Age-dependent changes in extracellular proteins, aminopeptidase and proteinase activities in *Frankia* isolate BR

A. MÜLLER,^{1,2} P. BENOIST,^{1,2} H. G. DIEM² and J. SCHWENCKE^{1*†}

¹Laboratoire d'Enzymologie, CNRS, 91198 Gif-sur-Yvette, France

²Laboratoire de Biotechnologie des Symbioses Forestières Tropicales ORSTOM/CTFT-CIRAD, 45 Bis Avenue de la Belle Gabrielle, 94736 Nogent-sur-Marne, France

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To investigate protein secretion by the nitrogen-fixing actinomycete *Frankia* isolate BR, we designed a rapid DEAE adsorption, salt elution and Biogel P6DG desalination method to concentrate protein from the growth medium. Secreted proteins reached a maximum concentration (5.6 mg l⁻¹) in the medium at growth arrest. Analysis by SDS-PAGE detected up to 63 extracellular polypeptides when *Frankia* cells were grown under stirred conditions in BAP medium supplemented with phosphatidylcholine and MES buffer and 65 proteins in stirred BAP media alone. The pattern of extracellular polypeptides changed during growth. Several extracellular proteolytic activities were detected and compared with intracellular ones. The substrate specificity of the extracellular and intracellular aminopeptidase activities were the same. Also, the electrophoretic migration patterns of secreted and intracellular aminopeptidases could not be distinguished. Secretion of the proline-specific aminopeptidase FAP 3.BR appeared to be the only one that arrested at the end of the exponential phase of growth. At least 15 proteinases (PF) were secreted: 10 had the same electrophoretic mobility as their intracellular counterparts after SDS-gelatin-PAGE while five (PF-39.5, PF-38.5, PF-36.5, PF-25.5 and PF-20.5 kDa) had a different electrophoretic mobility and, therefore, appeared to be exclusively extracellular. At least seven extracellular proteinases appeared to increase coordinately in activity shortly before growth arrest.

Introduction

Frankia is a soil-inhabiting actinomycete capable of forming nitrogen-fixing nodules on the root system of a number of non-legumes, now termed actinorhizal plants. Actinorhizal symbiosis was already a subject of interest in the last century (cited in Bond, 1967) but the first confirmed report of the isolation in pure culture of the microsymbiont *Frankia* was that of Callaham *et al.* (1978). The ability to cultivate *Frankia* *in vitro* has permitted a rapid increase in knowledge of its physiology, mainly of C- and N-metabolism, nitrogen fixation and related enzymes (Benson & Schultz, 1990). Very little is known about biochemical changes occurring during

hyphal growth and/or production of extracellular proteins, particularly proteases. The main problem in such studies is the heterogenous nature of the mycelial cultures due to unbalanced growth in the static conditions usually employed to cultivate *Frankia*. In such conditions young and old hyphae as well as sporangia are found together and cells show a varying degree of senescence as indicated by zones of lysis (Benson & Schultz, 1990). These inconveniences have been overcome for some strains by cultivation in stirred conditions (Murray *et al.*, 1984; Noridge & Benson, 1986; Diem & Dommergues, 1990), and also for three of our isolates from *Casuarina*, by growing them in stirred conditions in well-buffered, BAP mineral medium supplemented with phosphatidylcholine (Schwencke, 1991). Phosphatidylcholines are components of QMOD medium (Lalonde & Calvert, 1979), but growth of our *Frankia* strains in this rich medium proved to be quite slow.

Aminopeptidases have been described and proposed to be useful in the characterization of *Frankia* (Gardes *et al.*, 1987; Benoist & Schwencke, 1990), but the significance of *Frankia* proteases in host-microsymbiont

† Present address: Laboratoire de Biotechnologie des Symbioses Forestières Tropicales ORSTOM/CTFT-CIRAD, 45 Bis Avenue de la Belle Gabrielle, 94736 Nogent-sur-Marne, France.

