Subcellular localization and processing of the lytic transglycosylase of the conjugative plasmid R1

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Abstract Protein P19 encoded by the conjugative resistance plasmid R1, is essential for efficient conjugative DNA transfer and infection by the pilus-specific RNA phage R17. Based on sequence homologies P19 belongs to a family of lysozyme-like virulence factors which are found in type III and type IV secretion systems. In this report we describe the processing and subcellular localization of P19. Pulse-chase experiments were used to demonstrate the processing of P19 by the signal peptidase I of *Escherichia coli*. Translocation of P19 across the inner membrane was shown by gene *19-phoA* fusions. Cell fractionation studies of P19 expressing cells showed the presence of P19 in the membrane compartment. P19 was solubilized with the detergent Sarkosyl indicating an inner membrane localization. Using sucrose density gradient centrifugation to separate inner and outer membranes, P19 was found in both membrane fractions. Taken together, our data suggest that mature P19 is a periplasmic protein which may be attached to the proposed membrane-spanning DNA transport complex.

Key words: Bacterial conjugation; Plasmid R1; Protein P19; Protein translocation; Cell envelope; Lytic transglycosylase

1. Introduction

In order to cope with the high osmotic pressure most eubacteria have to maintain a rigid cell wall that is composed of a unique biopolymer called murein or peptidoglycan which surrounds the whole cell. In Gram-negative bacteria peptidoglycan comprises a single macromolecule which is embedded in the periplasm between the outer and inner membrane. Disruption of the peptidoglycan or the unbalanced action of murein-synthesizing and -degrading enzymes (for a recent review see [1]) is deleterious for the cell and leads to lysis and cell death. The successful treatment of infectious diseases by the use of β-lactam antibiotics is based on the above mentioned process. One of the glycan strand-degrading enzymes in *Escherichia coli* is the soluble lytic transglycosylase Slt70 [2]. Slt70 is an exomuramidase cleaving the β-1,4-glycosidic bond and has been crystallized recently [3]. Sequence database searches using the conserved residues present in the catalytic domain of the enzyme [4] revealed that the lytic transglycosylase motif is present in a large family of proteins [5–9]. Intriguingly, some members of this growing protein family are encoded by type III and type IV macromolecular transport systems, a finding implying that these systems utilize their own specialized lytic transglycosylases to facilitate efficient transenvelope transport of DNA and/or protein substrates.

P19 encoded by gene *19* of the F-like conjugative resistance plasmid R1 is a member of lysozyme-like virulence factors with the lytic transglycosylase motif [6–9]. Gene *19* is located next to the origin of conjugal DNA transfer, *oriT* of R1, and encodes a polypeptide of 169 amino acids (SwissProt: P17738) [10]. Expression of gene *19* is tightly controlled post-transcriptionally by RNase III [11]. Recent experiments demonstrated that protein P19 is essential for efficient conjugal DNA transfer and RNA phage infection [6]. The transmission of ssDNA during conjugation is believed to be achieved by a membrane-spanning transport complex comprising several transfer proteins. However, the nature and the composition of the complex are largely unknown [12]. Due to the sequence homologies of P19 to the lytic transglycosylase we hypothesized that P19 exhibits its function at the murein sacculus and might associate with the envelope-spanning transport complex to facilitate its correct assembly by catalyzing a controlled localized opening in the peptidoglycan. In order to test the validity of this hypothesis we investigated the subcellular localization and processing of P19. The results of these investigations are presented here.

2. Materials and methods

2.1. Bacterial strains, plasmids and growth conditions

E. coli K38 [pGP1-2] cells were used for protein expression experiments [13]. *E. coli* PN1 was used for random integration of Tn*phoA* and *E. coli* CC118 was used for alkaline phosphatase assays [14]. Plasmids pCS217 and pCK217 were described previously [11]. The construction of the recombinant plasmids pCK217ns and pCKBlaP19 is described below. *E. coli* cells were grown in 2×TY medium (16 g of tryptone, 10 g of yeast extract, 5 g of NaCl per liter) or in minimal medium M9 (6 g Na_2HPO_4, 3 g K_2HPO_4, 1 g NH_4Cl, 0.5 g NaCl per liter; 1 mM $MgSO_4$, 0.4% (w/v) glucose and 100 μg ml^{-1} thiamine). Antibiotics, when needed, were added at the following concentrations: epicillin (dihydroampicillin), 100 μg ml^{-1}; tetracycline, 15 μg ml^{-1}; kanamycin, 40 μg ml^{-1}.

