Purification of two *Bacillus subtilis* proteins which cross-react with antibodies directed against eukaryotic protein kinase C, the His HPr kinase and trigger factor

Naïla Zouari, Benoît Roche, Jos F. M. L. Seegers and Simone J. Sror

Author for correspondence: Simone J. Sror. Fax: +33 1 69 15 78 08. e-mail: Seror@igmors.U-psud.fr

Institut de Gntique et
Microbiologie, URA 2225,
Universit Paris XI,
Btiment 409, 91405 Orsay
cedex, France

As in eukaryotes, phosphorylation of Ser and Thr residues in proteins appears to be a common phenomenon in bacteria. Surprisingly, however, very few Ser/Thr protein kinases have been identified and in this study antibodies directed against mammalian protein kinase C (PKC) have been used in attempts to isolate conserved Ser/Thr protein kinases. Using the mAb M7 against rat brain PKC, a single 70 kDa band was identified in total cell extracts of *Bacillus subtilis* by Western blotting after SDS-PAGE, whilst using polyclonal antibody α -PKC1p against *Saccharomyces cerevisiae* PKC a single 67 kDa band was identified by the same procedure. The two proteins were purified independently on the basis of antibody recognition employing two-dimensional gel electrophoresis as a final step, which allowed subsequent microsequencing. The 70 kDa band was thus identified as the phosphoenolpyruvate-dependent His HPr kinase, Enzyme I of the phosphotransferase system. This identity was confirmed using a mutant deleted for *ptsI*, encoding Enzyme I. The 67 kDa protein was identified as a previously unknown *B. subtilis* 'trigger factor', homologous to an *Escherichia coli* protein-folding enzyme, peptidylprolyl *cis-trans*-isomerase implicated in cell division.

Keywords: His HPr kinase, Enzyme I, trigger factor, *Bacillus subtilis*, protein kinase C

INTRODUCTION

In the cell cycle of bacteria, several processes can be distinguished: DNA replication, chromosome segregation and cell division. These processes must be carefully controlled and co-ordinated both with respect to each other and with respect to the growth of the cell. The tight regulation of these processes implies the presence of an underlying timing or clock mechanism, which controls the temporal programme of cell cycle events. Initiation of chromosomal replication is a complex process, occurring at a specific site, *oriC* (for a review see Bramhill & Kornberg, 1988), when a certain initiation mass is apparently reached (Donachie, 1968; Wold *et al.*, 1994). The cellular concentration of DnaA,

essential for the initiation of replication at *oriC*, has been proposed as the principal regulator of initiation in *Escherichia coli* (Hansen *et al.*, 1991). However, definitive proof that DnaA is the master regulator has not yet been obtained (Nordstrm *et al.*, 1991; Vinella & D'Ari, 1995; Bremer & Churchward, 1991). In *Bacillus subtilis*, DnaA has been identified and shown to be specifically implicated in the initiation of DNA synthesis (Yoshikawa & Wake, 1993), but its role in regulation has not been analysed in detail. In addition, in *B. subtilis*, we have demonstrated a post-initiation control, depending upon replication terminator protein (RTP) and RelA, acting downstream of *oriC*. This appears to be a DNA checkpoint mechanism, coupling DNA replication and growth according to the nutritional status of the cell.

Since the specific mechanism controlling the timing of initiation of DNA replication in bacteria once per cycle remains unclear, we previously proposed that the level of intracellular free Ca^{2+} , mediated by Ca^{2+} -binding

Abbreviations: 2-D, two-dimensional; PKC, protein kinase C; SU, signal unit.

The EMBL accession number for the nucleotide sequence reported in this paper is Y09608.

proteins and a protein phosphorylation cascade, may provide such a control mechanism (Norris *et al.*, 1988; Holland *et al.*, 1990). Thus, it was proposed that cell cycle events in prokaryotic cells, as it appears to be the case in some eukaryotic systems, may be controlled by a Ca^{2+} flux, mediated, for example, by protein kinases, to trigger initiation of DNA synthesis, nucleoid segregation and cell division. Many phosphorylated proteins do indeed exist in bacteria (Cozzzone, 1988) and His phosphorylation, inherent to many two-component signal transduction processes, has been studied in detail (Stock *et al.*, 1989). In contrast, little attention has been paid to the functional significance of Ser/Thr protein phosphorylation, although large numbers of proteins apparently phosphorylated on these residues have been identified in *E. coli* (Cozzzone, 1988). Similarly, Ser/Thr protein kinases in bacteria have been little studied so far. The first reported kinase of this type, responsible for phosphorylation of isocitrate dehydrogenase in *E. coli*, was described by Cortay *et al.* (1988). More recently, Min *et al.* (1993) have described another Ser/Thr protein kinase which is an anti- σ factor involved in sporulation in *B. subtilis*. The gene for another Ser/Thr protein kinase, *prkA*, was recently cloned in *B. subtilis* (Fischer *et al.*, 1996), but its cellular role is unknown. Notably, none of these kinases shows homology with the conserved motifs of the catalytic domain present in all eukaryote protein kinases. Moreover, the bacterial Ser/Thr protein kinases so far identified do not fall into a homogeneous family. In contrast, conserved Ser/Thr protein kinases have been identified in *Myxococcus xanthus* (Muñoz-Dorado *et al.*, 1991; Udo *et al.*, 1995), in *Streptomyces coelicolor* (Urabe & Ogawara, 1995) and in cyanobacteria (Zhang, 1993). However, functional studies on these protein kinases have been limited.

In previous studies in this laboratory, we have isolated a mutant of *B. subtilis*, *spnA95*, resistant to the protein kinase C (PKC) inhibitor sphinganine. This mutant is a novel cell cycle clock mutant, with a reduced initiation mass at 30 °C, accompanied by a reduced cell size (Séror *et al.*, 1994). However, this mutant was not directly defective in a putative PKC, since *spnA* encodes the *B. subtilis* cysteinyl-tRNA synthetase, indicating an important but complex linkage between protein synthesis and cell cycle regulation.