Abbreviations: p-CMS, p-chloromercuriphenylsulphonate; EP, extracellular polypeptides; FAP, *Frankia* aminopeptidases; MNA, 4-methoxy β -naphthylamide; PF, *Frankia* proteinases; TEMED, N,N,N',N'-tetramethylethylenediamine.

interactions has not been explored. Extracellular proteases have been proposed as bacterial pathogenicity factors for *Xanthomonas campestris* pv. *campestris* which infects turnip plants (Daniels *et al.*, 1984). Also, several reports indicate that proteinase inhibitors are related to microbial resistance in a number of plants including tobacco, tomato, melon (for reviews see Lamb *et al.*, 1989; Ryan, 1990). These considerations, and others cited above, prompted us to study the secretion of proteolytic enzymes by *Frankia* *in vitro*.

Methods

Origin of *Frankia* material. *Frankia* isolate BR (ORS 020608) was isolated by one of us (H. G. D.) in 1983 from young nodules formed on plants of *Casuarina equisetifolia* after inoculation under axenic conditions with dried nodule samples collected in Brazil. *Casuarina* nodule lobes were superficially sterilized in a 3% (w/v) aqueous solution of osmium tetroxide for 4 min to eliminate contaminants harboured in the outer layer of the nodule (Lalonde *et al.*, 1981). Nodule lobes were then thoroughly rinsed in sterile water, chopped into small pieces and transferred into a double layer agar medium as recommended by Diem & Dommergues (1983). After 10–15 d, *Frankia* colonies grew out of nodule pieces and exhibited typical hyphae and sporangia. Nitrogen-fixing vesicles developed on nitrogen-free medium and, surprisingly, on medium supplemented with Casamino acids (Difco) as nitrogen source. Subsequent infection tests showed that BR was infective and effective on species of genus *Casuarina*, but not on members of the genus *Allocausarina* nor on members of the family *Elaeagnaceae*.

Growth conditions. Mycelial cells of *Frankia* isolate BR were disrupted by passing through a syringe and inoculated at 4 µg protein ml⁻¹ (Lowry) into 500 ml flasks containing 250 ml of either BAP mineral medium (Fontaine *et al.*, 1986) or BAP medium supplemented with phosphatidylcholine (4 mg l⁻¹) and MES/Tris buffer (10 mM, pH 6.8: BAP-PCM) as described by Schwencke (1991). Cells used as inoculum were sporangia-free. Cultures were grown at 28 °C without shaking or under magnetically stirred conditions.

Preparation of extracellular concentrates. All procedures were carried out at 4 °C using sterile solutions and laboratory ware. Cultures were harvested by centrifugation at 8000 g for 15 min. The supernatant was collected and vacuum-filtered through glassfibre filters (Whatman GF/F, 0.7 µm passing diameter) and adjusted to pH 8.0 with Tris base. We attempted to concentrate this cell-free medium by using two different methods.

In the first method, the cell-free filtrate was treated twice with 0.4 g l⁻¹ of pre-swollen DEAE-cellulose (Whatman DE-52) for 30 min under conditions of slow stirring. The cellulose was collected on Blutex linen cloth (10 µm mesh), resuspended in 20 mM-HEPES/Tris (pH 8.0), 10% (v/v) glycerol (HTG-buffer), and poured into a 10 ml plastic syringe previously stoppered with glass-wool. The syringe, placed inside a 50 ml plastic tube, was centrifuged in a swinging-bucket rotor at 200 g for 5 min. Unbound material was removed by washing and centrifuging the packed DEAE-cellulose three times with HTG-buffer. Bound proteins were then eluted by two successive additions of 2 M-NaCl in HTG-buffer to the packed DEAE-cellulose. After elution for 5 min, the eluate was recovered by centrifugation as above. Rapid desalination was obtained by passing the 2 M-NaCl eluate over a packed Bio-gel P6DG column built in a plastic syringe by centrifugation (200 g for 5 min). Before packing, the Bio-gel P6DG was equilibrated in 20 mM-HEPES/Tris (pH 7.0), 10% (v/v) glycerol,

25 mM-NaCl. The volume of 2 mM-NaCl used corresponded to 1/5th of the packed volume of Bio-gel P6DG.