2.2. Enzymes, chemicals and oligonucleotides

Restriction endonucleases and other enzymes used for standard cloning and DNA modification procedures were purchased from Roche Diagnostics. The thermostable DNA polymerases DynaZyme and Expand™ High Fidelity PCR System were purchased from Finnzymes and Roche Diagnostics, respectively. Radiochemicals were purchased from Amersham International or from Dupont, NEN Research Products. Oligonucleotides were purchased from the Vienna Biocenter or MWG Biotech.

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2.3. DNA manipulations

Recombinant DNA techniques were performed according to [15,16], or the manufacturers' protocols.

2.4. Site-specific mutagenesis

The signal sequence of protein P19 was removed by oligonucleotide directed deletion mutagenesis as described previously [17]. Oligonucleotide Gen19dlp, 5′-CCAGAGGTGGAAAAATGTGCTTTGATC-TTGCAGG-3′, and the plasmid pCK217 with the wild-type gene *19* were used to create an in-frame deletion of amino acids 2–21 of P19. The resulting plasmid, pCK217ns, was sequenced to confirm the mutation. The replacement of the signal sequence of protein P19 by residues 1–27 of TEM-1β-lactamase was carried out by cloning a 110 bp PCR fragment from pBluescript SK+ flanked by *Hin*dIII/*Sau*3a restriction sites into the *Hin*dIII/*Bgl*II restricted plasmid pCK217. The PCR fragments were produced by using the following oligonucleotides: BLAPRIMER1, 5′-TTTAAGCTTGAAAAAGGA-AGAGTATGAG-3′ and BLAPRIMER2, 5′-TTTGATCGTTTCTG-GGTGAGCAAAAAC-3′. The resulting recombinant plasmid was designated pCKBlaP19 and its sequence was verified. The gene *19-phoA* fusions were constructed by random integration of Tn*phoA* [14]. The recombinant plasmid pCK217 was transformed into the transposon donor strain *E. coli* PN1. Following the in vivo transposition event, plasmid DNA was isolated and digested with *Hin*dIII and *Dra*I for a first estimation of the position of the Tn*phoA* insertion. Automated DNA sequencing was then used to determine the exact Tn*phoA* integration site. Plasmid DNA was used for the transformation of competent CC118 cells and alkaline phosphatase activity was measured according to [18].

2.5. Protein expression

Protein expression experiments were performed as described previously [19]. *E. coli* K38 [pGP1-2] cells harboring the P19, P19ns or BlaP19 expressing plasmids pCK217, pCK217ns or pCKBlaP19, respectively, were used for pulse-chase experiments. Proteins were pulse-labeled for 45 s with [^{35}S]methionine/cysteine and chased with an excess of cold methionine/cysteine (final concentration of 0.1%). After the addition of the chase 120 μl aliquots of cell suspension were taken at the times indicated. Ice-cold trichloroacetic acid (10% final concentration) was added and the cells were collected by centrifugation at 17 400×*g* for 10 min at 4°C. The samples were resuspended in SDS–PAGE sample buffer (60 mM Tris–hydrochloride, pH 6.8, 1% SDS, 1% 2-mercaptoethanol, 10% glycerol, and 0.01% bromophenol blue) and boiled for 10 min. The extracted proteins were separated by 15% SDS–PAGE and visualized afterwards using a phosphorimager. The radioactivity in specific bands was quantitated using the ImageQuant software (Molecular Dynamics). As a control, the signal peptidase I inhibitor 2-phenylethanol [20,21] was added to a final concentration of 0.3%.

