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Molecular cloning and characterization of a proline iminopeptidase gene from *Neisseria gonorrhoeae*

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Summary

Proline iminopeptidase (Pip) is a hydrolase elaborated by virtually all strains of *Neisseria gonorrhoeae* that selectively removes *N*-terminal proline residues from peptides. *Escherichia coli* clones expressing the gonococcal gene coding for Pip were identified in a genomic cosmid library using a synthetic colorimetric substrate. Nucleotide sequence determination and analyses of polypeptides detected by coupled *in vitro* transcription/translation reactions revealed that Pip is a 311-amino-acid polypeptide with a M_r of 35 kDa and a pI of 5.4. Southern hybridization showed that the *pip* gene is present in a single copy on the chromosome of *N. gonorrhoeae* strain MS11 which maps immediately upstream of the previously identified *opaA* locus. The transcriptional start site of *pip* in *E. coli*, determined by primer extension analysis, was characteristic of an NtrA or sigma-54-dependent promotor. Complementation of an *E. coli* mutant deficient in both proline biosynthesis and dipeptide uptake confirmed that Pip is capable of releasing biologically active proline from peptides. Pip expression was found to be non-essential for *in vitro* growth of *N. gonorrhoeae*, based on the viability of a Pip$^-$ gonococcal mutant.

Introduction

Neisseria gonorrhoeae, the causative agent of gonorrhoea, is a highly successful parasite of man. Gonococcal isolates taken from infections often display a proline-requiring phenotype: as many as 20 to 40%, depending on geographical source (Knapp and Holmes, 1975). The isolation of a proline auxotrophic strain is conclusive evidence of its ability to colonize successfully the human host, suggesting that either exogenous proline is present or that the bacterium has alternative means of acquiring this amino acid critical to its viability. Chen and Buchanan (1980) have described two gonococcal enzymes implicated in proline acquisition: (i) an aminopeptidase P which cleaves peptides having the structure X–Pro–Y–Z to yield X + Pro–Y–Z where X, Y, and Z denote amino acid residues, and (ii) proline iminopeptidase (Pip) which hydrolyses peptides having the structure Pro–Y–Z to yield free proline. Pip was found to release only proline from each of 14 peptides with the structure Pro–X, Pro–X–NH_2 or Pro–X–Y, although its activity appeared to decrease with increasing chain length of the substrate (Chen and Buchanan, 1980).

Proline iminopeptidase activity was first identified in *Escherichia coli* based on the ability of proline auxotrophs to utilize poly-L-proline as a source of proline (Sarid *et al*., 1959). The authors described the activity as being highly specific for *N*-terminal L-prolyl residues, regardless of the chain length of the substrate (Sarid *et al*., 1959; 1962). Thereafter, the enzyme has been detected in eukaryotic sources, in *Bacillus megaterium* (Yoshimoto *et al*., 1980), *Bacillus coagulans* (Yoshimoto and Tsuru, 1985), and in *Lactobacillus casei* isolated from the human oral cavity (Mäkinen, 1969). Recently, the gene encoding proline iminopeptidase in *B. coagulans* has been cloned and sequenced (Kitazono *et al*., 1992).

The detection of Pip activity along with other enzymatic activities using synthetic chromogenic substrates has been used in clinical studies to differentiate *N. gonorrhoeae* from other diplococci (D'Amato *et al*., 1978; Sperry *et al*., 1986; Yajko *et al*., 1984). Results from these studies suggest that Pip may perform an important function for gonococci *in vivo* since all isolates tested express Pip activity, whereas both Pip$^+$ and Pip$^-$ strains of *Neisseria meningitidis* and *Neisseria lactamica* have been described (Sperry *et al*., 1986; Yajko *et al*., 1984). In this study, we describe the cloning, mapping and characterization of the gene encoding proline iminopeptidase (Pip) from *N. gonorrhoeae*.

Results and Discussion

Cloning and nucleotide sequencing of the pip *gene*

Six Pip$^+$ *E. coli* clones were identified within a gonococcal DNA cosmid bank using a standard assay for Pip activity.