In the second method, the cell-free medium was concentrated through Millipore 10 kDa Ultrafree filtration units to the desired final protein concentration. Extracellular concentrates were used immediately or stored at -80 °C. Protein concentration was determined by the bicinchoninic acid method according to the manufacturer's (Pierce) instructions using bovine serum albumin as standard.

Electrophoretic conditions. The mixture of secreted proteins was analysed by SDS-PAGE according to the method described by Laemmli (1970) in 14% (w/v) acrylamide/0.37% bisacrylamide slab gels containing 0.1% SDS. The polypeptides were visualized on the gel by the silver stain method (Morrisey, 1981). Proteinase activities were revealed after electrophoresis under denaturing conditions in gelatin/polyacrylamide gels (Heussen & Dowdle, 1980) modified as follows. The running gel contained 0.1% gelatin (freshly prepared), 11% polyacrylamide, 0.29% bisacrylamide, 0.375 mM-Tris/HCl, pH 8.8, 0.1% SDS, 0.05% ammonium persulphate and 0.025% TEMED. The stacking gel was 4.5% acrylamide, 0.23% bisacrylamide, 0.125 mM-Tris/HCl, pH 6.8, 0.1% gelatin, 0.1% SDS, 0.05% ammonium persulphate and 0.1% TEMED. Following acrylamide polymerization at room temperature, solidification of gelatin was allowed to proceed for 24 h at 4 °C. Gels were submitted to a pre-electrophoresis at 4 °C with running buffer (0.05 M-Tris, 0.384 M-glycine, pH 8.3, 0.1% SDS, 1 mM-DTT) for 1 h at 20 mA. The electrophoresis buffer was then changed. Samples were incubated for 15 min at 37 °C with denaturing buffer (2.5% (w/v) SDS, 1% (w/v) sucrose, 10% (v/v) glycerol and 0.5% bromophenol blue), and loaded immediately. Electrophoresis was done at 12 mA constant current (4 °C) until the cytochrome c marker reached the bottom of the resolving gel. Then the gels were gently shaken in 2.5% (v/v) Triton X-100 in water for 1 h at room temperature. After six to eight rapid washes with distilled water, the gels were incubated three times for 1 h at 37 °C with revealing buffer (100 mM-glycine/NaOH, pH 8.3, 0.1% Triton X-100, 1 mM-DTT) and then for 20 h in fresh revealing buffer. Finally, the gels were washed in distilled water and stained/desained as described by Heussen & Dowdle (1980). Amino-peptidase activities were revealed after ultralow gelling point agarose-PAGE in native conditions as described by Benoist & Schwencke (1990). In our experiments, 2 × crystallized Serva (10675) acrylamide gave satisfactory results. The following modifications were used for aminopeptidases 1 and 2 from *Frankia* BR (FAP 1.BR and FAP 2.BR). After washing the gel for 15 min with 100 mM-HEPES/KOH, pH 7.0, 100 mM-NaCl, 50 µM-CoSO₄, bands of FAP activities 1 and 2 were revealed with 1 mM-L-leucine 4-methoxy-β-naphthylamide (L-Leu MNA), 50 mM-MES/TES/KOH (pH 7.2), 100 mM-NaCl, 50 µM-CoSO₄ and 0.025% Fast Garnet GBC. All other FAP activities were revealed as previously described (Benoist & Schwencke, 1990). Details of salts used for revealing other FAPs are indicated in Table 1. We stored L-Leu MNA and L-Pro MNA as a 200 mM solution in DMSO at -20 °C.

