Volume 305, number 1, 67–73 FEBS 11189 June 1992

Characterization of the *pcp* gene encoding the pyrrolidone carboxyl peptidase of *Bacillus subtilis**

A. Awadé, Ph. Cleuziat, Th. Gonzalès and J. Robert-Baudouy

Laboratoire de Génétique Moléculaire des Microorganismes, Bâtiment 406, Institut National des Sciences Appliquées, 69621 Villeurbanne Cedex, France

Received 30 May 1992; revised version received 4 May 1992

Pyrrolidone carboxyl peptidase (EC 3.4.11.8) (Pcp) is an enzyme that catalyzes the removal of the N-terminal pyroglutamyl group from some peptides or proteins. Its value in protein chemistry and bacterial diagnosis makes this enzyme an interesting subject of study. The present paper reports for the first time the cloning and characterization of a pyrrolidone carboxyl peptidase gene (*pcp*). This gene is present in a single copy in the genome of *Bacillus subtilis* as indicated by Southern blot hybridization analysis. The *pcp* transcripts were analyzed in *Escherichia coli* by Northern blot hybridization and S1 nuclease mapping. The deduced amino acid sequence predicts a protein of 215 amino acids with a calculated molecular weight of 23,777 Da. The *pcp* gene has been over-expressed in *E. coli*, allowing the identification and partial characterization of Pcp protein.

Peptidase; *pcp* gene; Pcp protein; Gene expression; *Bacillus subtilis*

1. INTRODUCTION

Pyrrolidone carboxyl peptidase (Pcp) (EC 3.4.11.8) is an N-terminal exopeptidase widely distributed in many bacteria [1,2] and to a lesser extent in plant, animal and human tissues [3]. This enzyme catalyzes the removal of the N-terminal pyroglutamyl group from some peptides or proteins [4]. Such a property is used [4] to unblock pyroglutamyl peptides or proteins which are naturally resistant to Edman [5] sequential degradation prior to their sequencing. Moreover Pcp activity has been particularly used in bacterial diagnosis [6].

Since Pcp was discovered [1] it has been partially purified from different organisms, and its enzymatic and biochemical properties have been studied [7–9]. However, despite the importance of this enzyme for protein chemistry and for bacterial diagnosis no study on the characterization of this class of peptidase genes has been reported.

The present paper describes the cloning and characterization of the *B. subtilis pcp* gene. The gene has been over-expressed in *E. coli*, allowing partial characterization of the protein.

*The nucleotide sequence presented here has been submitted to the EMBL/GenBank database under the accession number no. X66034.

Abbreviations: Pcp, pyrrolidone carboxyl peptidase; pBS, plasmid Bluescript; ORF, open reading frame.

Correspondence address: A. Awadé, ENSBANA, Laboratoire de Biotechnologie, Campus universitaire Montmuzard, 21100 Dijon, France. Fax: (33) 80 39 66 11.

2. MATERIALS AND METHODS

2.1. *Bacterial strains, plasmids and growth conditions*

Bacterial strains and plasmids are listed in Table I. *Escherichia coli* and *B. subtilis* were usually grown in LB medium or in synthetic M63 medium supplemented by glycerol (0.1%) [15]. When needed the cells were grown on a solidified Difco agar (15 g/l) plate. In some cases ampicillin (50 μg/ml) was used for selection. For genomic DNA preparation *B. subtilis* was cultured as described by Rodriguez and Tait [16]. Cells were routinely grown at 37°C.

2.2. *DNA manipulations*

Preparation of genomic DNA was performed as described by Ausubel et al. [17].

Plasmid DNA isolation, DNA digestion, dephosphorylation, ligation, electrophoresis and bacterial transformation were carried out as described by Sambrook et al. [18].

DNA fragments were isolated, after digestion and electrophoresis on agarose gel, with the Geneclean II kit (Bio-Rad) or with electrophoresis on DEAE-cellulose membrane (NA 45, Schleicher and Shuell) as described by Sambrook et al. [18].

2.3. *Enzyme assays*

The detection of Pcp activity in bacteria plated on LB agar was conducted by a colorimetric test based on the method previously described by Mulczyk and Scewczuk [19]. The β-glucuronidase qualitative test was conducted in situ on agar-plated cells. After their lysis with toluene vapours, and addition of drops of a *para*-nitrophenyl-β-D-glucuronide solution as substrate, the colonies displaying activity exhibited a yellow colour due to the release of *para*-nitrophenol.