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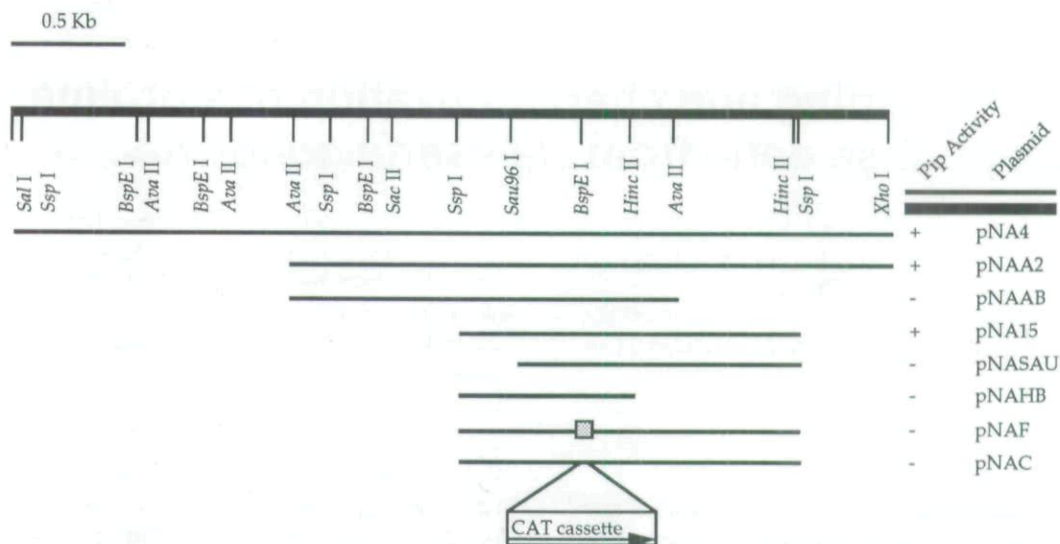

Fig. 1. Partial restriction endonuclease map of the 4 kb *Sal*I–*Xho*I fragment carrying Pip activity and subclones generated to assay for Pip in *E. coli* transformants. Pip activity in transformants was determined by the standard assay. The shaded box indicates the *BspE*I site that was filled in and made blunt-ended with Klenow to generate an 8 bp insertion (pNAF). The chloramphenicol acetyl transferase gene cassette (CAT) was also ligated into the *BspE*I site as a blunt-ended fragment (pNAC), giving rise to Cm-resistant Pip⁻ transformants having the CAT gene with the transcriptional orientation as shown.

Digestion of the cosmid DNAs with *Cla*I revealed a common 18 kb fragment which, when subcloned, was capable of conferring Pip activity on *E. coli*. Further subcloning localized Pip activity to a 4 kb *Sal*I–*Xho*I fragment that conferred full Pip activity on the host *E. coli* when cloned in pACYC184 or pBSK (pNA4, Fig. 1). This fragment was cloned into pBSK in both orientations with respect to *lacZ* transcription. By measuring Pip activity (rate of proline-*p*-nitroanilide hydrolysis) per mg total cell protein, a preferential expression of the gene in one orientation was found when transcription was induced by the addition of IPTG (not shown). To further localize the gene, and to construct clones for nucleotide sequencing, both nested deletions and subcloning were used to construct plasmids carrying smaller inserts of gonococcal DNA. Resulting clones were analysed for Pip activity, and a 1.5 kb *Ssp*I fragment was found to confer full Pip activity on the *E. coli* host (pNA15, Fig. 1). A unique *BspE*I site was identified on pNA15, which was filled-in and made blunt ended using Klenow enzyme and deoxyribonucleoside triphosphates. *E. coli* transformants for the religated plasmid were Pip$^-$. Similarly, when a promoterless chloramphenicol acetyltransferase (CAT) gene cassette from pCM7 (Close and Rodriguez, 1982) was cloned into the *BspE* I site, the *E. coli* transformants were Pip$^-$ and chloramphenicol (Cm) resistant (Fig. 1).

Nucleotide sequencing of the *Ssp*I fragment indicated the presence of a single large open reading frame (ORF) (Fig. 2). The deduced amino acid sequence from this ORF would correspond to a protein of 34.5 kDa having a pI of 5.4. Upstream of the translational start, a sequence characteristic of a ribosome-binding site (AGAAG, −8 bp, Fig. 2) was found. The ORF corresponds to 311 amino acids and, following the translational stop codon, there is a 12 bp inverted repeat (948–977 bp, Fig. 2) — a motif suggested to act as a transcriptional attenuator or terminator (Yager and von Hippel, 1987). The consensus gonococcal DNA uptake sequence (Goodman and Scocca, 1988) was also found internal to the ORF (406–417 bp, Fig. 2).

Sequence analysis of the region surrounding *pip* revealed no significant ORFs that appeared to be co-transcribed with *pip*. Northern blot analysis using gonococcal RNA and the *Ssp*I fragment as probe indicated that the size of the transcript was similar in size to the DNA needed to encode the ORF (not shown), thus *pip* does not appear to belong to an operon.