2.6. Cellular fractionation

For all fractionation studies protein P19 was expressed in *E. coli* K38 harboring plasmid pCS217 (wild-type) or pCS260 (negative control) as described earlier [11]. After labeling the proteins with [^{35}S]methionine, cells were harvested, broken by sonication and the cell debris was removed by centrifugation at 3000×*g* for 15 min. The membrane fraction was collected by centrifugation at 48 000×*g* for 1 h. The pellet was treated with the ionic detergent Sarkosyl (*N*-lauroyl sarcosinate) to solubilize the inner membrane proteins as described by Filip [22]. On the other hand inner and outer membranes were separated according to their buoyant densities by centrifugation through layers of 53% and 70% sucrose (w/v), containing 3 mM EDTA, at 25 000 rpm for 16 h in a swinging bucket rotor at 4°C. After collecting the membrane fractions, a purification step with Sarkosyl was added. Proteins in various fractions of the sucrose gradient were resuspended in SDS–PAGE sample buffer, boiled for 10 min, separated by 15% SDS–PAGE and visualized by autoradiography. To enhance the intensity of autoradiographic images, gels were treated with dimethylsulfoxide/2,5-diphenyloxazole (DMSO/PPO) [23], dried and exposed to X-ray films.

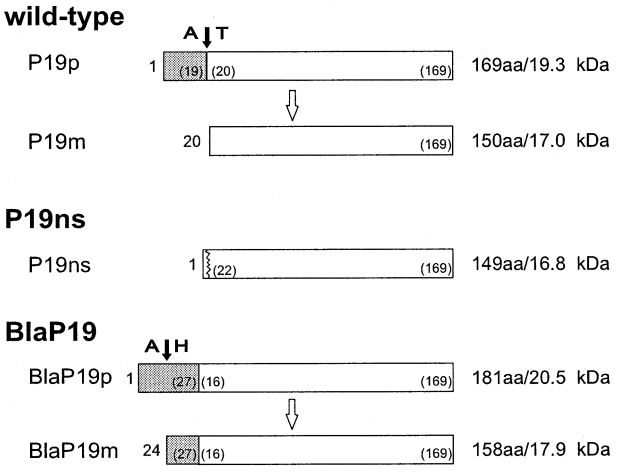

Fig. 1. Schematic representation of gene *19* polypeptides. P19p is the immediate translation product of gene *19* possessing a typical signal sequence located at its N-terminus. The wild-type protein has a calculated molecular weight of 19.3 kDa (SwissProt: P17738). Signal peptidase I cleavage (open arrow) between alanine (A) at position 19 and threonine (T) at position 20 produces the mature protein P19m consisting of 150 amino acids (17.0 kDa). P19ns is a modified protein without signal sequence. The calculated molecular weight of P19ns is 16.8 kDa. In BlaP19 the first 15 amino acids of protein P19 are replaced by the amino-terminus of TEM-1β-lactamase. 27 amino acids from TEM-1β-lactamase were fused to 154 C-terminal amino acids of protein P19 to give a preprotein with 181 amino acids (BlaP19p). The result of signal peptidase I cleavage (open arrow) between alanine (A) at position 23 and histidine (H) at position 24 gives rise to the mature protein BlaP19m consisting of 158 amino acids.

3. Results and discussion

3.1. P19 and P19 fusion constructions

Using the signal sequence prediction tool SignalP [24] analysis of the P19 sequence revealed a typical prokaryotic signal sequence with a predicted cleavage site between residues A_{19} and T_{20}. The proposed cleavage and processing of the P19 preprotein (P19p) to the mature form of P19 (P19m) is schematically illustrated in Fig. 1. To investigate the physiological relevance of the signal sequence the N-terminal part of P19 was removed leading to a construct named P19ns. In order to investigate the export of P19 by a known export sequence, the P19 signal sequence was replaced by residues 1–27 of the signal sequence of TEM-1β-lactamase. The fusion protein generated from this construction was designated BlaP19. Its proposed processing is shown in Fig. 1 together with the β-lactamase signal sequence cleavage site between residues A_{23} and H_{24} [25].