Radiolabelling of gelatin. [¹⁴C]Acetic anhydride [540 µCi (20 MBq)] was added to 10 ml 5% (w/v) gelatin in sterilized 0.1 M-borate/NaOH buffer (pH 9.0) and the reaction was allowed to proceed for 2 h at room temperature. Unincorporated ¹⁴C was removed by extensive dialysis at 4 °C against 10 mM-Tris/HCl, pH 7.5, 100 mM-KCl, 5% (v/v) glycerol, 2 mM-Na₃N. The specific activity of the [¹⁴C]gelatin was 1500 c.p.m. (µg protein)⁻¹. The labelled gelatin was divided into aliquots and stored in liquid nitrogen until use.

Assay of proteolytic activities. Proteolysis of [¹⁴C]gelatin was measured by adding 10 µl of labelled substrate (46 µg protein, 69 × 10³ c.p.m.) to 10 µg extracellular protein. The volume was adjusted to 0.1 ml with 100 mM-glycine/NaOH, pH 8.3, 0.1% Triton X-100, 1 mM-DTT buffer and incubated for 16 h at 37 °C. The reaction

Fig. 1. Kinetics of growth (●) and protein secretion (○) in BAP medium (a, c) and in BAP-PCM medium (b, d). Cells were inoculated at 4 mg l^{-1} and grown at 28°C . Duplicate samples were taken at the indicated times and protein concentration was determined using the bicinchoninic acid method for the total cellular fraction (a, b) and extracellular concentrates (c, d).

was stopped by adding 0.1 ml 16% (w/v) cold TCA after which the tube was kept on ice for 10 min . The TCA-insoluble material was centrifuged at $9800 g$ for 15 min at 4°C and 0.1 ml of the supernatant was mixed with 5 ml of Aquasol and counted.

Assay of aminopeptidase activities in extracellular concentrates. Leucine aminopeptidase activities were assayed by incubating $2.5 \mu\text{g}$ extracellular protein at 37°C for 30 min in $100 \mu\text{l}$ (final volume) of 1 mM -L-Leu-*p*-nitroanilide, $50 \mu\text{M}$ - CoSO_4 , 100 mM -NaCl, 50 mM -HEPES/Tris (pH 7.0). Proline-specific FAP 3.BR was assayed using $10 \mu\text{g}$ of extracellular protein in $100 \mu\text{l}$ (final volume) 1 mM -L-Pro-*p*-nitroanilide, $200 \mu\text{M}$ - CoSO_4 , 200 mM -NaCl, 50 mM -HEPES/Tris (pH 7.0) for 180 min at 37°C . The reaction was stopped with $100 \mu\text{l}$ cold 100 mM -EDTA, 2 mM -*o*-phenanthroline, pH 8.0. The amount of *p*-nitroanilide released was then measured at 405 nm . One unit of aminopeptidase activity was defined as that which released $1 \text{ mol p-nitroanilide min}^{-1}$. The molar absorption coefficient is $9900 \text{ l mol}^{-1} \text{ cm}^{-1}$ at 405 nm (Masuda *et al.*, 1975).

Inhibition assays of aminopeptidase activities. After electrophoresis and washing with buffer, the gels were cut in slabs and placed into plastic bags. Reversible inhibitors were incubated for 30 min at room temperature before adding the substrate. Irreversible inhibitors were pre-incubated for 15 min at 30°C with the extracellular protein samples before electrophoresis.

Materials and reagents. Bio-gel P6DG was from Bio-Rad. Ultrafree filter units were obtained from Millipore and Centricon units from Amicon. Bactogelatin (no. 0143-01) was from Difco. Phosphatidylcholine (P4279, type XIII), and Fast Garnet GBC were from Sigma. Ultralow gelling point agarose was from FMC Bioproducts. Antipain and leupeptin were from Peptide Institut Inc. (Osaka, Japan). L-Leucine chloromethylketone, β -naphthylamide, *p*-nitroanilide and 4-methoxy- β -naphthylamide derivatives of amino acids were from Bachem. Captopril was a gift from Squibb. All other chemicals used were commercial products of analytical grade.

