of YfkN share only 23% identity, 38% positives and 24% gaps. These data strongly suggest that *yfkN* resulted from gene fusion.

DISCUSSION

This work provides information concerning the kinetics of release and the multiple catalytic activities of YfkN, the largest protein exported by *B. subtilis*.

Release of the processed form of YfkN is a slow event. Our previous studies of the secretion kinetics of various model proteins (*22–24*) have shown that the last stage of the secretion process is always rate-limiting. However, for exocellular proteins with molecular masses ranging around 60 kDa, the half-time of release is 1 to 2 min. Therefore, this big difference may be ascribed to the greater size of YfkN. As demonstrated previously by Demchick and Koch (*25*) who analyzed the sieving properties of the *B. subtilis* cell wall, passive diffusion through this compartment may be not possible for proteins with molecular masses higher than 60 kDa. Such proteins could pass into the external medium only *via* the inside to outside growth process underlying turnover of the cell wall (*26*), which takes nearly a generation to complete.

The efficiency with which YfkN hydrolyses the four major 2′,3′ cyclic nucleotides and 5′, or 3′, or 2′ monophosphate nucleosides may indicate that this enzyme plays a role in the recovery of inorganic phosphate by *B. subtilis* rather than a role in the regulation of intercellular signalling. This hypothesis is supported by the observation that proteolytic processing by subtilisin releases a polypeptide fragment that has lost its 5′ phosphonucleotidase activity but retains its 2′,3′ cyclic nucleotide phosphodiesterase and 2′ (or 3′) nucleotidase activities. This latter can hydrolyze 3′ phosphonucleotides, which are produced by the action of exocellular RNase on single stranded RNA and produce nucleosides that can easily enter the cell (*16*). Subtilisin processing of YfkN can occur *in vivo* during the stationary phase of growth when the protease is released (*27*).

Among the enzymes coding for nucleotidase activities, YfkN is the only example of a trifunctional enzyme (2′,3′ cyclic nucleotides phosphodiesterase 2′ (or 3′) nucleotidases or 5′ nucleotidase) most probably resulting from gene fusion as we can infer from the comparison of protein sequences.

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