3.2. Processing of P19 and BlaP19

To evaluate the processing of P19 and BlaP19 pulse-chase experiments were performed. Within the period of observation the translation product, P19p, is converted to the mature form, P19m (Fig. 2). However, during this time the larger preprotein form of P19 never completely disappeared. Equivalent amounts of P19p and P19m were observed approximately 340 s after addition of the chase (Fig. 2B). We attribute the incomplete processing of P19 in the pulse-chase experiment to the rapid formation of inclusion bodies in the cytoplasm that are readily formed when P19 or P19 derivatives are over-expressed in *E. coli*. It is reasonable to assume

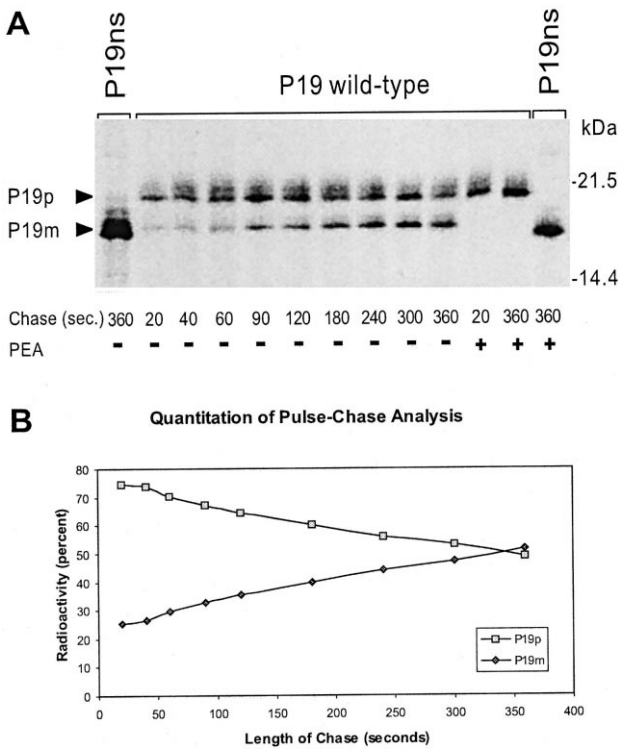

Fig. 2. Processing of protein P19wt. *E. coli* K38 [pGP1-2] cells expressing the indicated proteins were pulse-labeled for 45 s with [^{35}S]methionine/cysteine in the absence (−) or in the presence (+) of 2-phenylethanol (PEA) and chased with an excess of unlabeled methionine/cysteine. A: Labeled proteins were separated by SDS–PAGE and visualized using a phosphorimager. The duration of the chase time in seconds is indicated below each lane. P19wt, wild-type P19; P19ns, P19 without its signal sequence. The upper arrow indicates the position of the preprotein, P19p, the lower arrow that of the mature product, P19m. B: The percentage of the total radioactivity present in either precursor (squares) or mature (diamonds) form as a function of chase time is presented graphically.

that P19 present in these insoluble aggregates is not accessible for transport and processing. The processing of protein P19 was completely inhibited by the presence of 2-phenylethanol. Because of the presence of a cysteine residue near the proposed cleavage site of P19, we also investigated whether a prolipoprotein-specific signal peptidase (signal peptidase II) is involved in the processing of P19. Since the processing of P19p to P19m also occurred in the presence of the specific signal peptidase II inhibitor globomycin [26], this possibility could be excluded (data not shown). In the case of P19ns, only one protein band corresponding to the expected molecular weight of 16.8 kDa was detected. The failure of P19ns to be processed suggests that it is not transported across the inner membrane. In vivo analysis of the N-terminally truncated polypeptide indicated that the presence of a signal sequence in P19 is essential for the biological activity. It can be, however, replaced by the β-lactamase signal sequence without a loss of function of P19 [27]. As shown in Fig. 3, the conversion of the unprocessed form of the hybrid protein BlaP19p to mature BlaP19m occurred more rapidly than in the case of the wild-type P19 protein. 110 s after the addition of the chase 50% of the original preprotein was processed (Fig. 3B). As in the case of wild-type P19 processing of BlaP19p was completely blocked by the addition of 2-phenylethanol indicating that the processing of the hybrid protein is dependent on the activity of signal peptidase I. This result also showed that the signal sequence cleavage site of P19 is recognized less efficiently by the preprotein processing signal peptidase I of *E. coli* than that of the artificially created BlaP19 hybrid protein. The size of the BlaP19m protein as estimated from the SDS–PAGE shown in Fig. 3 suggests that BlaP19p is cleaved at the position of the signal sequence cleavage site which has been determined for β-lactamase [25].

