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PepN is the major aminopeptidase in *Escherichia coli*: insights on substrate specificity and role during sodium-salicylate-induced stress

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PepN and its homologues are involved in the ATP-independent steps (downstream processing) during cytosolic protein degradation. To obtain insights into the contribution of PepN to the peptidase activity in *Escherichia coli*, the hydrolysis of a selection of endopeptidase and exopeptidase substrates was studied in extracts of wild-type strains and two *pepN* mutants, 9218 and DH5αΔ*pepN*. Hydrolysis of three of the seven endopeptidase substrates tested was reduced in both *pepN* mutants. Similar studies revealed that hydrolysis of 10 of 14 exopeptidase substrates studied was greatly reduced in both *pepN* mutants. This decreased ability to cleave these substrates is *pepN*-specific as there is no reduction in the ability to hydrolyse exopeptidase substrates in *E. coli* mutants lacking other peptidases, *pepA*, *pepB* or *pepE*. PepN overexpression complemented the hydrolysis of the affected exopeptidase substrates. These results suggest that PepN is responsible for the majority of aminopeptidase activity in *E. coli*. Further *in vitro* studies with purified PepN revealed a preference to cleave basic and small amino acids as aminopeptidase substrates. Kinetic characterization revealed the aminopeptidase cleavage preference of *E. coli* PepN to be Arg > Ala > Lys > Gly. Finally, it was shown that PepN is a negative regulator of the sodium-salicylate-induced stress in *E. coli*, demonstrating a physiological role for this aminoendopeptidase under some stress conditions.

INTRODUCTION

The mechanisms involved in cytosolic protein degradation are central to regulating various aspects of cell biology, including cell cycle, disease progression, transcriptional regulation, antigen processing, stress responses, etc. Proteins targeted for degradation are unfolded and cleaved in an ATP-dependent manner to release large peptides, ranging from 3 to 25 amino acids (Maurizi, 1987; Coux *et al.*, 1996; Gottesman, 1996; Kisselev *et al.*, 1999; Zwickl *et al.*, 2000). These are further trimmed, cleaved and/or degraded by several endopeptidases, tri- and di-peptidyl peptidases (Tamura, T. *et al.*, 1996; Fukasawa *et al.*, 1998; Osmulski & Gaczynska, 1998; Tamura, N. *et al.*, 1998; Geier *et al.*, 1999; Wang *et al.*, 2000), aminopeptidases and carboxypeptidases (Conlin & Miller, 1995; Gonzales & Robert-Baudouy, 1996; Chandu & Nandi, 2002; Franzetti *et al.*, 2002) in an ATP-independent manner, also known as downstream processing. This general scheme of cytosolic protein degradation is conserved in all organisms, although the enzymes involved are distinct in different organisms. Most of the enzymes and their homologues that are upstream in the proteolysis pyramid, i.e. endopeptidases (e.g. Tricorn, tripeptidyl peptidase II, bleomycin hydrolase, thimet oligopeptidase), are present in selected organisms. However, the enzymes and their homologues involved in the latter steps of downstream processing, for example, leucine aminopeptidase and puromycin-sensitive aminopeptidase, are present in most organisms (Chandu & Nandi, 2002). Also, prokaryotes display greater redundancy than eukaryotes in the key enzymes involved in protein degradation. For example, 20S proteasomes, which are essential in eukaryotes (Coux *et al.*, 1996), are not essential in *Mycobacterium smegmatis* (Knipfer & Shrader, 1997). However, they are important only during heat shock in *Thermoplasma acidophilum* (Ruepp *et al.*, 1998). Similarly, Lon and Clp, the key ATP-dependent proteases in *Escherichia coli*, are not essential but are required during some stress conditions (Gottesman, 1996; Kuroda *et al.*, 2001).

Recently, characterizing enzymes involved in the ATP-independent process and studying their functional role has gained importance. Many of the studies have been done on archaeal and eukaryotic organisms. Enzymes involved in downstream processing, for example, tripeptidyl peptidase II, thimet oligopeptidase, bleomycin hydrolase, leucine aminopeptidase and puromycin-sensitive aminopeptidase,

Abbreviations: AMC, 7-amino-4-methylcoumarin; βNA, β-naphthylamide; Boc, butoxycarbonyl; Cbz, benzoyloxycarbonyl; NaSal, sodium salicylate; *p*NA, *p*-nitroanilide.

are also important in the trimming and degradation of major histocompatibility complex (MHC) class I binding peptides in mammals (Beninga *et al.*, 1998; Stoltze *et al.*, 2000; Saric *et al.*, 2001; York *et al.*, 2003). In *T. acidophilum*, this proteolytic process has been reconstituted *in vitro* (Tamura, N. *et al.*, 1998) and crystal structures have been determined for most of the key enzymes (Lowe *et al.*, 1995; Brandstetter *et al.*, 2001; Goettig *et al.*, 2002). Peptides released by 20S proteasomes are further degraded by Tricorn endoprotease to short peptides which are in turn broken down into amino acids by the Tricorn interacting exopeptidases, F1, F2 and F3. In fact, a model of the Tricorn–F1 complex suggests that the β7 propeller of Tricorn is used for substrate entry whereas the β6 propeller is for product egress and may act as a docking site for binding the Tricorn interacting factor F1 (Goettig *et al.*, 2002). Methionine aminopeptidases are essential in *E. coli* and *Saccharomyces cerevisiae*, demonstrating that cleavage of the N-terminal methionine from some proteins is critical for cellular function (Chang *et al.*, 1989; Bradshaw *et al.*, 1998). Peptidases in *Lactococcus lactis* are required for proteolysis and growth in milk (Mierau *et al.*, 1996). The turnover of cellular proteins is reduced in multiple-peptidase mutants, but not in single-peptidase mutants, in *Salmonella typhimurium* (Yen *et al.*, 1980) and *E. coli* (Miller & Schwartz, 1978; Conlin & Miller, 1995). Due to the redundancy of peptidases in prokaryotes, there is little information available on the physiological roles of these enzymes.