A database search using the deduced amino acid sequence showed that proline iminopeptidase from *N. gonorrhoeae* and *B. coagulans* share limited regions of homology. In addition, stronger similarities were found between Pip and the active site region of a family of hydrolases from *Pseudomonas* spp., including atropinesterase (Hessing, 1983) and hydroxymuconic semialdehyde hydrolase (Horn *et al.*, 1991; our unpublished results).

The pip *gene product is 35 kDa and has a pI of 5.4*

Analysis by *in vitro* transcription/translation was used to identify a gene product that could be correlated to Pip activity and the ORF. The results from this analysis on

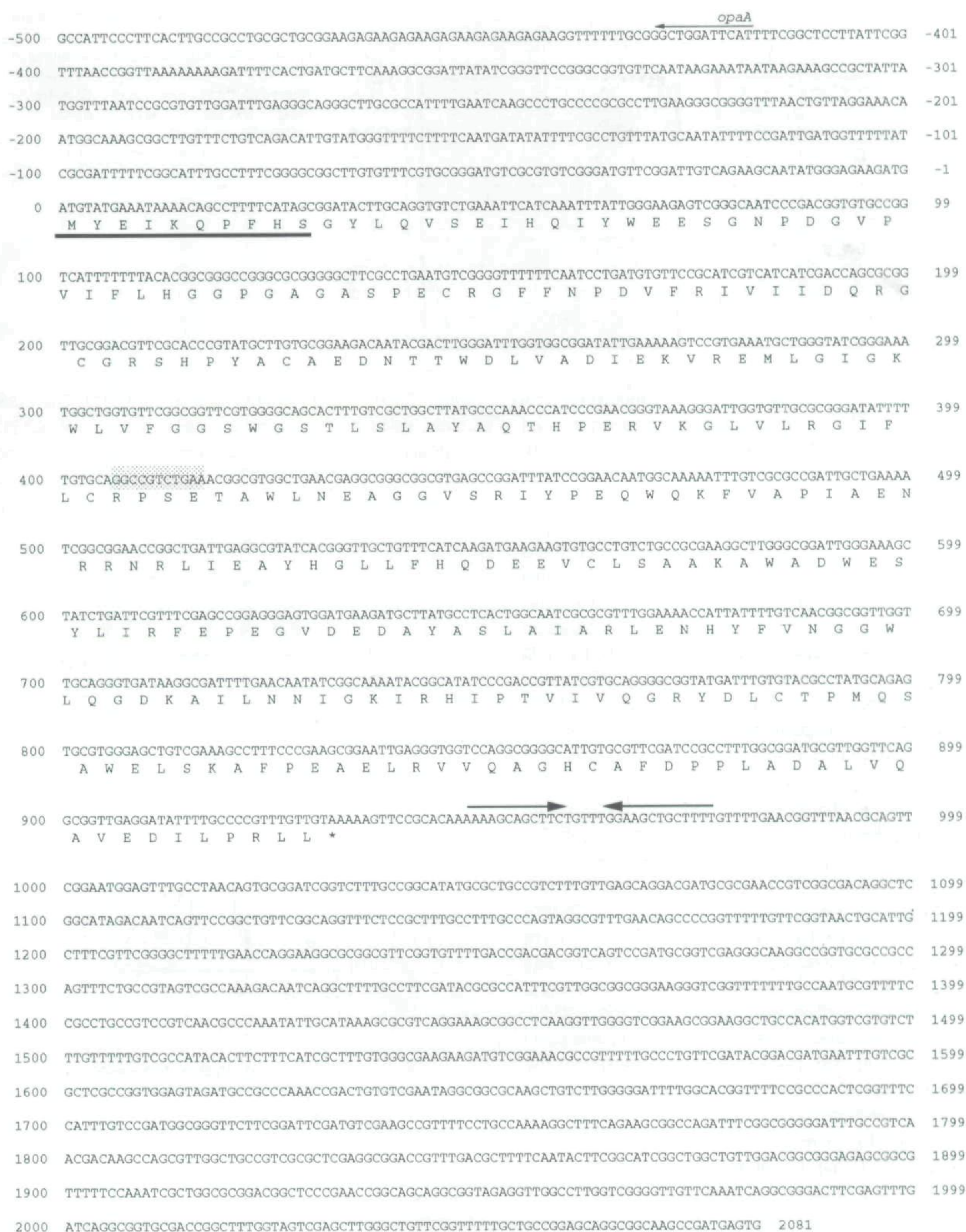

Fig. 2. Nucleotide sequence of the proline iminopeptidase gene and the deduced amino acid sequence of the ORF. The nucleotide sequence is numbered from the 5' end of the sequenced fragment. Amino acid sequence of the protein determined by Edman degradation is underlined. Translational start of the *opaA* gene is at position –419 relative to *pip*. The 10 bp gonococcal uptake sequence is shaded. A putative transcriptional terminator is indicated by arrows. These sequence data appear in the EMBL/GenBank/DDBJ Nucleotide Sequence Data Libraries under the accession number Z25461.