2.4. *Over-expression of Pcp*

Plasmid pTG1 was introduced into *E. coli* K38 [11] harbouring plasmid pGP1-2, which includes the DNA encoding bacteriophage T7 RNA polymerase [12]. Cells were grown at 30°C in a LB medium containing ampicillin and kanamycin (50 μg/ml) until the optical density at 595 nm reached approximately 1.5. The synthesis of bacteriophage T7 RNA polymerase from pGP1-2 was then induced by shifting the culture temperature to 42°C. After 25 min growth, rifampicin (200 μg/ml) was added to the medium to shut off transcription by *E. coli* RNA polymerase, and cells were cultured for 2 h at 37°C.

2.5. *Nucleotide sequence and computer analysis*

After isolation of nested deletions by exonuclease III and Mung bean nuclease [20] the DNA fragments inserted into pBS KS$^+$ and KS$^-$ were sequenced by the dideoxy chain termination method [21] using the T7 sequencing kit from Pharmacia. DNA fragments obtained by restriction endonucleases digestion were also sequenced for confirmation. The sequencing of both strands was performed using single-stranded DNA obtained from phagemid recombinants which were cultured as proposed by Pharmacia.

The Mac Molly program (SoftGene, Berlin) was used to analyze nucleotide and amino acid sequences.

2.6. *Preparation of radioactive probes*

For Southern or Northern blot hybridizations appropriate DNA fragments isolated from agarose gels were labeled by random priming using a kit from Boehringer.

To map the initiation site of *pcp* transcripts by S1 nuclease digestion the DNA fragment used as probe was dephosphorylated and labeled at its 5' ends by using T4 polynucleotide kinase as described by Sambrook et al. [18]. For the analysis of the 3' ends of the transcripts, the probe was labeled at its 3' ends with [α-^{32}P]dTTP by using the Klenow fragment of *E. coli* DNA polymerase I, as described by Sambrook et al. [18].

To be sure of the probes' purity DNA fragments were isolated after one and a second run on agarose gels.

2.7. *Other techniques*

For Southern blot hybridization chromosomal DNA digested with restriction endonucleases was separated by agarose gel electrophoresis (0.7%) and transferred to a nylon membrane (Hybond N$^+$, Amersham). Pre-hybridization, hybridization and washings were performed as proposed by the manufacturer.

Total RNA was prepared by a method based on the procedure described by Shimotsu et al. [22]. Cells were cultured in 25 ml of LB medium until exponential growth phase. After extraction and precipitation the RNA pellet was washed with 70% ethanol, dried and resuspended in a DNase buffer for a DNase digestion as described by Ausubel et al. [17]. After DNA digestion the RNA was extracted by one vol. of phenol/chloroform (v/v) and one vol. of chloroform. The RNA contained in the aqueous phase was then ethanol precipitated and resuspended in Milli Q (Millipore) water treated with 0.2% DEPC.

Northern blot analysis was conducted as described by Ausubel et al. [17].

To identify the ends of mRNAs by S1 nuclease mapping we essentially used the method described by Brakhage et al. [23].

3. RESULTS AND DISCUSSION

3.1. *Characterization of* B. subtilis pcp *gene encoding pyrrolidone carboxyl peptidase*

Since *E. coli* does not display Pcp activity the strategy used for cloning the *B. subtilis pcp* gene was to select recombinant clones displaying the Pcp activity in *E. coli*.

E. coli NM522 was transformed with a *B. subtilis* QB25 (derived from *B. subtilis* 168) genomic library [24]. Six of the approximately 5,000 recombinants clones screened exhibited Pcp activity. Restriction mapping of the six corresponding plasmids showed that they contained common DNA fragments. We chose the clone with the shortest DNA insert, pAWPY4, for further analysis. From pAWPY4 a 1.75 kb-pair (kbp) *Pst*I-*Hin*dIII fragment containing the *pcp* gene was subcloned into the appropriate sites of plasmid pBluescript KS (pBS KS). A more precise restriction map of the resulting plasmid, pAWPY414, was established (Fig. 1). Further subcloning experiments indicated that the *B. subtilis pcp* gene overlapped the *Eco*RV site (Fig. 1). The smallest fragment encoding Pcp activity was the 1.15 kb *Rsa*I fragment of pAWPY4141. This fragment was cloned in the two opposite orientations in plasmid pBS; there was no apparent orientation effect, with respect to pBS *lac* promoter, on *pcp* gene expression. This indicates that we had cloned the *pcp* gene with probably its own promoter region.