In this study, we attempted to identify conserved protein kinase genes by PCR techniques, but these proved to be unsuccessful. As an alternative, we then attempted to identify and isolate *B. subtilis* protein kinases with possibly conserved eukaryote-like features, by using anti-PKC antibodies. Two *B. subtilis* proteins were identified and purified, P70 and P67. P70 was found to be a His protein kinase (Enzyme I) of the phosphotransferase system, involved in sugar transport (Saier, 1994). The second protein, P67, was identified as a protein homologous to the *E. coli* trigger factor. Trigger factor in *E. coli* is involved in protein folding (Stoller *et al.*, 1995) and, when depleted or overproduced, promotes filament formation, which can be overcome by FtsZ overproduction (Guthrie & Wickner, 1990).

METHODS

Bacterial strains. The *B. subtilis* strain GP283 was used for purification of P70 and P67. GP283 is a derivative of GP279 (*amyE met*), and carries deletions in the protease genes *apr*, *nprE*, *isp-1*, *epr*, *bpr*, *mpr* and *vpr*, as well as a deletion in the regulatory gene *hpr* (Sloma *et al.*, 1991). W168 (*trpC*) was also used as a reference strain. Strains GM152 (*trpC2 sacB'::lacZ amy::sacXYΔ3*), its derivative GM287 (*ΔptsGHI*; Crutz *et al.*, 1990), GM1209 [*sacBAΔ23 sacTΔ4 sacXYΔ3 Δ(bglX)::ery^R*; Aymerich & Steinmetz, 1992], and its derivative GM1218 (*ΔptsGHI::tet^R*) were all gifts from S. Aymerich and M. Steinmetz (INRA, Thiverval-Grignon, France).

Antibodies. mAb M7, kindly provided by K. Leach (Leach *et al.*, 1988), was raised against highly purified rabbit brain type 3 PKC. M7 probably recognizes an epitope common to types 2 and 3, but not to type 1 PKC. M7 binds type 3 PKC under denaturing conditions and cross-reacts with the catalytic fragment of PKC in Western blots. mAb CK 1.20, recognizing the regulatory fragment of rat brain PKCs, was obtained from Y. Milner (see Mochly-Rosen & Koshland, 1987). A polyclonal antibody PC10 was made against rat brain PKCs (Huang & Huang, 1986). A polyclonal antibody against PKC 1 from *S. cerevisiae*, α -PKC1p, was obtained from G. Paravicini (Glaxo, Plau-les-Quates, Switzerland) and used for immunoprecipitation and Western blotting experiments at a 1/3000 dilution (see Antonsson *et al.*, 1994).

SDS-PAGE. Protein samples were analysed by SDS-PAGE, essentially as described by Laemmli (1970), using gels 0.75 mm thick containing 10% *N,N'*-methylene bis-acrylamide (ratio 37:1). The stacking gel was 7% acrylamide. Electrophoresis was carried out in SDS running buffer (0.025 M Tris-base; 0.192 M glycine; 1% SDS) at 25 mA until the dye reached the bottom of the gel. Gels were fixed and coloured with Amido blue-black, silver-stained or blotted for immunodetection. Molecular mass marker proteins were purchased from Amersham (Rainbow molecular mass marker, 14.3–220 kDa).

Western immunoblotting. After electrophoresis (see above), Western blotting was performed according to the immunoblotting procedure of Towbin *et al.* (1979). The transfer of proteins was conducted at 80 V for 50 min at 4 °C in electrophoresis buffer (0.025 M Tris-base; 0.192 M glycine, pH 8.3). After transfer, the nitrocellulose membrane (Schleicher & Schuell) was incubated with a 15% solution of Ponceau S for 10 min and rinsed briefly with water. In the case of detection with M7 antibodies, unreacted sites were blocked in 0.2% Tween in Tris-buffered saline (TBS: 0.5 M Tris, pH 7.5; 1.5 M NaCl) overnight at 4 °C. The membrane was then washed 4 × 5 min with TBS containing 0.1% Tween (TBS-T) and incubated with primary antibodies (1/200 dilution in TBS-T) for 90 min at room temperature with gentle shaking. The secondary antibody, peroxidase-conjugated anti-mouse (Promega), was used at a 1/7000 dilution in TBS-T after washing 2 × 5 min with the same buffer and incubated for 60 min at room temperature. Finally, the membrane was washed 2 × 5 min and 2 × 10 min with TBS-T. To reveal M7 fixation, the membrane was briefly washed with water, incubated for 1 min in luminol detection reaction mixture (ECL Amersham) and analysed by autoradiography against an X-ray film until a suitable exposure was obtained. In the case of detection with anti-PKC1p, 4% dried milk dissolved in TBS was used as blocking solution with incubation for 60 min at room temperature. The membrane was washed 4 × 5 min with TBS. Primary antibodies were added (1/500 dilution in TBS) and incubated for 90 min at room temperature with gentle shaking. The secondary antibody, alkaline-phosphatase-

conjugated anti-rabbit (Promega), was added at a 1/7000 dilution in TBS and incubated for 60 min at room temperature. The membrane was then washed 4 × 5 min with TBS. Finally, the membrane was washed for 10 min in detection buffer (100 mM Tris, pH 9.5; 10 mM NaCl; 5 mM MgCl₂), followed by the addition of the detection solution (66 µg nitro blue tetrazolium ml⁻¹, 33 µg 5-bromo-4-chloro-3-indolyl phosphate ml⁻¹ in detection buffer).

Estimation of P70 and P67 levels on Western blots. Gels or Western blots were first photographed with a CCD camera and scanned by using the GelScan program to quantify the relative intensity of the P70 and P67 bands. A reference signal unit (SU) was used: 1 SU P70 and P67 is equivalent to the signal obtained with 30 µg crude cell extract protein in a Western blot.

Phosphatidylserine fixation. After transfer of proteins from SDS-PAGE gels to nitrocellulose as described above, the nitrocellulose membrane was preblocked for 30 min in 30 g BSA l⁻¹, 0.2 g phosphatidylserine/phosphatidylethanolamine (1:1, w/w) l⁻¹, 130 mM NaCl and 20 mM HEPES/NaOH (pH 7.4) at room temperature. After washing for 2 × 5 min in 130 mM NaCl and 20 mM HEPES/NaOH (pH 7.4), the blot was incubated with 10 µM [¹⁴C]phosphatidylserine present in the washing buffer for 30 min at room temperature, washed for 10 min in 130 mM NaCl and 20 mM HEPES/NaOH (pH 7.4), dried and exposed to an X-ray film (Perin *et al.*, 1990).