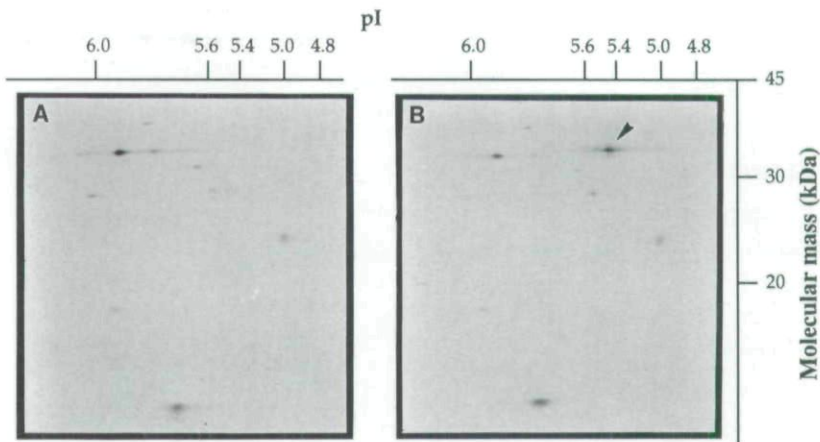

Fig. 3. *In vitro* coupled transcription/translation analysis of pBSK (A) and pNA15 (B) as analysed by 2-D SDS–PAGE. Molecular mass and pI of known proteins are indicated. Proteins were labelled by the incorporation of [^{35}S]-methionine using an S30 extract. The *pip* gene product is indicated by an arrow.

pBSK (A) and pNA15 (B) as analysed by two-dimensional (2-D) SDS–PAGE are shown in Fig. 3, indicating that the gonococcal DNA insert encodes a protein with a molecular mass of 35 kDa, having a pI of 5.4. Furthermore, *in vitro* transcription/translation products were analysed for Pip activity by the addition of pro-pNA to the *in vitro* assay. Only in the presence of pNA15 was there detectable Pip activity (not shown). To confirm the identity of the Pip protein with the predicted ORF, we overexpressed Pip by IPTG induction of an *E. coli* strain harbouring pNA15, separated the proteins by 2-D SDS–PAGE, and performed automated *N*-terminal sequencing directly on the protein blotted to a PVDF membrane (see the *Experimental procedures* for details). The *N*-terminal sequence obtained from the analysis (M Y E I K Q P F H S G) matched perfectly the ORF predicted in Fig. 2. The Pip gene product was also identified on Coomassie brilliant blue-stained one-dimensional SDS–PAGE gels from protein extracts of *E. coli* cells carrying pNA15 and induced by IPTG (Fig. 4B). In comparison with cells carrying only the vector plasmid, a new 35 kDa protein band was detected. Proteins from identical gels were blotted to a nitrocellulose membrane. The membrane was then sliced into fractions based on molecular weight, which were then individually assayed for Pip activity. Maximum activity was found to coincide with the band corresponding to the overexpressed product on the stained gel (Fig. 4A). Together, these results clearly indicate that the gene encoding Pip is present on the *Ssp*I fragment, that the gene product has a molecular mass of 35 kDa, and that no additional gonococcal DNA sequences are required for its expression in *E. coli*.

pip *maps adjacent to* opaA *on the MS11 chromosome*

Computer-assisted database searching revealed that the region upstream and on the opposite DNA strand from the *pip* gene had been previously characterized, with the DNA sequence found here being identical to that of the *opaA* gene of strain MS11 (Bhat *et al.*, 1991). The nucleotide sequence comprising the characteristic CTTCT repeats and the translational start of this particular *opa* locus (482 bp upstream of *pip*, Fig. 2) is previously unpublished. The DNA homology between *opaA* and the other