Table I

Bacterial strains and plasmids

Strains and plasmids	Genotypes and characteristics[a]	Origin or reference
Strains		
Bacillus subtilis 168	*trpC2*	[10]
Escherichia coli NM522	*SupE,thi,Δ(lac-proAB),Δhsd5, (r$_{K^-}$, m$_{K^-}$, [F', proAB, lacI^{q}ZΔM15]*	Stratagene
E. coli K38	*HfrC,λ+,phoA4,pit− 10,tonA22,ompF627,relA1*	[11]
Plasmids		
pAWPY4	pMK4 with 5.7 kbp *Sau*3A–*Sau*3A fragment containing *pcp* gene of *B. subtilis* QB25	This work
pAWPY414	pBluescript with 1.75 kbp *Pst*I–*Hin*dIII fragment containing *pcp* gene of *B. subtilis* QB25	This work
pAWPY4141	pBluescript with 1.15 kbp *Rsa*I–*Rsa*I fragment containing *pcp* gene of *B. subtilis* QB25	This work
pAWPY4143	pBluescript with 1.33 kbp *Sca*I–*Hin*dIII fragment containing *pcp* gene of *B. subtilis* QB25	This work
pBluescript (pBS)	Ap^R, *lacZ'*	Stratagene
pGP1-2	Km^R, PI-T7 gene *1*, P*lac-c*1857	[12]
pMK4	Ap^R, Cm^R, *lacZ'*	[13]
pT7-5	Ap^R, T7 ϕ10	[12]
pTG1	pT7-5 with the 1.33 kbp *Sca*I–*Hin*dIII fragment from pAWPY4143	This work
pUIDK31	pBR322 derivative vector containing *uidA* as reporter gene; Cm^R, Km^R	N. Bardonnet (pers. commun.)

lacZ' indicates that the 3' end of this gene is truncated. Ap^R, resistance to ampicillin; Cm^R, resistance to chloramphenicol; Km^R, resistance to kanamycin.

[a] Genotype symbols are according to Bachmann [14]

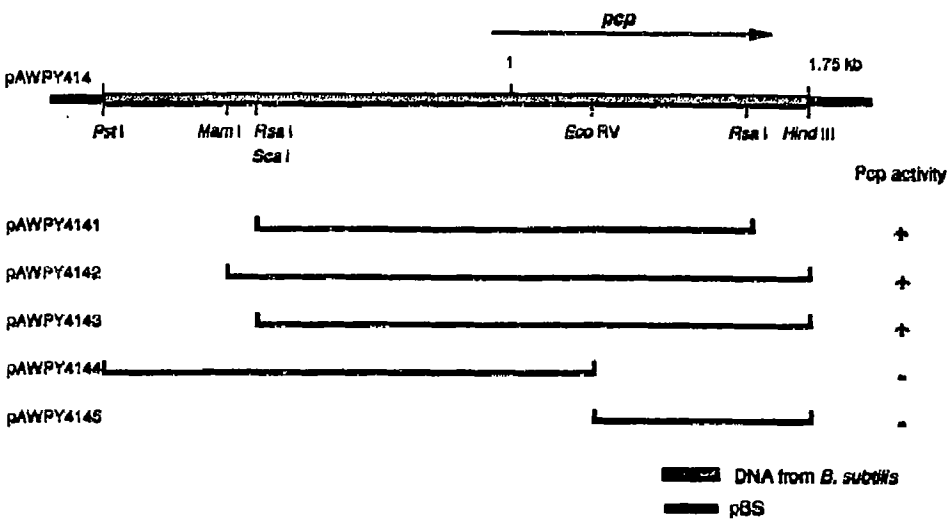

Fig. 1. Partial physical map of a 1.75 kb *Pst*I–*Hin*dIII DNA fragment (pAWPY414) containing the *pcp* gene from *B. subtilis* and subclone derivatives. The Pcp activity was checked as described in section 2.3. pAWPY4141 was constructed by insertion of the *Rsa*I–*Rsa*I fragment into the *Eco*RV site of pBluescript KS (pBS KS); pAWPY4142 by insertion of the *Mam*I–*Hin*dIII fragment into the *Eco*RV–*Hin*dIII sites of pBS KS; pAWPY4143 by insertion of the *Sca*I–*Hin*dIII fragment into the *Eco*RV–*Hin*dIII sites of pBS KS; pAWPY4144 by insertion of the *Pst*I–*Eco*RV fragment into the *Pst*I–*Eco*RV sites of pBS KS; pAWPY4145 by insertion of the *Eco*RV–*Hin*dIII fragment into the *Eco*RV–*Hin*dIII sites of pBS KS. *E. coli* NM522 harboring pAWPY4141, pAWPY4142 or pAWPY4143 displayed Pcp activity, whereas pAWPY4144 and pAWPY4145 could not provide this activity. The transcriptional orientation of the *pcp* gene is indicated by the arrow.