Results

Kinetics of protein secretion and its relation to microbial growth

Due to the known difficulties with growth of *Frankia* under static conditions (Benson & Schultz, 1990), our study was done using *Frankia* cultures grown under

controlled stirred conditions in BAP mineral medium supplemented with phosphatidylcholine and MES buffer (BAP-PCM) as described by Schwenke (1991). A culture under static conditions in BAP medium was also run for comparison.

Results shown in Fig. 1(a, b) indicate that under stirred conditions growth of *Frankia* showed a clearly defined exponential phase. Maximal biomass yield was obtained at day 6 in BAP alone (Fig. 1a) and at day 4 in BAP-PCM (Fig. 1b); after this the hyphal protein content slowly decreased. Vital staining with acridine orange showed that hyphal lysis started 1 or 2 d after the end of the exponential growth phase (results not shown). Proteins were secreted throughout the exponential growth phase and showed a significant increase just before growth stopped (Fig. 1c, d). Virtually no protein was secreted in the stationary phase. The slight increase in extracellular protein observed may be due to cell lysis.

Secreted protein amounted to 5.5 mg l^{-1} at maximal mycelial density (day 6) in BAP medium, and to 5.6 mg l^{-1} at maximal density (day 4) in BAP-PCM. Similar results were obtained with the two concentration systems employed. We had chosen the DEAE/salt elution desalination system described here because it was far less expensive, more rapid and could easily be adapted for simultaneous processing of multiple samples. Protein secretion by *Frankia* was quantitatively quite large, amounting to 8.5% of the total protein in the culture (mycelial + secreted) for BAP-grown cells and 7.0% for BAP-PCM-grown cells.

Polypeptide patterns of secreted proteins

A complex pattern of secreted polypeptides was observed after analysis of extracellular concentrates by SDS-PAGE. Under stirred conditions, up to 65 extracellular polypeptide (EP) bands were visualized in

Fig. 2. SDS-PAGE polypeptide pattern of proteins of *Frankia* sp. BR secreted into stirred BAP medium (a), BAP-PCM medium (b) or unstirred BAP medium (c). Variations in protein bands secreted are represented as follows: ●, decreasing; ○, increasing; ■, no change. Main bands present in all media are indicated with an arrowhead (►). ND, Polypeptides that could not be detected; s, polypeptides specific to each medium. Molecular mass markers in kDa: β -galactosidase, 116; phosphorylase b, 92.5; bovine serum albumin, 66; chicken egg albumin, 45; L-lactic dehydrogenase, 36.5; β -lactoglobulin, 18.4; and cytochrome c, 12.4.

concentrates from cells grown in BAP medium (Fig. 2a), while 63 bands were present in concentrates from cells grown in BAP-PCM medium for 4 d (shown schematically). In static conditions, in BAP medium, 59 extracellular polypeptides bands were found (shown schematically).

Qualitative and quantitative changes in the bands of secreted polypeptides were observed as growth proceeded under the different conditions as shown by the analysis of extracellular concentrates of *Frankia* cells grown in stirred BAP medium (Fig. 2a). These changes could be classified as follows. (a) Increase to a certain

Table 1. Specificity of intracellular and extracellular aminopeptidases of *Frankia*

Gel slabs were pre-washed with HEPES buffer as indicated in Methods, and then activities were revealed using a 5 mM concentration of each substrate (naphthylamide or methoxynaphthylamide derivatives). All the substrates were tested for each FAP and only reactive substrates are indicated. Thus, for FAP 3.BR only L-Pro-4-methoxy- β -naphthylamide was reactive. Relative intensity is indicated by the number of plus signs. Amino-terminal amino acids inside a square box are equally reactive towards intracellular and extracellular FAPs; the others have not been tested for extracellular FAPs.