Crude extract. For purification of P70 and P67, an overnight culture of *B. subtilis* strain GP283 was diluted to an OD₅₇₀ 0.05 into 3 l Brain Heart Infusion (BHI; 37 g l⁻¹; Difco) medium. Cells were grown at 30 °C to an OD₅₇₀ 1.5 and harvested by centrifugation at 4 °C, washed three times with lysis buffer kept at 4 °C [20 mM Tris/HCl, pH 8; 10 mM NaCl; 10 mM EGTA; 5 mM EDTA; 50 mM β-mercaptoethanol (β-Me)] and stored at -80 °C overnight. The cell pellet was resuspended in 15 ml lysis buffer containing 1 mM PMSF, 1 mM benzamidine, 10 µg trypsin inhibitor ml⁻¹, 10 µg pepstatin ml⁻¹ and 0.5 µg leupeptin ml⁻¹. The cell suspension was lysed by three passages through a chilled French pressure cell at 103 500 kPa. The cell lysate was clarified by centrifugation at 10000 g for 10 min at 4 °C, followed by precipitation of nucleic acids with 0.18 mg protamine sulphate [2.5 mg (g wet wt cells)⁻¹], and centrifuged at 100 000 r.p.m. (Beckman, TLA 100.2) for 30 min.

Ammonium sulphate precipitation. Proteins were precipitated with increasing amounts of ammonium sulphate. Fractions were collected at 25, 40, 55 and 70% saturation. Precipitated proteins were resolubilized in DEAE buffer A (50 mM Tris/HCl; 0.5 mM EDTA; 0.5 mM EGTA; 1 mM β-Me; 50 mM pepstatin; 10% glycerol) containing protease inhibitors and dialysed by microfiltration. The protein solutions were concentrated in a Centricon-30 (Amicon) at 5000 g for 20 min at 4 °C and diluted with buffer A containing protease inhibitors. The concentration step was repeated six times to eliminate ammonium sulphate completely. After measuring the protein concentration using the Bradford (1976) method, an aliquot was mixed with SDS sample buffer and tested by Western blotting.

DEAE chromatography. An HPLC DEAE-C18 column (Protein-pak DEAE 8HR, Waters AP-1 column, 100 × 10 mm) was equilibrated overnight with buffer A (see above). Different parameters were controlled by computer using the Maxima Waters program. Crude extract, in the case of P67, and the 55–70% ammonium sulphate fraction (AS 55–70), in the case of P70, were filtered on Millex (Millipore) and loaded onto the column in buffer A. The column was washed for 30 min (1 ml

min⁻¹) with the same buffer. Absorbed proteins were then eluted from the DEAE column by a NaCl gradient (Figs 2 and 5). Salt gradients were generated by addition of buffer B (1 ml min⁻¹; buffer A with 2 M NaCl): 0–25% in 20 min or in 35 min for P70 and P67, respectively (0–0.6 M), then 25–50% in 30 min (0.6–1 M). Fractions were collected (one fraction ml⁻¹), concentrated by microfiltration (Centricon-30; Amicon) and an aliquot was tested by Western blotting.

Two-dimensional (2-D) electrophoresis. High-resolution 2-D gel electrophoresis was performed essentially as described by Garrels & Fanza (1989) with a Millipore Investigator 2-D Electrophoresis System. Prior to isoelectrofocusing (IEF), samples were precipitated with cold 5% trichloroacetic acid (TCA) and washed three times with acetone. The pellet was resuspended in 100 µl buffer I (0.3% SDS; 200 mM DTT; 28 mM Tris/HCl; 22 mM Tris-base); this was incubated for 5 min at 100 °C, placed for 8 min on ice, mixed with 10 µl buffer II (24 mM Tris-base; 476 mM Tris/HCl; 50 mM MgCl₂; 1 mg DNase I ml⁻¹; 0.25 mg RNase A ml⁻¹) and precipitated with 80% acetone, then centrifuged in a microfuge (5 min at 4 °C). The pellet was dried and resuspended in 5 µl buffer I and 20 µl buffer III [9.9 M urea; 4%, v/v, Nonidet P40; 2.2%, v/v, ampholytes; 100 mM DTT]. Samples were stored at -80 °C after measuring the protein concentration.

IEF was performed in a 4% T/2.6% C polyacrylamide tube gel (1 mm inner diameter × 18 cm) prepared from a 30.8% T/2.6% C stock solution (30%, w/v, acrylamide; 0.8%, w/v, N,N'-methylene bis-acrylamide stock solution). The IEF gel also contained 9.5 M urea, 2% (v/v) Nonidet P40, 4.1% polyacrylamide stock solution, 5 mM CHAPS and 6.2% (v/v) Millipore 2-D optimized carrier ampholytes (pI 3–10). IEF gels were prefocused at 1500 V with the current limited to 110 µA per gel. The cathode buffer was 0.1 M NaOH and the anode buffer was 0.6% phosphoric acid. After loading samples prepared as described above on the top of each IEF gel, focusing was conducted for a total of 18000 V h (17 h at 1000 V, 30 min at 2000 V). When focusing was complete, IEF gels were extruded by an adapted syringe into the equilibration buffer (3 M Tris-base; 0.075 M Tris/HCl; 50 mM DTT), incubated at room temperature twice for 2 min. Each equilibrated IEF gel was loaded on the top of a second dimension of 10% polyacrylamide gel (gel dimensions 190 × 160 × 1 mm). The second dimension analysis was performed using 2000 V over a temperature range of 15–20 °C; the electrode buffer was the SDS running buffer. After completion of electrophoretic separation, gels were coloured with Amido blue-black, silver-stained or Western blotted. For the measurement of the pI profile, carbamylated protein standards (BDH) co-run on a duplicate gel and molecular mass markers were similarly employed.