To determine the transcriptional orientation of the *pcp* gene a *uidA* Km^R cassette (from pUIDK31) was introduced into the *Eco*RV site of plasmid, pAWPY4141. In this cassette the *uidA* gene is deleted from its own promoter but retains its Shine and Dalgarno sequence, and thus it was possible to generate transcriptional fusion. Insertion of the *uidA* cassette into the *Eco*RV site led to the loss of Pcp activity whatever the cassette orientation. Restriction mapping of the clones which displayed β-glucuronidase activity, encoded by *uidA*, indicated that the *pcp* gene was transcribed from the *Pst*I to the *Hin*dIII site of plasmid, pAWPY414 (Fig. 1).

To confirm the insert origin Southern blot hybridization against *B. subtilis* and *Streptococcus pyogenes* genomic DNA digested with *Rsa*I and *Hin*dIII was performed (Fig. 2). The 1.15 kbp *Rsa*I fragment from plasmid pAWPY4141 (Fig. 1) was used as probe. A specific 1.15 kbp *Rsa*I restriction fragment and a 2.2 kbp *Hin*dIII fragment from *B. subtilis* 168 genomic DNA hybridized with the probe, indicating that the *pcp* gene is present in a single copy on the *B. subtilis* genome. No hybridizing bands were detected with *Strep. pyogenes* DNA under the stringent conditions described by Amersham.

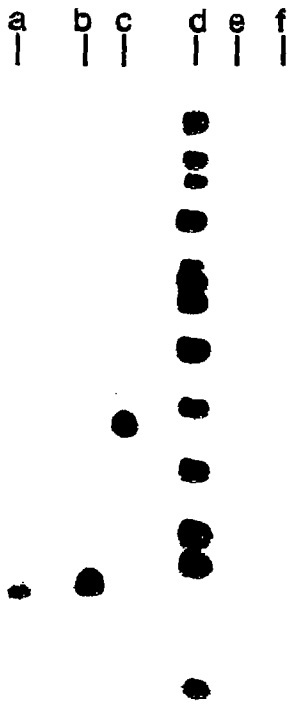

Fig. 2. Genomic Southern blot analysis. (a) Positive control, *Rsa*I–*Rsa*I fragment of pAWPY4141 used as probe for the hybridization; *B. subtilis* genomic DNA digested by *Rsa*I (b), and *Hin*dIII (c); (d) molecular weight markers, from bottom to top, 0.686, 1.255, 1.416, 1.810, 2.319, 2.938, 3.609, 3.988, 4.360, 5.634, 7.378, 9.007 and 14.98 kbp; *S. pyogenes* DNA digested by *Rsa*I (e) and *Hin*dIII (f).

3.2. *Sequence analysis of the* pcp *gene*

The 1.33 kbp *Sca*I–*Hin*dIII DNA fragment from plasmid, pAWPY4143 (Fig. 1), was sequenced on both strands. The complete nucleotide sequence of this fragment, including the *pcp* gene, is presented in Fig. 3.