PepN, also known to be the sole alanine aminopeptidase in *E. coli*, was identified 28 years ago (Lazdunski *et al.*, 1975a, b). Recently, we initiated studies on enzymes involved in downstream processing in eubacteria. In the course of our studies, we identified PepN from *E. coli* to be responsible for cleaving Suc-LLVY-7-amido-4-methylcoumarin (AMC), a substrate cleaved by the 20S proteasome from all sources. PepN was further characterized with respect to its amino-endopeptidase activities (Chandu *et al.*, 2003). PepN and its homologues are well conserved in all kingdoms and play a role in downstream processing during cytosolic protein degradation. We wished to study, using synthetic peptidase substrates, the substrate specificity of pure PepN. In addition, we wished to evaluate the contribution of PepN to overall cellular peptidase activities in extracts of wild-type and *pepN* mutant strains of *E. coli*. Previous studies had failed to demonstrate a phenotype for *E. coli* strains lacking *pepN* (McCaman *et al.*, 1982; Bally *et al.*, 1983). Therefore, we wished to investigate the role of PepN during different stress conditions. In this study, we demonstrate that PepN is responsible for the majority of the aminopeptidase activity in *E. coli* and that it prefers to cleave basic and small amino acids at the amino terminus of substrates. Furthermore, we show that PepN plays a negative role during sodium salicylate (NaSal)-induced stress.

METHODS

Strains, plasmids and overexpression of PepN. All *E. coli* strains used in the study were maintained in Luria–Bertani (LB) medium in the absence or presence of appropriate antibiotics: ampicillin (100 μg ml^{-1}), tetracycline (30 μg ml^{-1}) and kanamycin (30 μg ml^{-1}). *E. coli* 9218 is an *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine-derived *pepN* mutant (Bally *et al.*, 1983) and *E. coli* DH5α*ΔpepN*, a targeted deletion mutant of *pepN*, has been described previously (Chandu *et al.*, 2003). *pepN* was overexpressed from pBM15, a pBR322-based vector harbouring *pepN* under the control of its endogenous promoter (Bally *et al.*, 1984). In another construct, the *E. coli* K-12 *pepN* open reading frame was PCR-amplified and cloned into pBAD24, an L-arabinose-inducible vector, and referred to as pBAD/K12*pepN* (Chandu *et al.*, 2003). *pepN* was PCR-amplified from *E. coli* 9218 using gene-specific primers (Chandu *et al.*, 2003) and cloned under the control of the L-arabinose-inducible promoter of pBAD24; this construct is referred to as pBAD/9218*pepN* in the text. Strain DH5α*ΔpepN*(pBAD24, pBAD/K12*pepN* or pBAD/9218*pepN*) was grown in the presence of 0·4–2 mg L-arabinose ml^{-1} for overexpression studies. The DNA sequence of K12*pepN* and 9218*pepN* was determined using M13 and *pepN*-specific primers. *E. coli* strains with Tn*5* insertions in their peptidase genes (*pepA*, *pepB* and *pepE*), constructed in *E. coli* MG1655, were obtained from the Mutant strain collection, *E. coli* Genome Project, University of Wisconsin – Madison, WI, USA (http://www.genome.wisc.edu/).

Generation of antiserum to PepN and Western analysis. Antiserum was raised against purified PepN by injecting 200 μg of the pure protein per rabbit as an emulsion with complete Freund's adjuvant subcutaneously. Two booster injections of 100 μg PepN per rabbit were given with incomplete Freund's adjuvant, after gaps of 2 weeks. One week after the second booster, the serum was obtained and stored at −70 °C until further use. This antiserum against PepN specifically detects the enzyme from purified preparations as well as crude extracts, by both ELISA and Western blotting. However, pre-immune serum collected from the same rabbit before PepN immunization displayed negligible reactivity (data not shown). Western analysis was performed by separating the cellular extracts on a 10 % SDS-PAGE gel and transferring them to a nitrocellulose membrane. After blocking overnight with 1 % gelatin in 50 mM PBS (0·01 M NaH_2PO_4, 0·04 M Na_2HPO_4, 0·15 M NaCl, pH 7·4), the pre-immune serum and PepN-specific antiserum were used at a dilution of 1 : 10 000. Goat anti-rabbit antibody conjugated to horseradish peroxidase (Bangalore Genei) was used as secondary antibody (1 : 2000 dilution). Antibodies bound to PepN were visualized by staining with hydrogen peroxide and 3,3′-diaminobenzidine (Sigma) in 50 mM PBS. This antiserum specifically recognized PepN in cytosolic extracts from wild-type *E. coli* but not in cytosolic extracts from two strains lacking *pepN*, namely, 9218 and DH5α*ΔpepN* (Fig. 2a, b). Also, no cross-reactivity was found in cytosolic extracts from *M. smegmatis* or mouse liver with this antiserum (data not shown).

PepN purification, substrate hydrolysis assays and kinetic characterization. Overnight cultures of *E. coli* (OD_{600} of ∼2) were used as 0·5 % (v/v) inoculum and were grown for ∼10 h at 37 °C, washed, sonicated and centrifuged at 100 000 ***g*** for 1 h at 4 °C to obtain cell-free extracts. For experiments using the pBAD24 system, cultures (1 % inoculum) were grown for 4 h in the presence of appropriate amounts of L-arabinose. Assays with cellular extracts were performed using 25 μg total protein, for both aminopeptidase and endopeptidase substrate hydrolysis. Results shown are representative of at least two independent extract preparations. PepN was purified from *E. coli* DH5α transformed with pBM15 using conventional chromatographic procedures, as described previously (Chandu *et al.*, 2003). Single-band purity was checked by separating PepN on SDS-PAGE and staining with Coomassie Brilliant blue R-250. Enzyme assays were performed as described previously (Chandu *et al.*, 2003). Briefly, endopeptidase (0·5 mM) and aminopeptidase (1 mM) substrates were incubated with purified PepN in 20 mM phosphate

buffer, pH 8·0, at 37 °C. The endopeptidase substrates (Sigma) used in the study, with cleavage specificity in parentheses, are as follows: Boc-Leu-Arg-Arg-AMC (trypsin-like enzymes), Cbz-Ala-Arg-Arg-AMC (trypsin-like enzymes), Cbz-Gly-Gly-Leu-β-naphthylamide (βNA) (ClpYQ protease substrate), Cbz-Leu-Leu-Glu-βNA (post-glutamyl peptidyl hydrolytic activity), Suc-Ala-Ala-Phe-AMC (chymotrypsin-like enzymes), Suc-Leu-Leu-Val-Tyr-AMC (chymotrypsin-like enzymes), Suc-Leu-Tyr-AMC (porcine calpain I- and II-like enzymes). The tripeptidyl and exopeptidase substrate used was Ala-Ala-Phe-AMC and the aminopeptidase substrates used were L-Ala-*p*-nitroanilide (*p*NA), L-Arg-AMC, L-Asp-βNA, L-Asn-βNA, L-Gly-*p*NA, L-Leu-*p*NA, L-Lys-*p*NA, L-Met-*p*NA, L-Phe-*p*NA, L-Pro-βNA, L-Thr-AMC, L-Tyr-AMC and L-Val-βNA. Purified PepN was used at 1 μg for 2 h and 7·5 ng for 1 h for endopeptidase and aminopeptidase assays, respectively, and reactions were terminated with 100 % ethanol. Free AMC or βNA released after cleavage of peptide substrates was measured, using a spectrofluorimeter (Shimadzu), with excitation wavelengths of 370 and 335 nm and emission wavelengths of 430 and 410 nm for AMC-based and βNA-based substrates, respectively. Similar assays with chromogenic substrates were performed and free *p*NA released was measured by studying the absorbance at 410 nm in the spectrophotometer (Shimadzu). The net increase in AMC or *p*NA released was calculated after subtraction with appropriate controls. Standard curves were plotted using known amounts of free AMC or *p*NA to calculate the amount released. Protein amounts were calculated using Bradford's reagent and known amounts of BSA (Sigma) were used to generate a standard plot. Specific activity was calculated as nanomoles of AMC or *p*NA released per microgram of protein per hour at 37 °C. Kinetic characterization of a particular substrate was done by performing enzyme assays with different concentrations of the substrate. Kinetic constants were obtained from the direct linear plots (Eisenthal & Cornish-Bowden, 1974).