Microsequencing. For microsequencing proteins separated on 2-D gels, an excised section of a gel stained with Amido blue-black, containing the identified protein (P70 or P67), was incubated in 0.1 M Tris/HCl (pH 8.8), 0.03% SDS containing 0.2% (v/v) endoprotease Lys-C for 18 h at 36 °C. The resulting peptides were separated on HPLC-DEAE-C18 with a linear acetonitrile gradient (2–45%) containing 0.1% trifluoroacetic acid. The chosen fraction was purified on the same column with an acetonitrile gradient from 10 to 35% in acetate buffer (pH 5.5) and then sequenced with an Applied Biosystems 473A sequencer with a standard run.

Cloning and sequencing. Oligonucleotides were synthesized on the basis of the P67 microsequences (see above). These had the composition 5'-CCCCCTTAAGGARGTNACNTTYC-CNGARGARTA-3' and 5'-CCCCCTTAAGTTNGTNARY-TCNGCRTCNACYTC-3', where R can be either G or A, Y

can be either T or C and N can be either G, A, T or C (from Oligoexpress). PCR was performed with chromosomal DNA, isolated from *B. subtilis* strain W168 (100 ng), using these oligonucleotides as primers (1.5 µg). The PCR mixture also contained 10% (v/v) incubation buffer (10 mM Tris/HCl, pH 9; 0.1% Triton X-100; 1.5 mM MgCl₂; 0.2 mg BSA ml⁻¹) and 0.5 units *Taq* DNA polymerase (Appligene). DNA denaturation was carried out at 94 °C for 4 min, annealing with oligonucleotides at 55 °C for 1 min, and primer elongation at 72 °C for 1.5 min. This cycle was repeated 25 times. A 580 bp PCR product was obtained and the ends of the fragment were blunted with mung bean enzyme. This fragment was then ligated into the pBluescript SK(+) vector, linearized by *EcoRV* and dephosphorylated by alkaline phosphatase. *E. coli* DH5α cells, transformed with the ligation mixture, were screened using a blue/white selection system on LB agar containing X-Gal, IPTG and ampicillin. Thirty-three transformants exhibiting the white phenotype were screened by PCR using universal and reverse primers. Ten of these transformants exhibiting the expected size were selected. Plasmid DNA was isolated from the transformants and was used as a template for sequencing. DNA sequencing was performed by the method of Sanger *et al.* (1977) with a Pharmacia kit, using universal and reverse oligonucleotides as primers.

PCR analysis. Degenerate oligonucleotides for PCR were synthesized on the basis of conserved subdomains within the catalytic domain of eukaryotic kinases. Primer 1 (5'-GGGGA-TCGIGATTTIAARCCIGAI AAY-3', where I is inosine) was designed to match the consensus sequence of subdomain VI, RDLKPEN. Primer 2 (5'-GGGGATCCIARIATTTTCIGGN-GC-3', where I is inosine) was designed to match the consensus sequence of subdomain VIII, APEIL. Four additional primers were used, designed to match conserved regions specific for PKCs. The sequences of these primers were: 5'-GGGGAT-CCTAYTTYGYIATGGART-3' (primer 3); 5'-GGGGATC-CGAIGGICAYATIAAIATIGCNGA-3' (primer 4); 5'-GGG-GATCCTCIGGIGTICCCICARAANGT-3' (primer 5); and 5'-GGGGATCCYTCITCYTCITCNC-3' (primer 6). Primer 3 matches subdomain V, primer 4 subdomain VII, primer 5 a second sequence from subdomain VIII and primer 6 subdomain X. Primers 1, 3 and 4 are oriented in the direction of transcription, while primers 2, 5 and 6 are oriented in the opposite direction. All primers carried a *Bam*HI site plus two additional bases to facilitate cloning of the PCR products. PCR amplification was performed with *Taq* DNA polymerase (Appligene) in a thermocycler (Hybaid). Each reaction contained 100 ng *B. subtilis* chromosomal DNA, 0.25 mM each dNTP and 400 pmol of pairwise combinations of each oligonucleotide in a final volume of 100 µl. Denaturation was carried out at 94 °C for 1 min, annealing at temperatures varying from 37 °C to 50 °C for 2 min, and elongation at 72 °C for 1 min. This cycle was repeated 30 times. PCR products were separated on 1.5% agarose gels and transferred onto Hybond-N+ membranes (Amersham). Southern analysis was performed either with a probe composed of a cloned DNA fragment of a region between subdomains VI and VIII from a yeast PKC or with total DNA obtained by PCR using *B. subtilis* DNA.

RESULTS

Search for conserved protein kinase genes by PCR

The catalytic domain of eukaryotic Ser/Thr kinases contains 11 highly conserved subdomains (Hanks *et al.*, 1988). For PCR analysis, oligonucleotide primers were

designed in this study to match subdomains V (primer 3), VI (primer 1), VII (primer 4), VIII (primers 2 and 5) and X (primer 6), dispersed at specific intervals throughout the catalytic domain. Primers 3, 4, 5 and 6 were successfully used by Toda *et al.* (1993) for the isolation of two novel PKC-related genes of fission yeast. PCR reactions were carried out as described in Methods with total *B. subtilis* DNA as template with all possible pairwise combinations of the six primers. Control PCR reactions were performed with chromosomal DNA of *Schizosaccharomyces pombe*. All control reactions resulted in fragments of the expected size that hybridized to yeast PKC DNA in Southern hybridization experiments. The fragments obtained with primers 1 and 2 as well as 1 and 6 were subcloned and sequenced. The sequences were identical to previously identified *S. cerevisiae* PKCs. The results with *B. subtilis* DNA, in most cases, yielded a number of products, two to ten fragments of varying sizes, rather than single bands of the expected or close to the expected size. Moreover, none of these fragments hybridized with yeast PKC DNA amplified using the same PCR technique, even at low stringency. The products from different PCR reactions were cross-hybridized and apparently a few homologous bands were identified although these did not correspond in size to that expected from an authentic PKC. Nevertheless, such sequences as well as different PCR products around the expected size were cloned into pBluescript SK(+) (Promega) and their nucleotide sequences determined. None of the resulting sequences showed any detectable similarity to sequences of the catalytic domain of eukaryotic protein kinase.