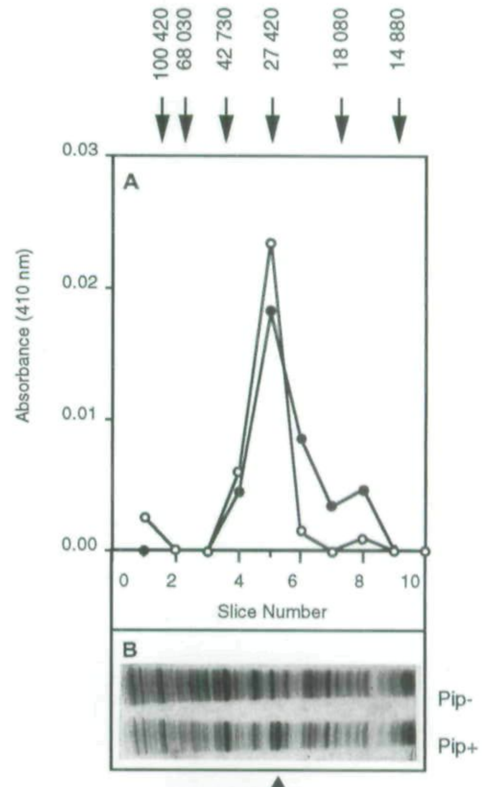

Fig. 4. Pip activity in discrete fractions of proteins separated by SDS–PAGE.
A. Identical gels were blotted to nitrocellulose and fractionated into 5 mm slices. Each slice was individually assayed for Pip activity by measuring the hydrolysis of pro-pNA at 410 nm. The absorbance of the corresponding slice of the control was subtracted from the Pip$^+$ samples. The molecular mass (Da) of size markers is indicated. The different symbols denote results from separate experiments.
B. One-dimensional SDS–PAGE gels of protein extracts from *E. coli* overexpressing pNA15 (Pip$^+$) and control (pBSK, Pip$^-$). The *pip* gene product is indicated.

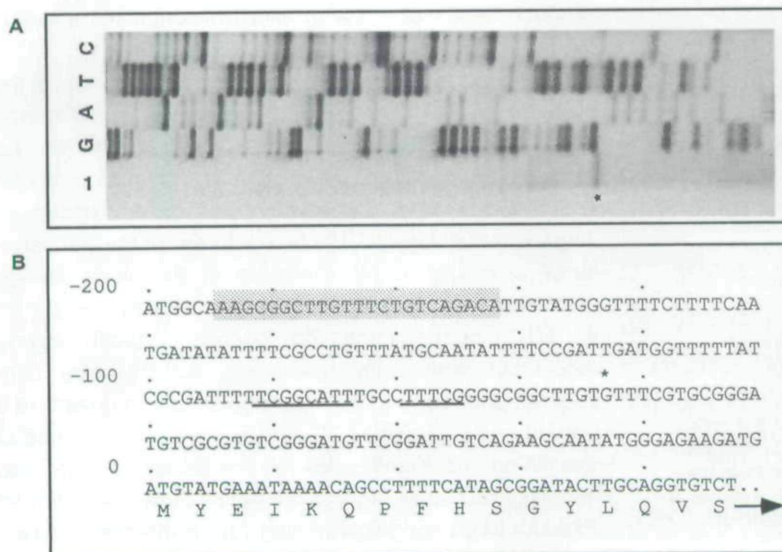

Fig. 5. Identification of the *pip* promoter.
A. Primer extension analysis of RNA isolated from log-phase *E. coli* expressing Pip from pNA4 (lane 1). Sequencing ladder generated from pNA4 using the same primer.
B. Sequence of the *pip* promoter and upstream activation site (UAS). The σ^{54} recognition site is underlined, the UAS is shaded, the transcription start site is indicated.

opa loci is conserved up to the –35 sequence of the putative promoter of the other *opa* genes (at –332 relative to *pip*), but *opaA* shares no further upstream sequence homology with the other 10 loci described in *N. gonorrhoeae* strain MS11 (Bhat *et al*., 1991). Like the other *opa* loci studied in an *E. coli* background, the translational start of *opaA* is out-of-frame with the mature protein. In addition, the *opaA* gene product was not seen on *in vitro* transcription/translation assays of pNA4, which contain the entire OpaA coding region (not shown). The *opaA* gene has been mapped to a 55 kb fragment on the chromosome of strain MS11-N198 (fragment nK; Bihlmaier *et al*., 1991), thus the *pip* gene was localized to this position on the gonococcal chromosome.

Transcription of pip *initiates near an* ntrA*-like promoter sequence in* E. coli