NaSal-induced stress. Single colonies of different *E. coli* strains were grown in 3 ml LB medium overnight, with appropriate antibiotics. Cell-free extracts of the strains were prepared after sonication and assayed for hydrolysis of Suc-LLVY-AMC or L-Ala-*p*NA, to confirm the authenticity of the cultures, as a standard practice. Each tested culture was streaked on one quadrant of appropriate plates and incubated at 37 °C in the absence or presence of increasing amounts of NaSal. In addition, a plate streaked with different strains was incubated at 42 °C to study the effect of growth at high temperature. After growth for ~10 h, the images of plates were obtained with the ALPHADIGIDOC documentation system (San Leandro, CA, USA).

RESULTS

Hydrolysis of peptidase substrates by pure PepN

First, the cleavage specificity of pure PepN was studied, using a panel of endopeptidase and aminopeptidase substrates. PepN hydrolysed three of the seven endopeptidase substrates tested (Fig. 1a). Interestingly, pure PepN hydrolysed eight out of the 13 aminopeptidase substrates tested (Fig. 1b), confirming that PepN is a broad-specificity aminopeptidase (McCaman & Villarejo, 1982). However, pure PepN hydrolysed basic (arginine and lysine) and small (alanine and glycine) amino acid substrates better than the rest (Fig. 1b), suggesting a preference for hydrolysis of these aminopeptidase substrates.

To obtain further insights into the cleavage preference of

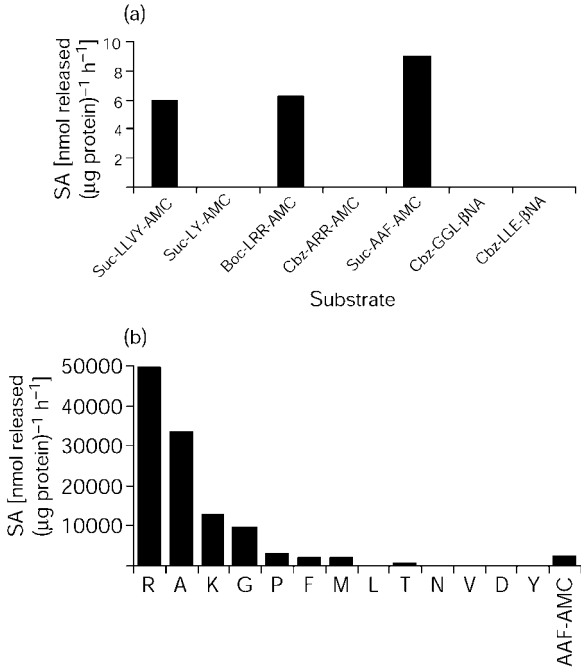

Fig. 1. PepN is a broad-specificity aminopeptidase. PepN was purified biochemically and assayed for its ability to hydrolyse a panel of endopeptidase (a) or exopeptidase (b) substrates. Pure PepN (1 μg) was incubated with different endopeptidase substrates (0·5 mM). To study the hydrolysis of exopeptidase substrates (1 mM), 7·5 ng of PepN was used. *x*-Axis labels in (b) represent standard single letter code for amino acids, which are present N terminus to the peptide bond hydrolysed. The free AMC, βNA or *p*NA released from these peptide substrates after hydrolysis by PepN was measured in a spectrofluorimeter (AMC and βNA) or spectrophotometer (*p*NA). SA, specific activity. Results shown are representative of at least two independent enzyme preparations.

PepN for synthetic substrates, kinetic characterization of selected peptidase substrates was performed (Table 1). Kinetic characterization revealed that PepN displays higher k_{cat}/K_m values for exopeptidase substrates than endopeptidase substrates. In our previous report (Chandu *et al.*, 2003), we demonstrated that PepN is a better aminopeptidase than endopeptidase using L-Ala-*p*NA and Suc-LLVY-AMC. As these substrates were not similar, we selected the exopeptidase substrate AAF-AMC and its cognate endopeptidase substrate Suc-AAF-AMC for kinetic comparison. Although the K_m values of these substrates were comparable, PepN hydrolysed AAF-AMC with an ~180-fold higher k_{cat}/K_m value than Suc-AAF-AMC (Table 1). These results corroborate our previous observation that PepN is a better exopeptidase than endopeptidase. Significantly, the sole alanine aminopeptidase of *E. coli*, PepN, displayed an ~8-fold higher k_{cat}/K_m value for arginine compared to alanine at the P1 position (i.e. the amino acid present N-terminal to the peptide bond that is hydrolysed by proteases/peptidases) of aminopeptidase substrates (Table 1). The preference of

Table 1. Kinetic characterization of peptidase substrate hydrolysis by pure PepN

Cleavage of different peptide and amino acid substrates by pure PepN was performed with varying concentrations of substrates. Kinetic parameters were calculated from the direct linear plots. Data are representative of two to six independent enzyme preparations and are shown ±SE.

Substrate	Kinetic parameter			
	K_m (μM)	V_{max} (μM s^{-1})	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} μM^{-1})
L-Arg-AMC	109±19·0	0·069±0·001	946·75	8·6800
L-Ala-*p*NA	638·6±13·8	0·05±0·0031	692·10	1·0830
L-Lys-*p*NA	533·33±40·8	0·013±0·005	181·60	0·3400
L-Gly-*p*NA	675±75·03	0·006±0·00002	84·70	0·1250
AAF-AMC	559·5±31·5	0·5±0·08	51·75	0·0924
Suc-LLVY-AMC	40·8±1·25	0·0007±0·00006	0·08	0·0019
Suc-AAF-AMC	339±39	0·001±0·0002	0·19	0·0005

PepN for arginine resulted from the high affinity for this substrate (low K_m) compared to alanine. Kinetic parameters demonstrated a hierarchy of preference for hydrolysis of aminopeptidase substrates: Arg > Ala > Lys > Gly. Albeit the broad specific nature, kinetic characterization revealed that PepN displayed a preference for basic and small amino acids as P1 residues in aminopeptidase substrates.