Detection of bands cross-reacting with anti-PKC antibodies in total cell extracts

In order to identify PKC-like proteins in *B. subtilis*, we tested whether *B. subtilis* contained proteins cross-reacting with antibodies against various eukaryotic PKCs. Several antibodies against PKC were first tested with total *B. subtilis* extracts by Western blotting. Four antibodies were found to cross-react with *B. subtilis* proteins (Fig. 1). Three of these antibodies appeared to recognize a 70 kDa *B. subtilis* protein: (1) mAb M7 IgG which recognizes the catalytic domain of type 2 and type 3 PKCs; (2) mAb CK 1.20, raised against the regulatory fragment of rat brain PKCs; and (3) PC10 polyclonal antibody to rat brain PKCs. In addition, a fourth polyclonal antibody (α -PKC1p), to *S. cerevisiae* PKC 1, recognized a protein of a similar size, 67 kDa. However, as described below, this protein is distinct from P70.

Cell extracts from *B. subtilis* strain W168 were prepared at different times during the growth phase and tested by Western blotting using M7 antibody. The results of this experiment with BHI showed that the amount of P70 was relatively constant during exponential phase but the level decreased in stationary phase. In contrast, in Spizizen minimal medium, the level of P70 was maximum in early exponential phase and then gradually declined (data not shown). Interestingly, Fig. 1 shows that radiolabelled phosphatidylserine, which binds the

Fig. 1. Detection of polypeptide bands in *B. subtilis* cross reacting with anti-PKC antibodies. A sonicate of *B. subtilis* strain W168 (30 µg protein) was separated by SDS-PAGE and transferred onto a nitrocellulose membrane for Western blotting. Lanes: 1, blotting with antibody M7; 2, CK 1.20; 3, PC10; 5, α -PKC1p. In lane 4, the nitrocellulose blot was preblocked with phosphatidylserine/phosphatidylethanolamine as described in Methods. The blot was finally reacted with [14 C]phosphatidylserine/phosphatidylethanolamine, dried and exposed to X-ray film.

mammalian PKC (Perin *et al.*, 1990), led to the appearance of several radioactive bands in *B. subtilis* extracts, including a band of 70 kDa. This provided further evidence that the 70 kDa band had some characteristics of PKC. Fig. 1 shows that the strongest signal for the 70 kDa protein was obtained with mAb M7, and, for convenience, this antibody was used for the subsequent purification of P70.

Validity of the quantification system

For the purpose of purification using antibodies to monitor fractionation of P70 and P67, it was first necessary to establish conditions for quantitative Western blotting with the antibodies. Therefore, immunoblotting with M7 was performed with different amounts of extracts containing P70. X-ray films exposed for different periods to immunoblots were submitted to densitometry, allowing the optimization of the conditions for establishing a linear signal/protein response.

Purification of P70

For the purification of P70, *B. subtilis* GP283, a strain which is deleted for nine protease genes to minimize proteolysis, was used. Additionally, cells were initially

broken by three passages through a French pressure cell at 103 500 kPa in a lysis buffer containing a mixture of protease inhibitors. About 50 % of P70, as detected by M7 antibodies, was found in the protamine sulphate pellet (see Methods). The soluble fraction containing 150 mg protein (18.75 mg ml⁻¹) and 5000 SU P70 was subsequently purified. The cell extract was then fractionated with ammonium sulphate and each fraction was tested by Western blotting. The protein fraction precipitated between 55 % (w/v) and 70 % (w/v) ammonium sulphate (AS 55–70) was found to have 60 % of P70 corresponding to an accumulated yield of 30 %. The enrichment was about 2.8-fold at this step (see Table 1).

The fraction containing P70 (AS 55–70) was subsequently loaded on a DEAE-cellulose column for HPLC separation. Two successive linear NaCl gradients (0–0.6 M; 0.6–1 M) were used for the elution (Fig. 2b). Fractions were collected and then analysed by Western blotting with M7 antibodies and the gels were quantified by densitometry as before. Fig. 2(a, b) shows that P70 was eluted from the column at 0.4 M NaCl primarily in two fractions, E31 and E32. Importantly, fractions containing P70 were separated from the majority of the protein (Fig. 2b), resulting in a substantial, more than 200-fold, purification factor, with a recovery of about 75 %. As shown in Table 1, the yield from the two purification steps was about 22.5 %. It is important to note that HPLC chromatography was carried out, for convenience, at room temperature. The P70 protein appeared to be sufficiently enriched in fractions E31 and E32, as demonstrated by SDS-PAGE and subsequent staining with Amido blue-black to allow microsequencing after 2-D gel electrophoresis.

Isolation of P70 by 2-D electrophoresis

Fractions E31 and E32 from HPLC (Fig. 2) were pooled and prepared, with ampholytes, for 2-D electrophoresis as described in Methods. Samples of varying amounts were loaded onto an IEF gel and focused for 17 h at 18 000 V h before loading onto the second dimension gel for SDS-PAGE. Two additional gels were run in parallel with the same sample, together with pI and molecular mass markers. One gel containing 500 SU was subsequently fixed and stained with Amido blue-black and the second (20 SU) was blotted onto a nitrocellulose

Table 1. Quantitative analysis of P70 purification

	Protein (mg)	Amount of signal (SU)*	Specific amount of signal (SU mg ⁻¹)	Apparent yield (%)	Purification factor (-fold)
Cell extract	150	5000	33.3	50	1
AS 55–70	32.2	3000	99.8	30	2.8
DEAE†	0.267	2254	8441	22.5	253.5

* One signal unit (SU) corresponds to 30 µg cell extract on a Western blot.

† Peaks 31 and 32 from DEAE-cellulose chromatography were pooled.

Fig. 2. Purification of P70 by DEAE-cellulose HPLC chromatography. The ammonium sulphate fraction AS 55–70 (35 mg; 1.5 ml) was applied to the HPLC DEAE-cellulose column as described in Methods. A NaCl gradient was applied for elution with continuous monitoring of protein concentration with a Waters 486 UV detector. Fractions (1 ml) were collected and 1/50th of each fraction was mixed with SDS sample buffer, separated by SDS-PAGE and a Western blot with M7 antibody was carried out. (a) Western blot with fractions E28–E34 and AS 55–70; the P70 signal was quantified by scanning densitometry (see Methods). (b) The solid line represents protein (A_{300}), the hatched line represents the NaCl gradient (M) and the histogram represents P70 (SU).