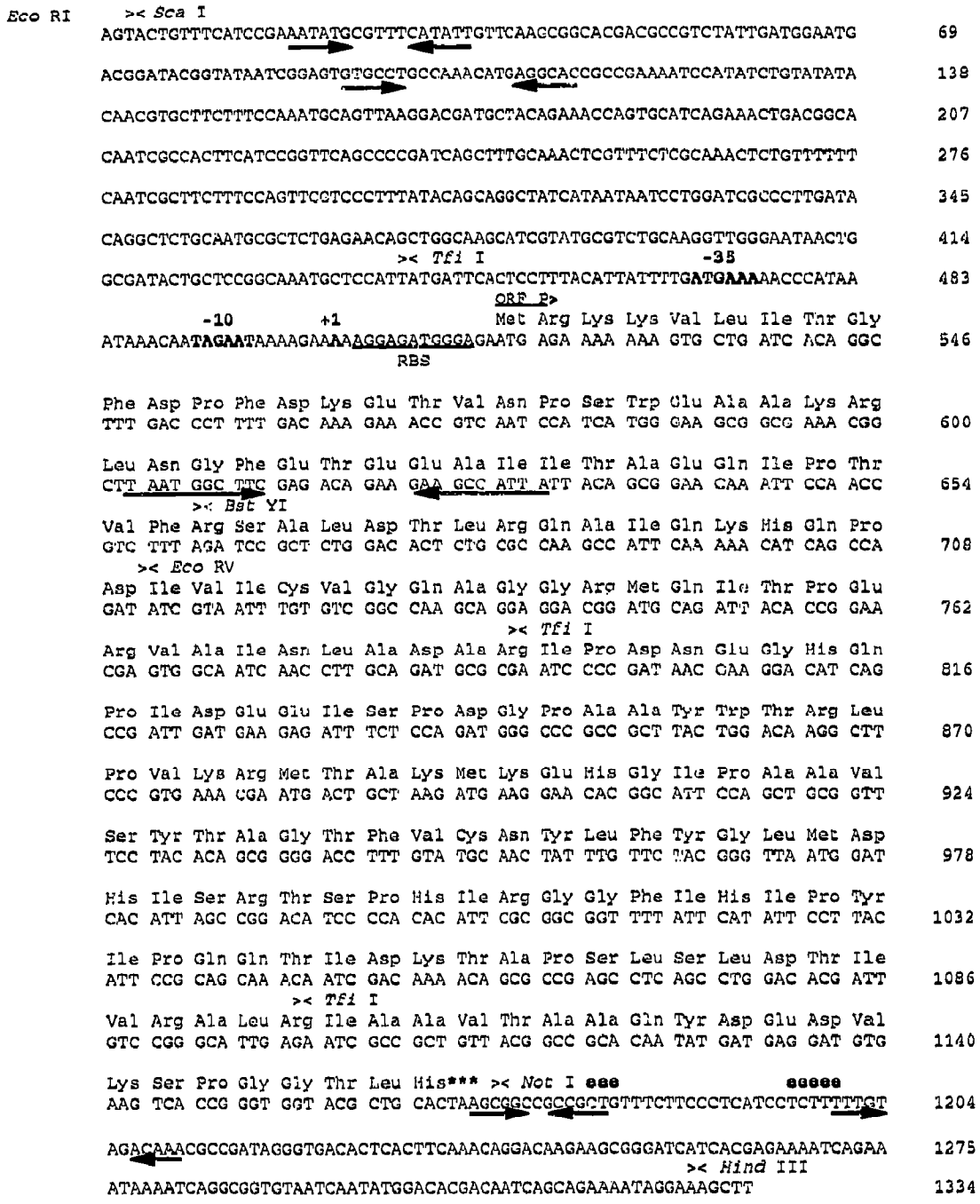

Fig. 3. Nucleotide sequence of the *Sca*I–*Hin*dIII fragment containing the *pcp* gene and its surrounding areas. The beginning of ORF P is shown. The promoter region of the *pcp* (−35, −10, +1) is in bold, and the transcription ends deduced from S1 nuclease mapping are marked (e). Inverted repeat sequences are indicated by arrows. A putative ribosome binding site (RBS) is underlined, and the stop codon for the ORF P is marked by asterisks. The deduced amino acid sequence of Pcp is given above the nucleotide sequence. Restriction endonuclease sites (><) used to generate probes for Northern blot hybridization and S1 nuclease mapping are also shown above the nucleotide sequence. The *Eco*RI site is located in the pBS polylinker.

Analysis of this sequence showed 6 open reading frames (ORFs) on the two strands. The larger one, that we called ORF P, is transcribed in the direction from the *Sca*I-to-*Hin*dIII site. ORF P starts at nucleotide (nt) 520 and ends at nt 1,165. It encodes a polypeptide of 215 amino acids with a calculated molecular weight of 23,777 Da. After reference to the molecular weights of the different pyrrolidone carboxyl peptidases studied, and particularly with the Pcp from *B. amyloliquefaciens* [9], whose molecular weight was estimated to be 24,000 Da by SDS-PAGE, we suggested that ORF P might correspond to Pcp. Analysis of the nucleotide sequence