Characterization and mapping of the mutation in *pepN* from *E. coli* 9218

In addition to displaying aminoendopeptidase activities (Chandu *et al.*, 2003), our current results with pure PepN demonstrated that it has the ability to hydrolyse a wide variety of aminopeptidase substrates. This prompted us to check the contribution of PepN for overall peptidase activity in cell-free extracts of two *pepN* mutants, 9218 and DH5αΔ*pepN*, with their respective parent strains. We demonstrated the construction of strain *E. coli* DH5αΔ*pepN*, with a targeted deletion in *pepN* and lacking PepN activity, previously (Chandu *et al.*, 2003). *E. coli* 9218, an *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine-derived *pepN* mutant, was isolated in 1983; however, the nature of the mutation was not identified at that time (Bally *et al.*, 1983). In this report, we wished to determine the nature of the *pepN* mutation in strain 9218. Towards this goal, we addressed whether PepN was detected in cytosolic extracts in different *E. coli* strains, by Western blotting using a PepN-specific antiserum. PepN expression was detected at low levels in the wild-type strain *E. coli* DH5α and the intensity of PepN increased in extracts from DH5α strains transformed with pBM15, the vector overexpressing PepN (Fig. 2a). Significantly, this band was not detected in extracts of strain 9218; however, PepN was detected in extracts of strain 9218 transformed with pBM15. Together, these results demonstrated that the band recognized by the antiserum was specific to PepN as it was detected in wild-type and in extracts overexpressing PepN but was not detected in *pepN* mutant strain 9218. To address the molecular nature of the mutation in strain 9218, we PCR-amplified and cloned *pepN* from 9218 under the control of the L-arabinose-inducible promoter pBAD24, referred to as pBAD/9218*pepN*. pBAD/K12*pepN* or pBAD/9218*pepN* was transformed into *E. coli* DH5αΔ*pepN* and induced with 2 mg L-arabinose ml^{-1} for 4 h. Previously, we demonstrated that PepN is responsible for the hydrolysis of Suc-LLVY-AMC and L-ala-*p*NA in *E. coli* (Chandu *et al.*, 2003). Accordingly, these activities were not detected in extracts of *E. coli* DH5αΔ*pepN* transformed pBAD24.

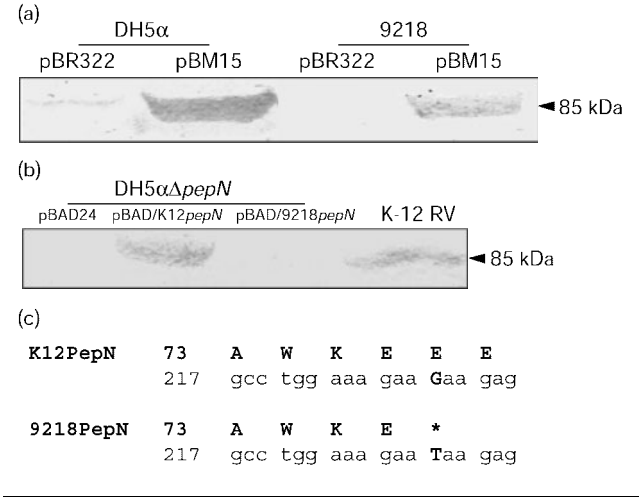

Fig. 2. Characterization and mapping of the *pepN* mutation in *E. coli* 9218. (a) Cytosolic extracts of *E. coli* DH5α and the *pepN* mutant 9218 transformed with pBR322 or pBM15, the vector overexpressing PepN, were prepared and subjected to Western analysis using the PepN-specific antiserum. (b) *pepN* was PCR-amplified from *E. coli* strains K-12 RV and 9218, cloned and expressed in the L-arabinose-inducible pBAD system. Wild-type PepN is represented as pBAD/K12*pepN* and 9218 PepN is represented as pBAD/9218*pepN*. The *E. coli* strain with a targeted deletion in *pepN*, DH5αΔ*pepN*, was transformed with pBAD24, pBAD/K12*pepN* or pBAD/9218*pepN* and the L-arabinose-induced extracts were analysed for PepN expression by Western analysis. (c) Amino acid and nucleotide sequences of *pepN* from *E. coli* K-12 RV and *E. coli* 9218 are represented.

Overexpression of wild-type K12*pepN* restored the ability to hydrolyse both Suc-LLVY-AMC and L-ala-*p*NA. However, overexpression of 9218*pepN* failed to restore the ability to hydrolyse both these substrates. Transformation and induction of all the three constructs displayed a similar ability to hydrolyse a control peptide substrate, Cbz-LLE-βNA (data not shown). These activity data have been further corroborated by Western analysis in Fig. 2(b). As shown in Fig. 2(a), PepN was detected at high levels on overexpression; therefore, we attempted to study the expression of wild-type K-12 and mutant 9218 PepN. PepN expression was not detected in the strain lacking *pepN*, DH5$\alpha\Delta$*pepN*, transformed with vector alone, pBAD24 (Fig. 2c). However, induction of wild-type PepN (pBAD/K12*pepN*), but not 9218 PepN (pBAD/9218*pepN*), resulted in detection of PepN expression in DH5$\alpha\Delta$*pepN*. It is most likely that detection of PepN activity is a more sensitive method compared to detection of PepN by Western analysis (Fig. 2b). Together, activity and expression data suggested that full-length PepN was not synthesized by 9218*pepN*. Finally, DNA sequence analysis of K12*pepN* and 9218*pepN* demonstrated that 9218*pepN* differed from the wild-type by a transversion of nucleotide 229 from G to T, resulting in Glu-77 of wild-type PepN being replaced by a stop codon (Fig. 2c). Significantly, the putative active site residues of PepN (Gly-261 to Glu-320) are present downstream to this mutation site. Previously, based on agar-based antigen–antibody precipitation studies, PepN in 9218 was thought to be synthesized (Latil *et al.*, 1976). It is not clear whether those results were due to cross-reactivity to truncated PepN in 9218 or non-specific reactivity. By PCR-amplifying *pepN* from strain 9218 and studying its expression and sequence, we demonstrated that PepN in 9218 is truncated due to a mutation that results in a premature stop codon. This protein is probably unstable and degraded rapidly, resulting in loss in ability to detect protein and enzymic activity (Fig. 2a–c).