Fig. 3. 2-D electrophoresis to identify P70. For IEF, samples were prepared by precipitation of the pooled fractions from Fig. 2, E31 and E32, with TCA, and then processed as described in Methods. In the right panel, 500 SU partially purified P70 was loaded onto the 2-D gel, which was then stained by Amido blue-black. In the left panel, 20 SU was loaded onto the 2-D gel; the gel was blotted and a Western blot was performed using the M7 antibody, as described in Methods.

membrane for immunodetection. In the left panel of Fig. 3, the Western blot shows the region recognized by the M7 antibody at the expected molecular mass (70 kDa)

Fig. 4. P70 is the HPr His kinase (Enzyme I) of the phosphotransferase system. Cells from strains deleted for *ptsI* (GM1218 and GM287) and the isogenic strains (GM1209 and GM152) were collected at OD₅₇₅ 1.5, broken by sonication and centrifuged at 100 000 r.p.m. (Beckman, TLA 100.2) for 15 min at 4 °C. The cell extract was added to SDS running buffer and analysed by SDS-PAGE. A Western blot was performed with M7 antibody.

with a major spot having a pI value between 4.4 and 4.8. The spot corresponding to the strongest signal on the Western blot (arrowed in Fig. 3) was excised from this gel and used for microsequencing. The other spots indicated by the antibody were not further analysed. In fact, in other experiments, we only observed a single spot with the Western blots.

P70 is a His HPr kinase

The P70 spot excised from the 2-D gel was digested with the endoprotease Lys-C. Peptides were separated by DEAE-cellulose chromatography on HPLC as described in Methods. The first 18 amino acids of an internal peptide were determined, giving the sequence NISDSEAEVXRFDEAIX. Comparison with the non-redundant NCBI protein database using the BLAST program revealed 89% identity with the Enzyme I sequence encoded by the *ptsI* gene of *B. subtilis* (Reizer *et al.*, 1992; Gonzy-Treboul *et al.*, 1989). In fact, the two residues in position 10 and in position 18 gave ambiguous results in the amino acid analysis. At all other positions, the sequence was identical. Enzyme I is part of the phosphoenolpyruvate:sugar phosphotransferase system and was previously identified as a kinase which phosphorylates the phospho-carrier protein HPr on the His₁₅ residue. The molecular mass of the *B. subtilis* Enzyme I (550 amino acids) was calculated to be 63.4 kDa (Reizer *et al.*, 1992). The apparent molecular mass, estimated by SDS-PAGE (10%) by Gonzy-Treboul & Steinmetz (1987), was 70 kDa, thus identical to the value obtained in our studies. The pI of Enzyme I measured by Reizer *et al.* (1992) by IEF was 4.5 ± 1 (theoretical value was 4.78), which corresponds closely to the results obtained in this study (Fig. 3). To confirm that P70 was Enzyme I from the phosphotransferase system, a cell extract was prepared from strains carrying deletions in the *pts* operon (*ptsGHI*) provided by M. Steinmetz, and analysed by Western blotting. Fig. 4 shows that M7 antibodies now failed to recognize any band equivalent to P70 in the strains deleted for Enzyme I ($\Delta ptsGHI$) while P70 could still be detected in the isogenic wild-type strains. Finally, a highly purified preparation of Enzyme I (kindly provided by J. Deutscher, IBCP, Lyon, France) specifically recognizes mAb M7 in Western blots (data not shown).

Table 2. Quantitative analysis of P67 purification

	Protein (mg)	Amount of signal (SU)*	Specific amount of signal (SU mg ⁻¹)	Apparent yield (%)	Purification factor (-fold)
Cell extract	30	1000	33.3	50	1
DEAE	0.134	682	5089	34	153

* One signal unit (SU) corresponds to 30 µg cell extract on a Western blot.

A *B. subtilis* mutant, *spnA95*, has previously been isolated as being resistant at 30 °C to the PKC inhibitor sphinganine, and temperature sensitive for growth. This mutant displays an alteration of the cell clock, initiating DNA replication early in the cell cycle, and *spnA* was shown to encode a cysteinyl-tRNA synthetase. At the non-permissive temperature, in *spnA95*, the synthesis and/or the stability of P70 (Enzyme I) was also greatly reduced (Séror *et al.*, 1994). Interestingly, Enzyme I of *B. subtilis* contains two cysteines, which could be the reason why it is not synthesized at high temperature in the *spnA95* mutant.

In retrospect, it was surprising that the mAb M7, recognizing the catalytic domain rather than the regulatory domain of PKC, also apparently detected P70. However, the cross-reactivity with CK 1.20 was not confirmed with the purified Enzyme I, since this antibody was no longer available. We cannot exclude the possibility, therefore, that the CK 1.20 cross-reacting band in Fig. 1 is distinct from P70 (Enzyme I).

Purification of P67

As shown in Fig. 1, a 67 kDa protein (P67), distinct from P70, was recognized by the α-PKC1p antibodies. This antibody was therefore used for the subsequent purification of P67. The GP283 *B. subtilis* strain was also used for the purification of P67. The initial cell lysate was obtained from 600 ml culture (OD₅₇₀ 1.5), using the same method as for the purification of P70. This initial cell extract contained 32 mg protein (16 mg ml⁻¹) and 1000 SU P67 as determined by a Western blot with α-PKC1p antibodies (Table 2). After filtration on Millex (Millipore), the cell extract was directly subjected to HPLC with a DEAE-cellulose column. After loading the samples, the column was eluted with two gradients. Initially, a shallow gradient, 0–0.6 M NaCl, was employed, followed by a second gradient, 0.6–1 M NaCl (Fig. 5d). Eluted fractions were collected (1 ml per fraction) and concentrated by microfiltration. An aliquot of each fraction was loaded onto three SDS-PAGE gels; following SDS-PAGE, two gels were blotted and tested with either M7 (detecting P70) or α-PKC1p (P67). A third gel was stained by Amido blue-black. Fig. 5(b, d) shows that P67 eluted at 0.43 M NaCl, essentially in fractions 90 and 91, separated from the majority of the proteins. As shown in Fig. 5(b, c), P67 was well separated from P70, which eluted at 0.37 M NaCl (Fig. 5c).