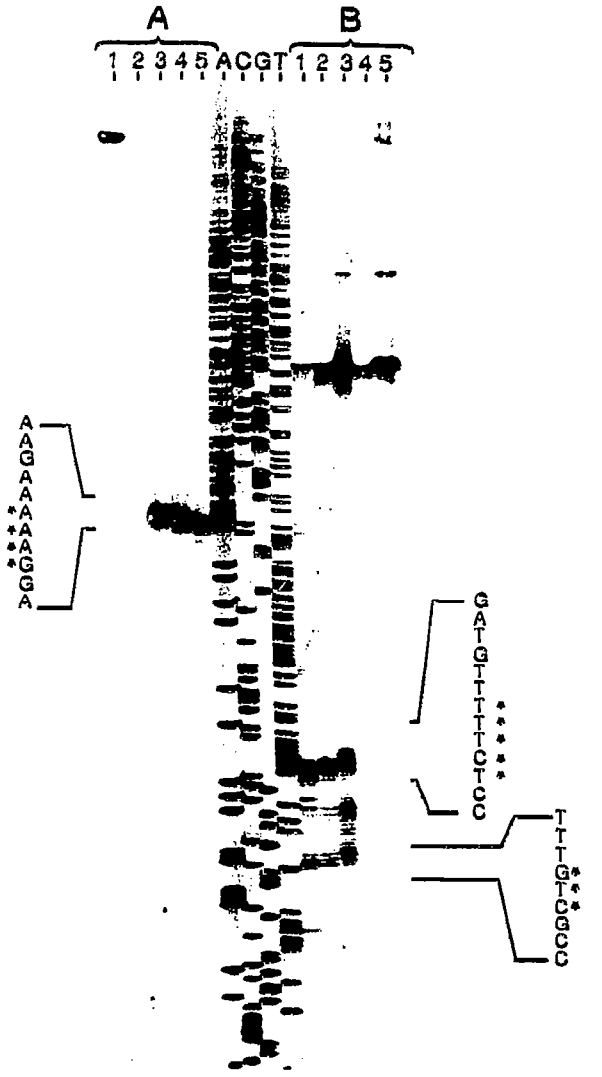

Fig. 4. S1 nuclease mapping of the 5′ and 3′ end of the *pcp* transcripts. Lanes ACGT correspond to a DNA sequence ladder obtained by sequencing a deletion of pAWPY4143 using a T3 primer. (A) Determination of the 5′ end: lane 1, the 666 bp *Bst*YI–*Eco*RI DNA fragment (Fig. 3) used for the hybridization; lane 2, experiments conducted with the RNA extracted from *E. coli* NM522 carrying pBS KS; lanes 3, 4 and 5, hybridization of the probe with RNA extracted from *E. coli* NM522/pAWPY4143 and digested with different concentrations of S1 nuclease, respectively, 125, 250 and 500 U/ml. Lane 4 gives the best results. Nucleotides with stars on the left of the figure indicate the 5′ ends deduced from the length of S1 nuclease digestion-resistant fragments shown in lane 4. (B) Determination of the 3′ end: lane 5, the 233 bp *Tfi*I–*Hin*dIII DNA fragment (Fig. 3) used for the hybridization; lane 4, experiments conducted with the RNA extracted from *E. coli* NM522 carrying pBS KS; lanes 3, 2 and 1, hybridization of the probe with RNA extracted from *E. coli* NM522/pAWPY4143 and digested with different concentrations of S1 nuclease, respectively, 125, 250 and 500 U/ml. Lane 2 gives the best results. Nucleotides with stars on the right of the figure indicate the 3′ ends deduced from the length of S1 nuclease digestion-resistant fragments shown in lane 2.

upstream from the ATG initiation codon of ORF P showed the presence of a purine rich sequence (AGGAGATGGGA) (Fig. 3) which could be a ribosome binding site (RBS) both in *E. coli* and *B. subtilis* [25,26]. The

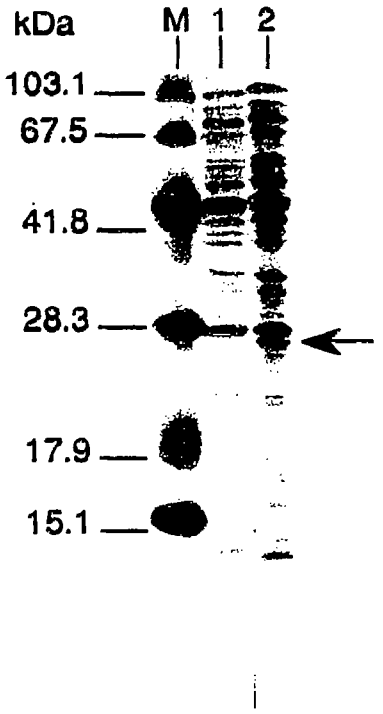

Fig. 5. SDS-PAGE of different cell-free extracts from (1) *E. coli* K38 harbouring pT7-5 plasmid and (2) *E. coli* K38 harbouring pTG1 plasmid, which were grown for protein over-expression, as described in section 2. M denotes molecular weight markers. The arrow indicates the over-expressed polypeptide which corresponds to the Pcp protein.

putative *pcp* RBS exhibits extensive complementarity to the 3′ region of *B. subtilis* 16 S rRNA [27]. Moreover a possible −35 and −10 promoter sequence was also identified between nt 469 and 474 and between nt 492 and 497, respectively. This promoter sequence shows homology to the canonical promoter sequence (TTGACA-17bp-TATAAT) typically recognized by σ^{70} RNA polymerase of *E. coli* and σ^{43} RNA polymerase of *B. subtilis* [28]. This sequence could act as a promoter in both *B. subtilis* and *E. coli*.