3.3. Gene 19-phoA fusions

Proteins equipped with a signal sequence are either destined for export or for a membrane location [28]. A periplasmic location in close association with other membrane anchored proteins would be consistent with the postulated role of P19 acting as a muramidase. To corroborate this hypothesis a random fusion of *phoA* to gene *19* was used as a sensor for the subcellular localization of P19. Generation of a fusion protein that exhibits alkaline phosphatase activity using this approach can only occur when the portion derived from the target protein possesses sufficient signals to drive localization of the catalytic domain of alkaline phosphatase to the periplasm. In a transposon mediated fusion approach three different constructions were obtained. In one case, the transposon inserted outside of gene *19*. In the second construct PhoA was fused to amino acid N55 of P19. The fusion position in the third construct was at amino acid Y134 of P19. The enzymatic activity of alkaline phosphatase expressed from the three different recombinant plasmids were 0.2 U, 1738 U and 1531 U, respectively. These values indicate unambiguously that the

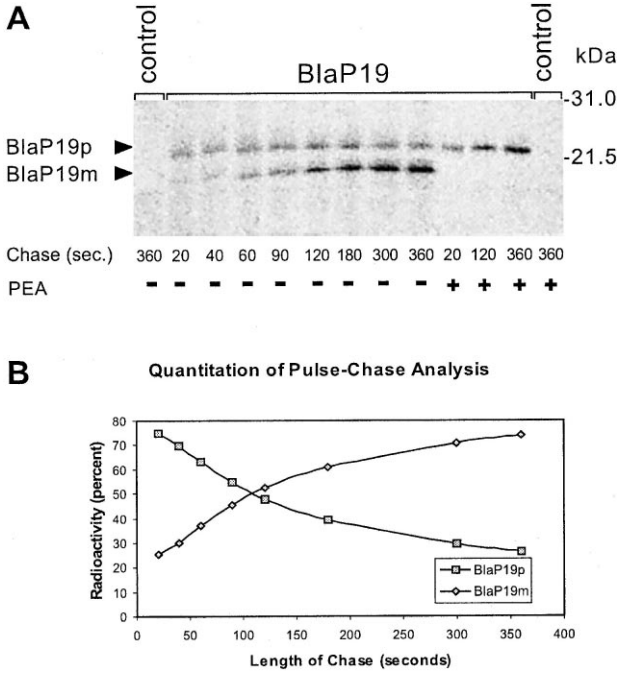

Fig. 3. Processing of the hybrid protein BlaP19. The pulse-chase experiment was performed as described in the legend to Fig. 2. A: A phosphorimage of an SDS–PAGE is shown. BlaP19, signal sequence of β-lactamase fused to P19; control, vector control. The upper arrow indicates the position of the preprotein, BlaP19p, the lower arrow that of the mature product, BlaP19m. B: The percentage of the total radioactivity present in either precursor (squares) or mature (diamonds) form as a function of chase time is presented graphically.

first 55 amino acids of P19 are sufficient to convey the hybrid protein to the periplasm. It has been shown by analogous experiments with VirB1 and IpgF, respectively, that these P19 homologues are also transported to the periplasm [29,30]. Together with the pulse-chase experiments, the data suggest that P19 is transported across the inner membrane by the utilization of a cleavable signal sequence. The results also imply that P19m is released into the periplasm.