Examination of the region upstream of the translational start of *pip* revealed no significant homology to typical –10 or –35 consensus promoter sequences. However, sequences reminiscent of promoters requiring the sigma factor encoded by the *ntrA* gene (σ^{54}) product for transcription were identified. This sequence, upstream of *pip* (GG–N_{10}–TC) is identical to the NtrA-dependent *glnHp2* promoter in *E. coli* (Claverie-Martin and Magasanik, 1991), but not to the consensus sequence (GG–N_{10}–GC) which has been found at –24 and –12, with respect to the transcription initiation site in almost all the other NtrA-dependent systems (Kustu *et al*., 1989). The transcriptional start of *pip* was determined by primer extension on RNA isolated from *E. coli* carrying pNA4, which has 2 kb of gonococcal DNA 5' of the *pip* gene. The primer used in the extension shown in Fig. 5 is complementary to bases –9 to –26 in Fig. 2. The result of this analysis indicated that the 5' end of the transcript initiated at position –63 and that the *ntrA*-like sequences were positioned –16 and –28 relative to the start site. The *pip* promoter sequence shows good homology to the pilin gene promoters in both *N. gonorrhoeae* (Meyer *et al*., 1984) and *Pseudomonas aeruginosa*, and which in the latter case has been shown to be dependent on NtrA for its transcription (Ishimoto and Lory, 1989). NtrA-dependent genes require, in addition to the sequences at –24 and –12, the binding of an activator protein (NtrC, NifA homologue) to upstream sequences for transcription to be initiated (Kustu *et al*., 1989). A sequence similar to the consensus (G/A–N_7–TGT–N_4–T–N_5–ACA) for NifA binding was found –194 bp relative to the mRNA start site of *pip* (A–N_7–TGT–N_3–T–N_5–ACA).

Gonococcal Pip mutants have no residual proline iminopeptidase activity

To determine if the *pip* gene identified from the cloning experiments was responsible for Pip activity detected in gonococci, gonococcal mutants carrying defined lesions in the *pip* locus were constructed. pNAC was digested to release a fragment containing the entire *pip* gene, including the 700 bp CAT gene cassette. When gonococci were transformed with this DNA, resultant Cm-resistant transformants were found to lack Pip activity, as determined by the standard assay. This indicates that Pip activity is non-essential for growth in the background of a gonococcal proline prototroph. Chromosomal DNA was isolated from the transformants and analysed by Southern hybridization using the *Ssp*I fragment as the probe (Fig. 6). In comparison with the wild-type DNA, the *Ssp*I probe hybridized to a larger *Cla*I fragment in the two mutants, and because

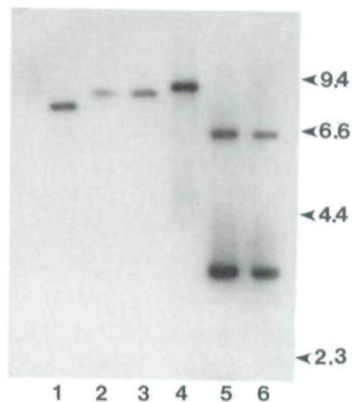

Fig. 6. Southern hybridization of gonococcal chromosomal DNA digested with *Cla* I (lanes 1–3) and *EcoR* I (lanes 4–6) from wild type (lanes 1 and 4) and Cm-resistant, Pip$^-$ transformants (lanes 2, 3, 5, 6). The filters were probed using the 1.5 kb *Ssp*I fragment from pNA15. The CAT cassette contains no *Cla*I sites and one *Eco*RI site. DNA size markers in kb are indicated.

of the *Eco*RI site within the CAT cassette, two fragments in the *Eco*RI digests in the mutants bound the probe. These results also show that only one copy of *pip* is present on the MS11 chromosome.

Pip releases biologically active proline from pro–gly

In addition to hydrolysing pro-pNA, the ability of Pip to hydrolyse the dipeptide pro–gly was investigated. An assay was devised based on the observation that *E. coli* proline auxotrophs that carry a mutation in the *dppA* (dipeptide permease) locus are unable to utilize the dipeptide pro–gly as a source of proline since they are unable to transport pro–gly into the cytoplasm (Olson *et al.*, 1991, Fig. 7B). These strains were transformed with vector (pACYC184, Fig. 7C) or Pip-expressing plasmids (pNAA2, Fig. 7D) and grown in M9 minimal media supplied with either proline or pro–gly as proline source. In comparison with the *proC* mutants (Fig. 7A), the *proC dppA* mutants were able to utilize pro–gly as a proline source only when transformed with Pip-expressing plasmids. This experiment might suggest that when expressed from a medium-copy-number plasmid (pACYC184 derivative), some Pip activity is located outside the cytoplasm in *E. coli*. This activity may instead be a result of the lysis of a few cells. Thus, stronger evidence points to the fact that Pip is a cytoplasmic protein in gonococci since cell fractions analysed for Pip activity indicated that the cytoplasm held the greatest activity of Pip per mg protein (not shown). Also, no typical *N*-terminal processing, as is common for proteins targeted for export from the cytoplasm, has been detected and the protein expressed in *E. coli* has the same molecular mass as the product identified in the *in vitro* transcription/translation assay.