Endopeptidase substrate hydrolysis profile of *pepN*-deficient *E. coli* extracts

Next, we studied the contribution of PepN to the overall endopeptidase activity in *E. coli*. The hydrolysis of a panel of endopeptidase substrates was studied in cytosolic extracts of the two *pepN* mutants, 9218 and DH5$\alpha\Delta$*pepN*, and their respective wild-type strains, K-12 RV and DH5α (Fig. 3a). The hydrolysis of three endopeptidase substrates, Suc-LLVY-AMC, Suc-AAF-AMC and Boc-LRR-AMC (partial effect), was reduced (Fig. 3a) in extracts of both *pepN* mutants. Overexpression of PepN in *E. coli* 9218 with pBM15 rescued all three activities (Fig. 3b). The rescue of Boc-LRR-AMC was partial as PepN and another uncharacterized serine peptidase (data not shown) were responsible for its hydrolysis. Although we were unable to infer any cleavage preference for endopeptidase substrates by PepN, these results demonstrated that PepN is responsible for hydrolysis of some endopeptidase substrates. As both the mutants were generated in different genetic backgrounds,

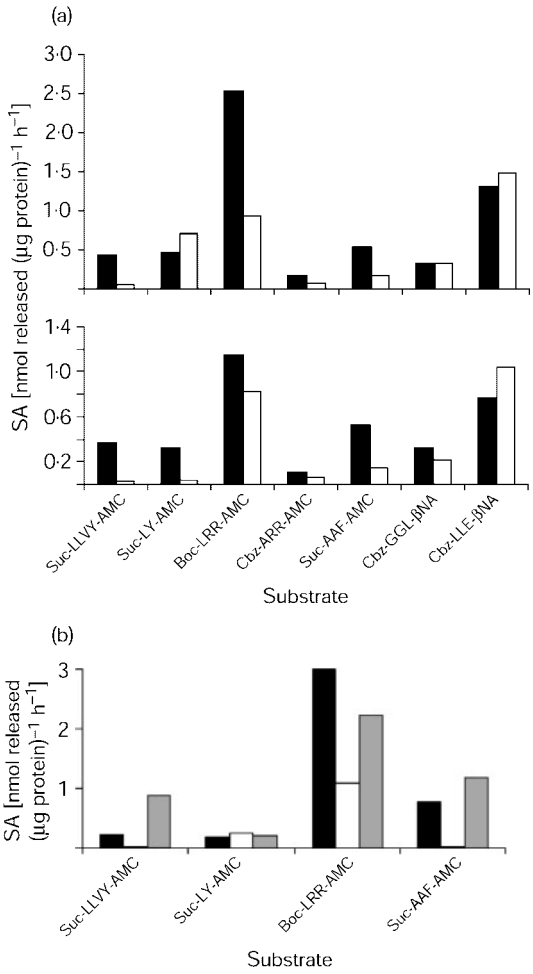

Fig. 3. Hydrolysis profile of endopeptidase substrates. (a) Cytosolic extracts of *E. coli* strains K-12 RV (top, solid bars), 9218 (top, open bars), DH5α (bottom, solid bars) and DH5$\alpha\Delta$*pepN* (bottom, open bars) were tested for their ability to hydrolyse a panel of endopeptidase substrates. (b) PepN overexpression under its endogenous promoter in *E. coli* 9218 [9218(pBM15), grey bars] compensated the reduced activities in *E. coli* 9218(pBR322), white bars. *E. coli* K-12 RV, black bars. SA, specific activity.

effects that were observed in both mutants only were considered as significant.

PepN is the major aminopeptidase in *E. coli*

To determine the contribution of PepN to the overall aminopeptidase activities in *E. coli*, we studied the hydrolysis of a panel of aminopeptidase substrates and an exopeptidase/tripeptidyl peptidase substrate, AAF-AMC, in wild-type and *pepN* mutant strains K-12 RV and 9218 (Fig. 4a, upper) and DH5α and DH5$\alpha\Delta$*pepN* (Fig. 4a, lower). Surprisingly, the hydrolysis of nine out of 13 aminopeptidase substrates was reduced in extracts of *pepN* mutants 9218 and DH5$\alpha\Delta$*pepN* compared with the extracts