Computer aided research revealed two inverted sequences located downstream from the TAA stop codon (Fig. 3). The first one is a short G/C-rich inverted sequence located between nt 1,167 and 1,178. This sequence could be involved in transcription termination since it is followed by a T-rich sequence [29]. In addition to these inverted sequences three others were detected. Two of them are located upstream far away from the *pcp* promoter and their role in the transcription of *pcp* is not discussed; the third one is located at the beginning of the ORF (Fig. 3).

The potential promoter and transcription termination signals detected by sequence analysis were verified by S1 nuclease mapping (Fig. 4) and Northern blot hybridization (data not shown) of *pcp* transcripts in *E. coli* harboring pAWPY4143. S1 nuclease mapping experiments were performed using the 5′ end-labelled 666 bp *Bst*YI–*Eco*RI fragment (Fig. 3) as probe to identify

the 5′ end and the 3′ end-labelled 233 bp *Tfi*I–*Hin*dIII fragment (Fig. 3) to define the 3′ limits of the *pcp* transcripts. The results of the *pcp* transcripts 5′ end mapping suggested that the transcription originated approximately 15 nt upstream from the Pcp protein translation initiation codon (Fig. 3). This short distance is rather unusual in Gram-negative bacteria such as *E. coli*, but seems to be more common in Gram-positive bacteria [26,30]. The position of the transcription start, deduced from S1 mapping experiments, is in agreement with the possible promoter detected by sequence analysis. S1 nuclease mapping of the 3′ ends of *pcp* transcripts suggested the presence of two possible transcription terminations (Fig. 3): a minor one lying after the G/C-rich inverted sequence located between nt 1,178 and 1,180, and a major one in a T-rich sequence located between nt 1,196 and 1,200. The second dyad symmetry sequence (Fig. 3) located downstream from the TAA stop codon, which is not G/C rich, is unlikely to be involved in the termination of *pcp* transcription. The 3′ ends of the transcripts could also correspond to the processing of a larger RNA which is not detected by Northern blot hybridization. In order to define the length of *pcp* transcripts we performed Northern blots of 5 μg total RNA from *E. coli* NM522 carrying pAWPY4143. Blots of these RNA were hybridized with the uniformly labelled 458 bp *Eco*RV–*Not*I fragment (Fig. 3) as a probe. Two *pcp* transcripts of very similar size were detected in the range of 700 nt. This is in good agreement with the distance of 676–696 nt which separates the potential transcription start and stop signals.

3.3. *Over-expression and partial characterization of* B. subtilis *Pcp protein*

The *Sca*I–*Hin*dIII DNA fragment including the *B. subtilis pcp* gene was cloned into pT7-5 plasmid, giving pTG1. In this derivative plasmid the *pcp* gene was under the control of the T7 Φ 10 promoter. Transformation of *E. coli* K38 with pTG1 led to over-expression of a polypeptide of approximately 26 kDa on SDS-PAGE (Fig. 5). This size is in agreement with the predicted molecular weight of the polypeptide encoded by ORF P (23,777 kDa). Moreover, a relatively high induction of Pcp activity was observed after T7 RNA polymerase thermo-induction (data not shown). The molecular weight of the protein, as estimated by gel filtration, was approximately 85 kDa. This suggested that the enzyme may be present as a tetramer.

3.4. *Analysis of the deduced Pcp amino acid sequence*

The deduced amino acid sequence from ORF P was compared with protein sequence databases (Swissprot. 18 and PIR.28). No significant similarity with any sequence in these databases was noticed, suggesting that Pcp belongs to a new class of peptidases. The amino acid sequence of the *B. subtilis* Pcp showed the presence of two cysteines. Probably these cysteines are not involved in disulfide bonds since it has been shown that this enzyme is inhibited by SH blocking reagents [7–9]. This observation suggests that a sulfhydryl group may form a part of the catalytic site of the Pcp enzyme.

The active enzyme should have a cytoplasmic location since no Pcp activity was detected in either *B. subtilis* and recombinant *E. coli* culture media. This suggests that Pcp does not have a post-translational processed signal sequence [31]. Thus the amino acid sequence deduced from the nucleotide sequence of the gene should correspond to the mature protein. Intracellular location of this protein has been previously mentioned by Tsuru et al. [9] for two strains of *B. subtilis* (DT-39 and ML-208), for *B. amyloliquefaciens* and for *B. circulans* IFO 3342. This is in accordance with the potential involvement of Pcp in cellular protein metabolism.