In summary, we have cloned and characterized the gene encoding proline iminopeptidase from *N. gonorrhoeae*. Primer extension analysis indicated that the *pip* gene in *E. coli* is transcribed from an NtrA-like promoter and a sequence similar to the NifA-binding consensus sequence was located 194 bp upstream of the transcriptional start site of *pip*. Because of the close linkage between *opaA* and *pip*, we were also able to map *pip* on the MS11 chromosome. Pip$^-$ gonococci, totally lacking detectable proline iminopeptidase activity were constructed and found to be indistinguishable in growth rate from the wild-type strain. Future studies will be aimed at examining the significance of the limited amino acid homology between Pip and the family of hydrolases from *Pseudomonas* spp., determining if *pip* transcription is regulated in response to growth conditions, and at understanding the possible role of *pip* in proline metabolism during growth and survival in the host.

Experimental procedures

Bacterial strains and plasmids

N. gonorrhoeae strain MSO1-1X (Zhang *et al.*, 1992), a derivative of $MS11_{ms}$ was cultivated as previously described (Swanson *et al.*, 1985). *E. coli* strains HB101 and DH5α were used as recipients for recombinant plasmids. *E. coli* W3110 derivatives E1769 *(proC)* and E1614 (*proC* and *dppA*) were kindly supplied by E. Olson. Vectors used were pACYC184 (NEB) and pBluescript SK/KS II+ (pBSK, pBKS, Stratagene). *E. coli* were grown in LB medium (Sambrook *et al.*, 1989) or M9 minimal medium (Miller, 1982) at 37°C supplemented with chloramphenicol (10 μg ml^{-1}) or carbenicillin (50 μg ml^{-1}) and X-gal (0.8 mg per plate) when appropriate. *E. coli* and gonococcal cells were fractionated into periplasmic, cytoplasmic and membrane fractions according to Achtman *et al.* (1983).

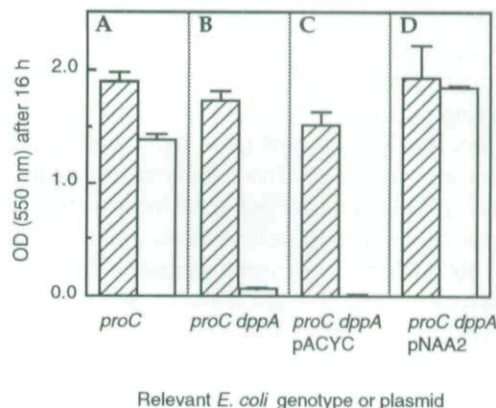

Fig. 7. Growth of *proC* (A) and *proC dppA* mutants of *E. coli* transformed with no (B), vector (C), or Pip-expressing (D) plasmids in M9 minimal media supplemented with proline (□) or pro–gly (□) at 0.4 mM.

Molecular cloning and sequencing procedures

DNA purifications, ligations, restriction analysis, gel electrophoresis, primer extension and Southern and Northern hybridizations were prepared according to Sambrook *et al.* (1989). RNA was extracted from gonococci according to Bergström *et al.* (1986). Double-stranded nested deletions were constructed using a kit from Pharmacia for that purpose. Templates for double-stranded DNA sequencing were prepared according to Ausubel *et al.* (1987), or by using Qiagen midi columns (Qiagen, Inc). The protocol for annealing primers to templates was performed according to Su *et al.* (1991). Briefly, 2 µg plasmid DNA in a microcentrifuge tube was dried in a speed vacuum concentrator. Two microlitres 0.20 N NaOH was added to the DNA pellet and the tube was maintained at room temperature for 2 to 3 min. The following were then added in sequence: 2.0 µl primer (2.5 ng µl^{-1}), 2.0 µl H_2O and 2.0 µl annealing buffer (833 mM Tris, pH 7.5, and 83 mM $MgCl_2$). After 2 min at room temperature, 2 µl 0.2 N HCl was added and the tube was incubated for 2 min at room temperature. DNA sequencing was performed directly on these samples using the Sanger dideoxy chain termination method with [^{35}S]-ATP and Sequenase (USB) according to the manufacturer's instructions. Sequences were stored, matched and analysed using MacVector software and the Wisconsin Genetics Computer Group package.

In vitro transcription/translation analysis of plasmids was performed using the Promega *E. coli* S30 coupled transcription/translation system. Non-radioactive assays were prepared by using the complete premix supplied in the kit.

One-dimensional SDS–PAGE

Conditions for SDS–PAGE and electroblotting were as described previously (Koomey *et al.*, 1991) using the Mini-Protean gel and transfer system (Hoeffer Scientific Instruments).