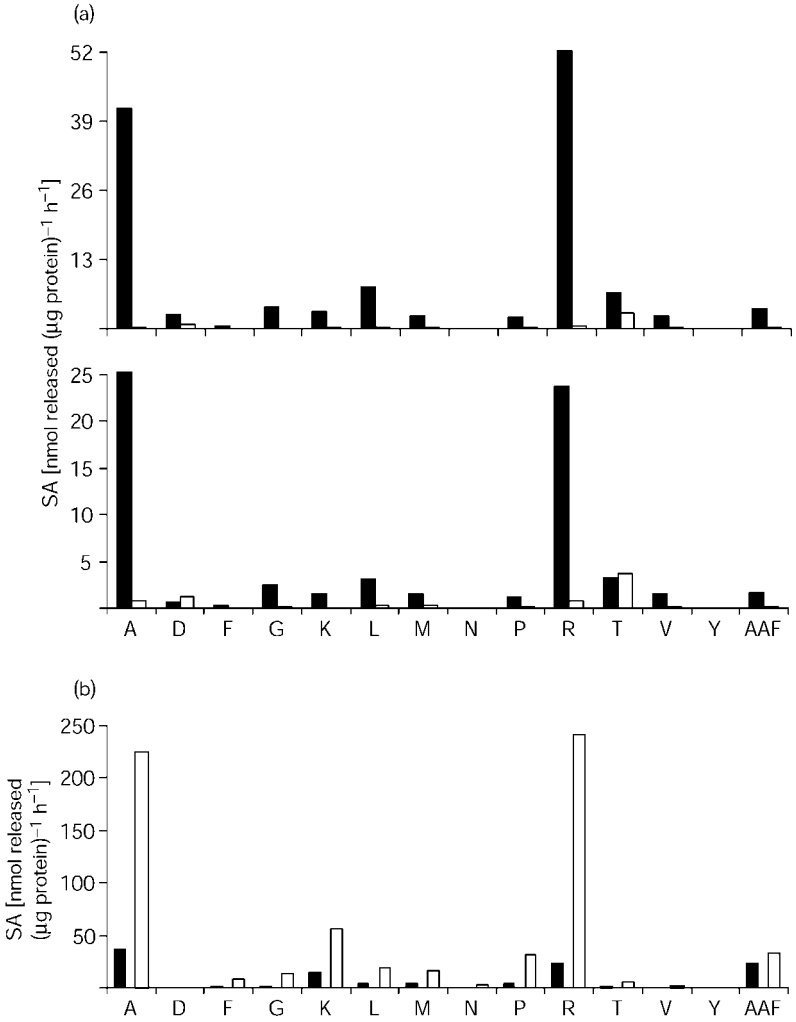

Fig. 4. PepN is responsible for the majority of aminopeptidase activity in *E. coli* extracts. (a) *E. coli* K-12 RV (top, solid bars) and DH5α (bottom, solid bars) with the respective *pepN*-deficient strains *E. coli* 9218 (top, open bars) and DH5$\alpha\Delta$*pepN* (bottom, open bars) were assayed with a panel of exopeptidase substrates. (b) Extracts of *E. coli* 9218(pBM15), white bars, were tested for ability to compensate the reduced aminopeptidase activities in *E. coli* 9218(pBR322), grey bars. As expected, extracts from *E. coli* 9218(pBR322) displayed negligible activity. *E. coli* K-12 RV, black bars. Standard single letter code is used for amino acids in the P1 position of aminopeptidase substrates. SA, specific activity.

of the respective parental strains. Similar loss in hydrolysis was also observed with AAF-AMC, an exopeptidase and tripeptidyl peptidase substrate. PepN overexpression in 9218 by transforming with pBM15 resulted in recovery of all the lost activities (Fig. 4b), suggesting that PepN is responsible for the majority of the aminopeptidase activity in *E. coli* extracts. Importantly, there was no major loss in hydrolysis of any of the aminopeptidase substrates tested in extracts of peptidase *pepA*, *pepB* or *pepE* mutant strains compared to the isogenic wild-type strain MG1655 (Fig. 5). Hence, the loss in ability to hydrolyse most of the exopeptidase substrates is specific to *pepN*.

pepN deficiency confers resistance to NaSal-induced stress

Previous studies failed to reveal a physiological role for PepN (McCaman *et al.*, 1982; Bally *et al.*, 1983). Recently, genome-wide transcriptional profiling identified induction of the *pepN* transcript on treatment with NaSal (Pomposiello *et al.*, 2001) but not heat shock (Richmond *et al.*, 1999). These observations led us to asses the role of PepN during stress using a genetic approach. *E. coli* strains

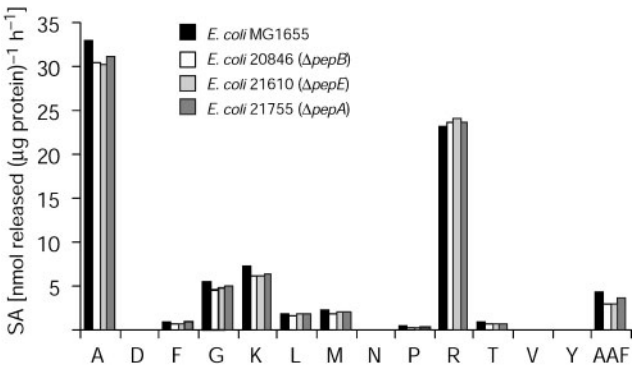

Fig. 5. *E. coli* PepA, PepB and PepE do not contribute significantly to cleave the exopeptidase substrates tested. Cellular extracts of mutants in *E. coli* peptidases *pepA*, *pepB* and *pepE* were tested for their ability to hydrolyse the panel of exopeptidase substrates. The parent strain *E. coli* MG1655 was used as a control. Standard single letter code for amino acid residues at P1 position of aminopeptidase substrates is represented. SA, specific activity.

DH5α and DH5αΔ*pepN* with or without PepN overexpression were subjected to different stress conditions, i.e. growth at high temperature (42 °C) or growth at 37 °C in the presence of increasing amounts of NaSal. The Δ*pepN* strain grew better than its isogenic wild-type strain in the presence of increasing amounts of NaSal (top two quadrants, Fig. 6a). However, both DH5α and DH5αΔ*pepN* strains overexpressing PepN displayed significant loss in ability to grow in the presence of NaSal (bottom two quadrants, Fig. 6a). To test if the observed effect was strain-specific, *E. coli* 9218 was subjected to growth in the presence NaSal. As observed in Fig. 6(b), *E. coli* 9218 transformed with pBAD24 displayed significantly better growth compared to 9218/pBADK12*pepN*, overexpressing wild-type K-12 PepN (Fig. 6b) from the L-arabinose-inducible promoter (Chandu *et al.*, 2003). Among different stress conditions tested, the role of PepN in NaSal-induced stress was prominent and consistent. Our results using two different strains of *E. coli* and two different expression systems (one constitutive and the other inducible) demonstrate clearly that PepN acts as a negative modulator during NaSal-induced stress.