Acknowledgements: We wish to thank M. Débarbouillé and A. Klier for providing us with a *B. subtilis* genomic library. We are grateful to L.F. Wu and M.G. Daney de Marcillac for assistance in data bank homology research. We thank W. Nasser for valuable discussions. We also thank G. Condemine, M. Moulard and S. Reverchon for reading the manuscript. This work was supported by grants from the Commission of the European Communities (Biotechnology Action Programme), from an Action d'Intervention sur Programme of the Centre National de la Recherche Scientifique, from the Etablissement Public Régional Rhone-Alpes, from the Ministère de la Recherche et de l'Enseignement Supérieur (Aide sur Fond de la Recherche), and from the Fondation Marcel Mérieux.

REFERENCES

[1] Szewczuk, A. and Mulczyk, M. (1969) Eur. J. Biochem. 8, 63–67.
[2] Doolittle, R.F. (1970) Methods Enzymol. 19, 555–569.
[3] Szewczuk, A. and Kwiatkowska, J. (1970) Eur. J. Biochem. 15, 92–99.
[4] Doolittle, R.F. and Armentrout, R.W. (1968) Biochemistry 7, 516–521.
[5] Edman, P. (1950) Acta Chem. Scand. 4, 283–293.
[6] Mantle, D., Lauffart, B. and Gibson, A. (1991) Clin. Chim. Acta 197, 35–46.
[7] Kwiatkowska, J., Torain, B. and Glenner, G.G. (1974) J. Biol. Chem. 249, 7729–7736.
[8] Sullivan, J.J., Muchnicky, E.E., Davidson, B.E. and Jago, G.R. (1977) Aust. J. Biol. Sci. 30, 543–552.
[9] Tsuru, D., Fujiwara, K. and Kado, K. (1978) J. Biochem. 84, 467–476.
[10] Lepesant, J.-A., Kunst, F., Lepesant-Kejzlarová, J. and Dedonder, R. (1972) Mol. Gen. Genet. 118, 135–160.
[11] Russel, M. and Mobel, P. (1985) J. Bacteriol. 159, 1034–1039.
[12] Tabor, S. and Richardson, C.C. (1985) Proc. Natl. Acad. Sci. USA 82, 1074–1078.
[13] Sullivan, A.M., Yasbin, R.E. and Young, F.E. (1984) Gene 29, 21–26.
[14] Bachmann, B.S. (1990) Microbiol. Rev. 54, 130–197.
[15] Miller, J.H. (1972) Experiments in Molecular Genetics, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
[16] Rodriguez, R.L. and Tait, R.C. (1983) Recombinant DNA Techniques: An Introduction. Addison-Wesley, Reading, MA.
[17] Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Smith, J.A., Seidman, J.G. and Struhl, K. (1987) Current Protocols in Molecular Biology, Wiley/Interscience, New York.
[18] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular

Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
[19] Mulczyk, M. and Szewczuk, A. (1970) J. Gen. Microbiol. 61, 9–13.
[20] Henikoff, S. (1984) Gene 28, 351–359.
[21] Sanger, F., Nicklen, S. and Coulsen, A.R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463–5467.
[22] Shimotsu, H., Kuroda, I., Yanofsky, C. and Henner, D.J. (1986) J. Bacteriol. 166, 461–471.
[23] Brakhage, A.A., Wozny, M. and Putzer, H. (1990) Biochimie 72, 725–734.
[24] Débarbouillé, M., Kunst, F., Klier, A. and Rapoport, G. (1987) FEMS Microbiol. Lett. 41, 137–140.
[25] Stormo, G.D., Schneider, T.D. and Gold, L.M. (1982) Nucleic Acids Res. 10, 2971–2995.
[26] Moran, C.P., Lang, N., LeGrice, S.F.J., Lee, G., Stephens, M., Sonenshein, A.L., Pero, J. and Losick, R. (1982) Mol. Gen. Genet. 186, 339–346.
[27] McLaughlin, J.R., Murray, C.L. and Rabinowitz, J.C. (1981) J. Biol. Chem. 256, 11283–11291.
[28] Reznikoff, W.S., Siegele, D.A., Cowing, D.W. and Gross, C.A. (1985) Annu. Rev. Genet. 19, 355–387.
[29] Friedman, D.I., Imperiale, M.J. and Adhya, S.L. (1987) Annu. Rev. Genet. 21, 453–488.
[30] Qi, F.-X. and Doi, R.H. (1990) J. Bacteriol. 172, 5631–5636.
[31] Kreil, G. (1981) Annu. Rev. Biochem. 50, 317–348.