Two-dimensional SDS–PAGE and sample preparation for protein microsequencing

Cultures of *E. coli* DH5α carrying pNA15 were grown in LB to log phase and induced for Pip synthesis by the addition of 0.4 mM IPTG for 1 h. Cell extracts for two-dimensional polyacrylamide gels were prepared by the method of O'Farrell (1975) with modifications (VanBogelen *et al.*, 1990). After electrophoresis, the gels were soaked in transfer buffer (10 mM 3-[cyclohexylamino]-1-propanesulphonic acid, 10% methanol, pH 11.0) for 10 min to reduce the amount of Tris and glycine contaminations. Polyvinylidene diflouride (PVDF; Immobilon, Millipore) membranes were rinsed with 100% methanol and stored in transfer buffer. The gels, sandwiched between sheets of PVDF membrane and several sheets of Whatman paper, were assembled into a blotting apparatus and electroeluted for 30 min at 50 V (170–100 mA) in transfer buffer. The PVDF membranes were rinsed in sterile deionized water for 10 min, then stained with 0.1% Coomassie brilliant blue in 50% methanol for 5 min. After destaining (50% methanol, 10% acetic acid), and rinsing with deionized water, the protein spot representing Pip was identified and excised from the blot. Determination of the *N*-terminal amino acid sequence of the Pip protein was performed by automated Edman degradation directly on the membrane fragment (University of Michigan Medical School core facility).

Primer extension

Oligonucleotide primers complementary to bases −9 to −26 (Fig. 2) were end-labelled by using 50 µCi of [γ-^{32}P]-ATP (5000 Ci mmol^{-1}) and T4 polynucleotide kinase as described by Sambrook *et al.* (1989). Labelled primer (50 pmol) was mixed with RNA (60 µg) prepared from *E. coli* DH5α harbouring pNA4, heated to 85°C for 10 min and annealed at 50°C for 12 h. The primer:RNA hybrids were precipitated and resuspended for reverse transcriptase reactions as described by Sambrook *et al.* (1989). The primer extension reaction mixtures were subjected to electrophoresis next to a sequencing ladder generated using the same primer in 8% polyacrylamide gels containing 8 M urea followed by autoradiography.

Proline iminopeptidase assay

Proline iminopeptidase was assayed by monitoring the hydrolysis of the chromogenic substrate proline-*p*-nitroanilide (pro-pNA, Sigma). Gonococci or *E. coli* transformants were assayed for Pip activity by resuspending cells in 200 µl PBS containing 0.019 mM pro-pNA in a microtitre well and looking for the visible change to a yellow colour. For kinetic analysis, the rate of change in absorbance at 410 nm owing to the hydrolysis of pro-pNA was assayed and normalized to the amount of protein in the assay. Although proline iminopeptidase activity has been described in *E. coli* K-12 (Sarid *et al.*, 1959), this activity was too low to interfere with these studies. The Pip activity of proteins blotted to nitrocellulose from a one-dimensional SDS–PAGE gel was determined by taking 5 mm slices of the membrane and placing them in tubes containing 1.5 ml 10 mM Tris (pH 7.8) containing 0.019 mM pro-pNA. After 24 h, the absorbance at 410 nm was measured, and compared with the activity of the corresponding slice from cells harbouring only pBSK.

Functional assay of Pip on pro–gly

To determine if the hydrolysis of Pip on the dipeptide pro-gly resulted in free, biologically active proline, *E. coli* strains mutant in proline biosynthesis *(proC)* and uptake of dipeptides (*dppA)* were utilized. Strain E1772 carries both mutations and is therefore unable to grow on pro–gly as its sole source of proline (Olson *et al.*, 1991). Pip-expressing plasmids (pACYC184 derivative pNAA2) were transformed into E1772, and assayed for Pip activity by the hydrolysis of pro-pNA, and these strains were then grown in M9-glucose medium containing either proline (0.4 mM) or pro–gly (0.4 mM) as proline source. Cultures were grown aerobically for 16 h at 37°C and the optical density at 550 nm was measured.

Gonococcal transformation

Gonococci were transformed with linearized pNAC (10 µg ml^{-1}) by resuspending cells grown for 15 h on plates in preincubated (37°C, 5% CO_2) liquid medium (10^8 cells ml^{-1}) containing 7 mM $MgCl_2$. After incubation at 37°C for 30 min, the suspension was

diluted 30-fold and incubated for 5 h with tumbling to allow for expression of Cm resistance. Cultures were plated on Cm gradient plates and incubated at 37°C. Transformants were identified as large colonies in the area of high concentrations of Cm, and streaked onto Cm (5 $\mu g\ ml^{-1}$) plates. These were tested for Pip activity, and chromosomal DNA was prepared from them for Southern hybridization.

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