3.4. Cell fractionation studies

Membrane separation was performed in two ways, namely by dissolution with the ionic detergent Sarkosyl and by sucrose gradient centrifugation. As expected both P19p and P19m were found to be present in the whole cell lysate (Fig. 4, lane A). The mature form P19m was also readily detectable in the Sarkosyl soluble fraction as well as a weak band corresponding to P19p which was only visible on the original autoradiogram (lane D). In the cytoplasm (lane B) and Sarkosyl insoluble fraction (lane C) no P19 protein could be detected. To confirm this finding inner and outer membranes were resolved according to their buoyant densities by sucrose gradient centrifugation. As shown in Fig. 5, both forms of the protein were found in the outer and inner membrane fractions (lanes D and G), although marker proteins, such as outer membrane proteins, were clearly separated (data not shown). When the fractions were subsequently treated with Sarkosyl the protein was consistently resolved in the soluble fraction (Fig. 5, lane H). These results suggested the intriguing possibility that the presence of the protein in both membrane fractions of the sucrose gradient is due to a membrane-spanning protein complex to which both forms of P19 (P19m and P19p) are attached. This complex could be composed of the envelope-spanning DNA transport proteins produced by the Hfr strain K38 which was used as a host. It has been shown that the mating pair formation (Mpf) components of the RP4 plasmid fractionated by density flotation gradients of membranes revealed an additional membrane fraction with the density close to that of the outer membrane [12]. The interpretation of these results was that the Mpf of RP4 connects the cytoplasm and the outer membrane. Similarly to our observations, the P19 homologous VirB1 protein from the Ti plasmid of *Agrobacterium tumefaciens* was more prominent in the outer membrane fraction of a sucrose gradient. Like P19, VirB1 was found to be Sarkosyl soluble and the fractionation behavior was attributed to association of VirB1 to a transport channel that spans both membranes [31].

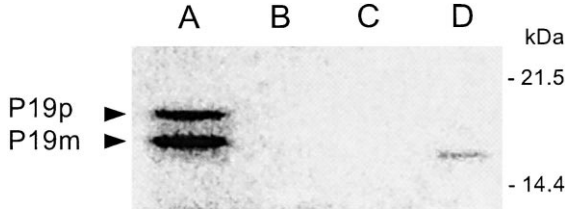

Fig. 4. Subcellular localization of protein P19. After T7 promoter driven expression of protein P19 using [^{35}S]methionine as a label, *E. coli* K38 [pGP1-2] [pCS217] cells were harvested, broken by sonication and the membrane fraction collected by centrifugation. The inner membrane proteins were separated from outer membrane proteins by solubilization with the ionic detergent *N*-lauroyl sarcosinate (Sarkosyl). Lane A, whole cell lysate; lane B, cytoplasm; lane C, Sarkosyl insoluble fraction; lane D, Sarkosyl soluble fraction.

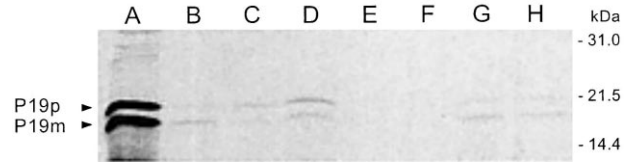

Fig. 5. Fractionation of P19-containing membranes by sucrose gradient centrifugation. Membranes of P19 expressing *E. coli* cells were prepared as described in Fig. 4. The inner and outer membranes were separated according to their buoyant densities. Gradient fractions were subsequently extracted with Sarkosyl. Lane A, whole cell lysate; lane B, cytoplasm; lane C, total membrane fraction; lane D, outer membrane (sucrose); lane E, Sarkosyl insoluble fraction of 'D'; lane F, low molecular weight standard; lane G, inner membrane (sucrose); lane H, Sarkosyl soluble fraction of 'G'. Proteins were separated electrophoretically and visualized by autoradiography.

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