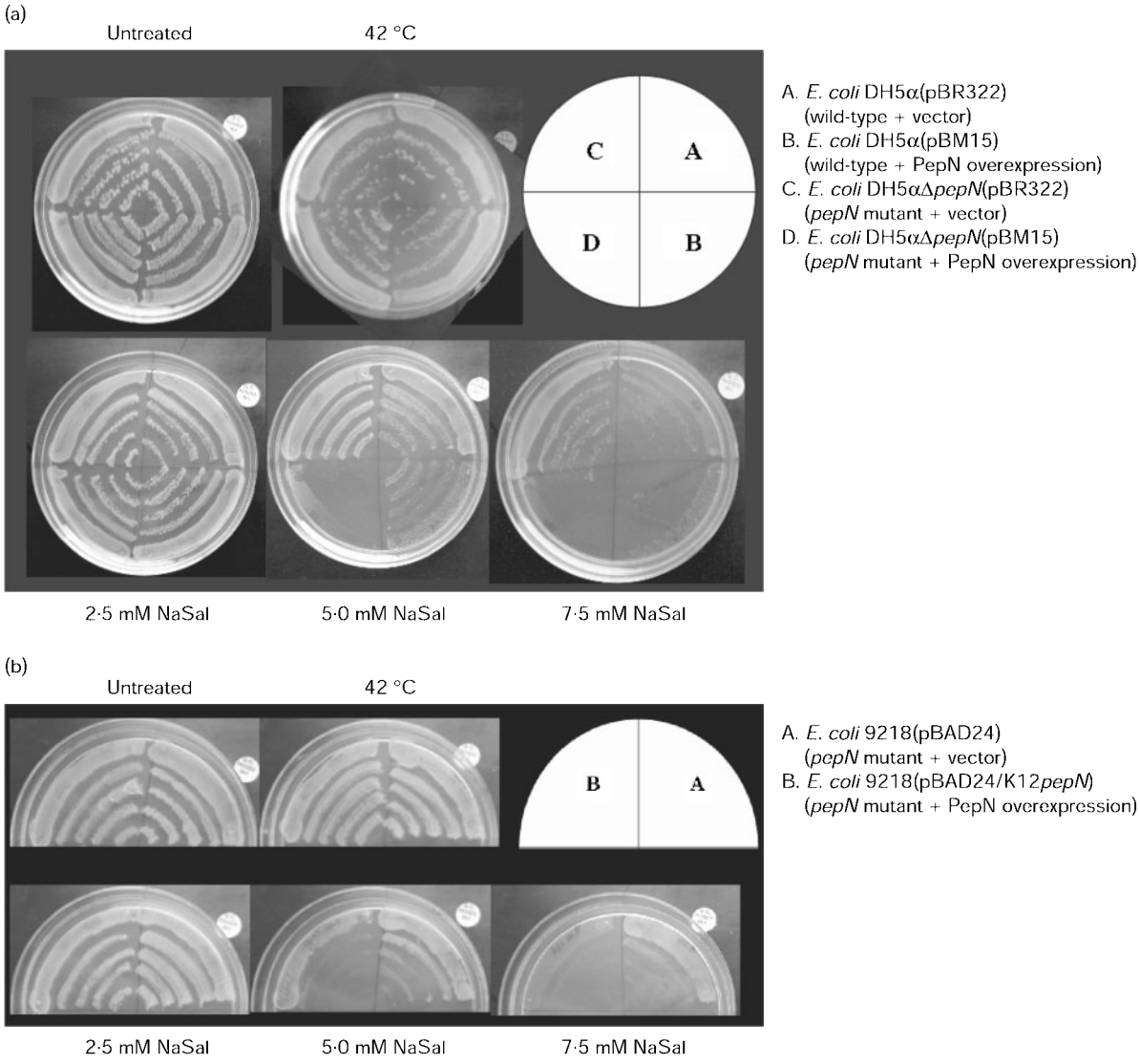

Fig. 6. PepN expression negatively modulates the response of *E. coli* to NaSal-induced stress. (a) *E. coli* DH5α and DH5αΔ*pepN* transformed with vector pBR322 or pBM15, overexpressing PepN, were plated onto LB agar plates supplemented with 30 μg tetracycline ml^{-1} and subjected to growth under different conditions. (b) *E. coli* 9218 transformed with pBAD24 or pBAD24/K12*pepN* was grown in the presence of 100 μg ampicillin ml^{-1}, 400 μg L-arabinose ml^{-1} and varying concentrations of NaSal. The schematic diagrams of the strains in different quadrants are also represented. The results are representative of multiple experiments.

DISCUSSION

Using genetic and biochemical approaches, we evaluated the contribution of PepN to the overall peptidase activity in *E. coli*. Importantly, our studies demonstrate that PepN, but not PepA, PepB or PepE, is responsible for the majority of aminopeptidase activity in *E. coli*. On the one hand, it is important to point out that the profiles obtained with purified enzyme reflect the ability of the enzyme to hydrolyse a particular substrate. On the other hand, profiles obtained with extracts from wild-type and mutant strains reflect the contribution of the enzyme for overall cellular hydrolysis. Pure PepN cleaved Boc-LRR-AMC as efficiently as Suc-LLVY-AMC (Fig. 1), whereas results with extracts revealed that PepN is the major Suc-LLVY-AMC-hydrolysing enzyme in *E. coli* (Chandu *et al.*, 2003) although it contributes partially to the hydrolysis of Boc-LRR-AMC (Fig. 3). Similarly, it is not clear as to the reason for the PepN-specific cleavage of Suc-LY-AMC by strain DH5α but not by strain 9218 (Fig. 3). However, purified PepN does not cleave Suc-LY-AMC (Fig. 1a). Therefore, comparison of the profiles obtained with both mutant and wild-type extracts, together with data obtained with the pure enzyme, is important in assessing the overall role of PepN in cleaving selected substrates. It is possible that the primary reason for PepN to constitute the majority of aminopeptidase activity in *E. coli* is the high steady-state levels of this constitutive enzyme. Consequently, the inability to detect differences in cleaving the panel of exopeptidase substrates by PepA, PepB and PepE (Fig. 5) suggests that the steady-state levels of these enzymes are low in *E. coli* grown in LB medium. Based on the ability to activate an antibiotic, albomycin, a study had predicted that PepA levels are much lower than PepN in *E. coli* (Braun *et al.*, 1983). Finally, a study has shown that PepN is responsible for ~1 % of total cellular protein in *E. coli* K-12 (McCaman & Villarejo, 1982).

Previously, PepN was known to cleave Ala, Lys, Gly, Leu (McCaman & Villarejo, 1982) and the dipeptide cysteinyl-glycine (Suzuki *et al.*, 2001). The wide range of substrates used in this study has not been used before and our systematic analysis of the cleavage preference of PepN revealed that it is a broad-specificity aminopeptidase, which cleaves basic and small amino acids substrates better than others (Fig. 1). There are few enzymes that can cleave proline-containing peptides; this activity is performed by dedicated enzymes, known as proline iminopeptidases and prolidases (Vanhoof *et al.*, 1995). Interestingly, PepN cleaves L-Pro-βNA, a proline aminopeptidase substrate. Although PepN is known as the sole alanine aminopeptidase, it cleaves arginine better than alanine. However, the alanine/arginine aminopeptidase-1 (Aap-1), the PepN homologue in *Saccharomyces cerevisiae*, cleaves both Arg and Ala (Caprioglio *et al.*, 1993). PepN homologues from *L. lactis* (van Allen-Boerrigter *et al.*, 1991), *Streptococcus thermophilus* (Chavagnat *et al.*, 1999) and *Aspergillus niger* (Basten *et al.*, 2001) are, primarily, lysine aminopeptidases. In general, aminopeptidases involved in protein degradation act on short, not long, peptides (Tamura, N. *et al.*, 1998; Franzetti *et al.*, 2002). It is possible that PepN preferentially cleaves peptides with basic or small amino acids as amino-terminal residues. Given the specificity of PepN, it is interesting to speculate that, perhaps, other aminopeptidases may be specific for hydrolysis of acidic amino acids, for example, PepB and/or PepE (Larsen *et al.*, 2001). This hypothesis may be justified as the cleavage specificities of aminopeptidases F1, F2 and F3 in *T. acidophilum* are distinct: F1 is a proline iminopeptidase, F2 prefers basic amino acid substrates and F3 prefers acidic amino acid substrates (Tamura, N. *et al.*, 1998).