Fig. 5. Purification of P67 by DEAE-cellulose HPLC chromatography. Cell extract (2 ml; 30 mg protein) was applied directly to an HPLC DEAE-cellulose column and subsequently analysed as described in Methods and in Fig. 2. (a, b, c) SDS-PAGE fractions E86–E92 and the initial cell extract (ET). (a) The gel was stained with Amido blue-black, (b) Western blots were carried out with α-PKC1p antibody and (c) Western blots were carried out with M7 antibody; the P67 and P70 signals were scanned by densitometry. (d) Solid line represents the A₃₀₀ protein profile, the broken line represents the NaCl gradient (M) and the histograms, stippled and hatched, represent P67 and P70 (SU).

On the gel treated with Amido blue-black (Fig. 5a), a major stained band migrating to the position of P67 was visible in the fraction giving the strongest antibody signal. The enrichment of P67 by this simple purification step was very efficient, corresponding to a purification

Fig. 6. 2-D electrophoresis to identify P67. Samples were prepared by precipitation of the fraction E91 from Fig. 5 with TCA, and processed for 2-D gels as described in Methods. In the right panel, 300 SU P67 was loaded onto the 2-D gel, the gel was then stained by Amido blue-black and the spot corresponding to P67 was excised for microsequencing. In the left panel, 20 SU was loaded onto the 2-D gel, the gel was blotted and a Western blot was performed using the α -PKC1p antibody.

factor of 153, as shown in Table 2. Also shown in Fig. 5, P70 is well separated from P67.

Separation by 2-D electrophoresis

Fraction 91 from the DEAE column (Fig. 5) was mixed with ampholytes as described in Methods. The sample was analysed by 2-D electrophoresis as described for P70 purification. One gel loaded with 300 SU was subsequently fixed and stained with Amido blue-black and the second (20 SU) was blotted onto a nitrocellulose membrane for immunodetection. The left panel of Fig. 6 shows a unique protein spot (indicated by the arrow), recognized by α -PKC1p antibodies in Western blots, which exhibits the expected molecular mass (67 kDa) and a pI of approximately 5.1. As shown in the right panel of Fig. 6, the spot corresponding to P67 appeared to be well separated from other spots. Therefore, the spot was excised from the stained gel and used for microsequencing.

P67 is homologous to the *E. coli* trigger factor

The P67 spot excised from the 2-D gel was treated with Lys-C endoprotease for internal microsequencing as described in Methods. Two internal peptides were sequenced. The amino acid sequence of the first fragment was LLLFAIAEVDALTK. The resulting sequence of the second was VVEVTFPEEYHAEDLAGK. Comparison with the combined NCBI protein database using the BLAST program revealed 20% and 72% identity, respectively, with the *E. coli* trigger factor. This protein was originally isolated because of its ability to promote translocation of certain polypeptides into membrane vesicles *in vitro* (Crooke & Wickner,

1987). Degenerate oligonucleotides were deduced from the *B. subtilis* microsequences and used as primers for PCR experiments. A fragment of 580 bp was cloned in pBluescript SK(+) and used for sequence analysis. The amino acid sequence deduced from this was compared with the *E. coli* trigger factor sequence. Fig. 7 shows that there was 44% homology (30% identical amino acids). Whilst this work was in progress, the *B. subtilis* trigger factor gene was sequenced in the course of the *B. subtilis* genome sequencing project (sequence kindly provided by C. Harwood, University of Newcastle upon Tyne, UK) and gave an identical result to the 580 bp sequenced here. The predicted pI value (4.73), deduced from the sequence provided by C. Harwood, corresponded approximately to the one determined by 2-D electrophoresis (5.1). In contrast, the molecular mass of the *B. subtilis* trigger factor, deduced from the sequence of 432 amino acids, 48 kDa, was different from the apparent molecular mass estimated on SDS-PAGE, 67 kDa, suggesting that the protein runs aberrantly under these conditions. Interestingly, the *E. coli* trigger factor also appears to migrate aberrantly in SDS-acrylamide gels (Lecker *et al.*, 1989; Guthrie & Wickner, 1990), although the reason for this is not clear from the sequence of either protein.

DISCUSSION

His protein kinase action is well established in prokaryotes as a key event in signal transduction, coupling the detection of environmental cues to appropriate intracellular changes in gene expression. Recent studies have demonstrated that similar two-component signal transduction systems are conserved in eukaryotes (Ota & Varshavsky, 1993). Additionally, however, Ser/Thr phosphorylation, mediated by a highly conserved superfamily of protein kinases, is present in eukaryotes, responsible for the regulation of a wide range of cellular activities (Hanks *et al.*, 1988). Surprisingly, although many proteins, for example in *E. coli*, appear to be subject to Ser/Thr phosphorylation (Cozzzone, 1988), studies of the functional significance and the identity of the responsible kinases have been limited in prokaryotes. Very little is known of the precise functional role of the few reported examples of Ser/Thr kinases in bacteria. Two important exceptions are isocitrate dehydrogenase kinase/phosphatase, essential for growth of some *E. coli* strains on minimal medium containing acetate as sole carbon source (LaPorte & Koshland, 1982; Cortay *et al.*, 1988), and SpoIIAB, which phosphorylates SpoIIAA, absolutely essential for regulation of sporulation in *B. subtilis* (Min *et al.*, 1993). These kinases have no significant homology with any of the 11 conserved subdomains of eukaryotic Ser/Thr kinases. In contrast, conserved protein kinases have been described in *M. xanthus* (Muñoz-Dorado *et al.*, 1991; Udo *et al.*, 1995), in cyanobacteria (Zhang, 1993) and in *S. coelicolor* (Urabe & Ogawara, 1995).

In this laboratory, we are interested in the identification and functional role of protein kinases in relation to cell cycle regulation and in this study we sought to identify

<i>B. subtilis</i> TF	VTFPEEYHAEEDLAGKPPAMFKKIKHEIKAKELPELDDFAKDIDEEVETLAEITKTKRLIEAKENEDADAKLREELVLKA	296
<i>E. coli</i> TF	VTFPEEYHAEEDLAGKPPAMFKKIKHEIKAKELPELDDFAKDIDEEVETLAEITKTKRLIEAKENEDADAKLREELVLKA	295
<i>B. subtilis</i> TF	SENAEIDVHQAAMVDTELDRLMLKEFEORLQMOGMNLELYTQFSCDEAALKEOMKEDNEKRVKSNUTDEATAKAENDEVSD	376
<i>E. coli</i> TF	VKANIDVHAALIDSELDVLRQAAQDFGG-----E-KALELPRELFEEONKRRVVVGLLGEVIRTNELKADE	360
<i>B. subtilis</i> TF	EEVDAAETKMAEAYNMEEVNIKQAIGS---TDA	406
<i>E. coli</i> TF	ERVKGLIEEMASAYEDEKRVIEFYSKNELMN	398

Fig. 7. Comparison of the deduced amino acid sequence equivalent to the 580 bp PCR product corresponding to an internal fragment of the P67 gene of *B. subtilis* (see text for details) with the sequence of the *E. coli* trigger factor. For the *B. subtilis* sequence, the amino acid residue number was deduced from the nucleotide sequence of the entire *B. subtilis* gene kindly provided by Dr C. Harwood. Identical and conserved residues are boxed.

conserved kinases of the Ser/Thr type in *B. subtilis*. However, using a PCR approach to detect DNA sequences encoding different conserved protein kinase motifs, particularly in subdomains VI, VII and VIII of the eukaryote protein kinase family, no such sequences were identified. Although it cannot be excluded that the obtained sequences encode part of a kinase, the absence of sequence homology, notably the absence of the highly conserved domain VII, make this unlikely. In addition, no such sequences are present in the currently available DNA genome sequences for *B. subtilis* and *E. coli* in the database. Similarly, highly conserved genes of this type have not been detected in the recently sequenced *Haemophilus influenzae* genome (Fleischmann *et al.*, 1995). These results did not, however, preclude the conservation of structurally related Ser/Thr protein kinases or kinases with much more limited primary sequence conservation in *B. subtilis* or other bacteria. We therefore used, as an alternative method, polyclonal and monoclonal antibodies to highly conserved regions of mammalian PKC to identify cross-reacting *B. subtilis* proteins. With this technique, we clearly detected two *B. subtilis* proteins that were subsequently purified. However, the identity of these two proteins, Enzyme I and trigger factor, provided results both intriguing and paradoxical. Enzyme I is a phosphoenolpyruvate-dependent His protein kinase which phosphorylates HPr on the His₁₅ residue, an essential component of the phosphotransferase system (Saier, 1994). This enzyme has no detectable primary sequence homology with eukaryotic protein kinases. It was particularly surprising, therefore, that P70, the protein purified on the basis of cross-reactivity with the M7 antibody, was Enzyme I of the phosphotransferase system. The fact that this cross-reacting protein was absent from the Enzyme I deletion strain and that M7 also specifically recognized a homogeneous preparation of Enzyme I purified independently from another laboratory provided unambiguous evidence that P70 is identical to Enzyme I. However, recent structural studies of a protein family with a conserved actin fold (Kabsch & Holmes, 1995) have led to the identification of distantly related prokaryotic relatives, for example FtsA, a cell division protein (Bork *et al.*, 1992) with little if any detectable primary sequence homology. The possibility remains, therefore, that the three-dimensional structure

of Enzyme I retains significant structural features conserved in other protein kinases.

Trigger factor, detected for the first time in *B. subtilis* in this study, was first shown in *E. coli* to be implicated *in vitro* in protein translocation (Crooke & Wickner, 1987) and *in vivo* in cell division, through a possible interaction with FtsZ (Guthrie & Wickner, 1990). More recently, it has been clearly established that trigger factor is a member of the large family of proteins, immunophilins, with a wide reactivity of sequences, present in both prokaryotes and eukaryotes and possessing peptidyl-prolyl *cis-trans*-isomerase (PPIase) activity (Stoller *et al.*, 1995). The possession of such an activity leads to the conclusion that such proteins are important as catalysts in protein-folding mechanisms. However, the diversity of this family of proteins and the evidence that PPIase activity may not be essential for their biological role leads to the conclusion that such proteins fulfil other major activities yet to be identified (Timmerman *et al.*, 1995). Nevertheless, as in the case of PtsI, we failed to detect significant homology with eukaryotic protein kinases at the primary sequence level. Also, as with PtsI, we cannot rule out the possibility at this stage that trigger factor and eukaryote kinases are related at the three-dimensional structural level, which is detected by the antibody, or indeed that trigger factor possesses latent protein kinase activity.

Notwithstanding these possibilities, our study using both PCR and the antibody approach appears to indicate that eukaryotic-like Ser/Thr protein kinases of the conserved PKC type may not exist in *B. subtilis* in an easily detectable form. In consequence, therefore, purification of such protein kinases based on phosphorylation of specific substrates should now be the most desirable approach to the identification of this type of enzyme in *B. subtilis* and probably other prokaryotes.

ACKNOWLEDGEMENTS

We would like to thank many colleagues for providing us with antibodies, in particular K. Leach, G. Paravicini, K. P. Huang and Y. Milner, and we are grateful to S. Aymerich and J. Pero for providing strains. We are especially grateful to C. Harwood for communicating the *B. subtilis* trigger factor DNA sequence before publication. We are pleased to

acknowledge support from CNRS, Université Paris XI, EU Science Stimulation Programme (no. SCT-CT91-0713), INSERM (no. 930110), Association pour la Recherche contre le Cancer, Ligue Nationale Française contre le Cancer, and Human Frontier Science Programme (no. RG-386/95). N.Z. wishes to acknowledge the receipt of a fellowship from the French Government and J.S. the receipt of an EU fellowship (Human Capital & Mobility, no. ERBCHBG CT93 0491). We are also very grateful to I. B. Holland for critical reading of the manuscript.

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Received 31 October 1996; accepted 5 December 1996.