Although there is some information on the role of ATP-dependent proteases, for example, Lon and Clp, under stress conditions (Gottesman, 1996; Kuroda *et al.*, 2001), not much is known about the physiological role of ATP-independent peptidases. PepA, PepB, PepD and PepN are thought to be redundant and deficiency in all four peptidases is required to demonstrate an effect on cytosolic protein turnover (Miller & Schwartz, 1978; Yen *et al.*, 1980). There is a linear relationship between PepN activity and bacterial growth (Bally *et al.*, 1983) and *pepN* transcription is induced on phosphate starvation, anaerobic conditions and growth in minimal medium (Gharbi *et al.*, 1985). As the significance of these observations is unclear, we resorted to a genetic approach to study the role of PepN under different conditions. The observation that the *pepN* transcript is induced on NaSal treatment (Pomposiello *et al.*, 2001), but not heat shock (Richmond *et al.*, 1999), prompted us to study the role of PepN under different stress conditions. As shown in Fig. 6, the lack of PepN resulted in increased growth, whereas PepN expression clearly reduced the growth of *E. coli*, in the presence of NaSal. Thus, PepN is a negative regulator of NaSal-induced stress. The observation that *pepN* is induced on NaSal treatment and our current result that PepN is a negative regulator may at first appear to be inconsistent. However, modulation of gene expression, especially microarray data, needs to be confirmed using genetic and/or biochemical approaches (Slonim, 2002). Also, NaSal inactivates the repressor *marR* and activates *marA*; however, both these genes are induced on NaSal treatment (Pomposiello *et al.*, 2001). The use of genetic mutants and overexpression studies, as shown in Fig. 6, is the appropriate approach to address the role of *pepN* under stress. NaSal does not modulate the activity of pure PepN and no difference in PepN activity was found in extracts of untreated or NaSal-treated *E. coli* (data not shown). Notably, *pepN* homologues in other organisms are known to play distinct roles. *L. lactis* lacking *pepN* displayed growth reduction by 20 % in medium containing casein as carbon source (Mierau *et al.*, 1996). *Saccharomyces cerevisiae* lacking *aap-1*, a *pepN* homologue, accumulates less glycogen, whereas, on transforming Δ*aap-1* cells with *aap-1*, there is more glycogen accumulation (Caprioglio *et al.*, 1993). As glycogen accumulation occurs just as glucose is being exhausted during diauxic growth, glycogen accumulation is considered a marker for stress in yeast. Male mice lacking puromycin-sensitive aminopeptidase, a mammalian PepN

homologue, are sterile due to impaired spermatogenesis and degenerative morphology of Sertoli cells (Osada *et al.*, 2001a). Female mice lacking puromycin-sensitive aminopeptidase are also sterile due to impaired formation of the corpus luteum (Osada *et al.*, 2001b). PepN and its homologues play distinct roles in the physiology of organisms from different kingdoms. Thus, enzymes involved in downstream processing may play specialized roles in cellular processes.

In mammals, NaSal acts as a non-steroidal anti-inflammatory agent. It is an inducer of heat shock and inhibits the production of inflammatory cytokines, whereas in plants it is part of the host defence system (Price *et al.*, 2000). In prokaryotes, NaSal is used to study the response to a xenobiotic compound. There are several mechanisms by which NaSal acts – dissipation of the proton gradient across the inner membrane, iron chelation, growth inhibition, induction of heat shock and the *marA* regulon – resulting in the modulation of several genes (Price *et al.*, 2000; Pomposiello *et al.*, 2001). NaSal binds and inactivates MarR and activates MarA, resulting in changes in the outer-membrane profile, decreased permeability to antibiotics and, consequently, increased antibiotic resistance (Cohen *et al.*, 1993; Ramani & Boakye, 2001). To understand the cellular role of PepN and its relation to certain stress conditions (induced by NaSal, etc.), it is important to consider that it acts as an aminoendopeptidase. In terms of protein turnover, the aminopeptidase function of PepN will be clearly important in the recycling of amino acids. However, we know that there are redundant aminopeptidases which can take over this role (Miller & Schwartz, 1978; Yen *et al.*, 1980). Therefore, during stress the aminopeptidase activity may be important in cleaving peptides or proteins containing basic or small amino acids which reduce the ability of *E. coli* to display resistance to stress. Arginine and lysine at the amino terminus destabilize proteins and such proteins are targeted for degradation (Gonzales & Robert-Baudouy, 1996). Also, aminopeptidase action may result in isoforms of proteins possessing different amino terminal amino acids (Ishino *et al.*, 1987). Alternatively, PepN via its endopeptidase activity may modulate the proteome of cells. In fact, PepN is required for the activation of the antibiotic albomycin inside *E. coli*; therefore, *pepN* mutant cells are resistant to albomycin action. The predicted cleavage sites in albomycin suggest that the endopeptidase activity of PepN plays a role in this cleavage (Braun *et al.*, 1983). It is possible that PepN cleaves cellular proteins that result in decreased ability of *E. coli* to resist NaSal-mediated stress. However, in the absence of PepN, these proteins may be present and help to withstand NaSal-induced stress. Comparison of the proteome of wild-type and *pepN* mutant strains may identify such cellular substrates of PepN. Further studies are in progress to understand the mechanisms by which the major aminopeptidase in *E. coli*, PepN, functions as an aminoendopeptidase to negatively modulate the response to NaSal-induced stress.

Thus far, seven aminoendopeptidases have been reported: α-*N*-benzoylarginine-β-naphthylamide hydrolase (Singh & Kalnitsky, 1980), hydrolase H (Okitani *et al.*, 1981; Nishimura *et al.*, 1983), PepN (Chandu *et al.*, 2003), bleomycin hydrolase (Koldamova *et al.*, 1998), cathepsin H (Turk *et al.*, 2001), multicorn (Osmulski & Gaczynska, 1998) and tripeptidyl peptidase II (Geier *et al.*, 1999). Of these, the last five are involved in cytosolic protein degradation, suggesting an important role for these enzymes in this process. Notably, PepN is the only bacterial aminoendopeptidase characterized (Chandu *et al.*, 2003). PepN is a better aminopeptidase than an endopeptidase, as indeed are other aminoendopeptidases, for example, multicorn (Osmulski & Gaczynska, 1998) and tripeptidyl peptidase II (Geier *et al.*, 1999). Although purified enzymes involved in downstream processing have been studied in *T. acidophilum* (Tamura, N. *et al.*, 1998), *Schizosaccharomyces pombe* (Osmulski & Gaczynska, 1998) and mouse cells (Geier *et al.*, 1999), there are no studies reported in organisms lacking these enzymes. The extensive substrate preference profile of pure PepN and its role in cellular extracts, together with its physiological role during some stress conditions, are unique to this study and will enhance our understanding of the role of the aminoendopeptidase PepN in cytosolic protein degradation in *E. coli*.

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