Amino-peptidase	Optimal salts conditions*	Terminal amino acid recognized	
FAP 1.BR	100 mM-NaCl 50 μ M-CoSO ₄	Leu ++	> Met > Phe = Ala + weak
FAP 2.BR	100 mM-NaCl 50 μ M-CoSO ₄	Leu ++++	> Met > Phe = Ala ++ weak
FAP 3.BR	200 mM-NaCl 200 μ M-CoSO ₄	Pro ++	
FAP 4.BR	200 μ M-CoSO ₄	Leu +++++	> Ala > Arg > Lys > Pro > Phe = Ser = Met = Orn ++ weak
FAP 5.BR	200 mM-NaCl 200 μ M-CoSO ₄	Leu +++++	> Ala > Lys > Arg > Phe > Glu > Pro = Ser > Met = Orn > Val > His +++ ++ ++ ++ ++ ++ weak
FAP 6.BR	200 μ M-CoSO ₄	Leu +++++	> Ala > Arg > Lys > Pro = Phe = Met = Orn +++ ++ + weak

* For revealing the relevant FAP activity.

point in the growth curve and stabilization at this level. Some of these extracellular polypeptides were: a 55 kDa polypeptide (EP-55-0) and also EP-29-5, EP-20-5, EP-18-8 and EP-16-0. (b) Continuous increase starting at some point on the growth curve, i.e. EP-17-0, EP-12-8 and EP-12-5. (c) Increase for a few days then decrease and disappearance, i.e. EP-52 and EP-38. (d) Continuous decrease, i.e. EP-92 (not detectable at day 10), EP-48 and EP-43-5, (not detectable at day 9), EP-36 (not detectable at day 7) and EP-20-3 (not detectable at day 6). Some polypeptides, i.e. EP-75, EP-70 and EP-43 did not disappear during the period of our experiment. (e) Unchanged bands: the majority (33 bands), particularly the two main bands, EP-24-0 and EP-22-0, marked in Fig. 2. Some changes have also been detected among intracellular proteins under each of the different growth conditions (unpublished results). A summary of all the variations in band intensity of extracellular polypeptides is shown in Fig. 2.

Clearly, even when analysing a single strain, extracellular polypeptides showed significant variations depending on growth conditions. Furthermore, some of them (as detailed in Fig. 2), appeared to be specific to a particular growth condition, i.e. EP-115-0, EP-112-5, EP-39-5, EP-33-5, EP-18-0 and EP-16-5 for stirred BAP, while EP-119-0, EP-41-5, EP-32-0, EP-30-0, EP-28-0,

EP-23-2, EP-19-8, EP-16-3, EP-12-6, EP-12-2 and EP-10-8 were specific to stirred BAP-PCM, and EP-31-5 was detected only in BAP medium under static conditions.

Electrophoretic patterns of extracellular aminopeptidases

After ultralow gelling point native agarose-PAGE (Benoist & Schwencke, 1990), six extracellular FAPs were detected in concentrates of both BAP and BAP-PCM media. No qualitative differences among FAPs were noted for extracellular concentrates obtained from *Frankia* grown in different media nor in static versus stirred growth conditions. The relative mobility of each of the extracellular FAPs was indistinguishable from that of the corresponding intracellular activity as can be seen in Fig. 3. Furthermore, the specificity of each extracellular FAP was identical with that of its intracellular form (summarized in Table 1).

Secretion of *Frankia* aminopeptidases with the exception of FAP 3.BR appeared to proceed continuously throughout the period of the experiment. Secretion of FAP 3.BR appeared to be constant up to day 4, then its activity slowly decreased until it was no longer detectable. Intracellular FAP 3.BR activity was detectable up

Fig. 3. Electrophoretic pattern of aminopeptidase activities from *Frankia* cells grown in stirred BAP-PCM medium. G1, G3, G5, extracellular concentrates; G2, G4, G6, intracellular fraction. The position of the prestained fumarase marker (FUM) is indicated.

to day 10 (Fig. 3, gels G1 and G2). Increased secretion of FAP 3.BR starting at day 4 appeared to correspond to a similar increase for the intracellular enzyme (Fig. 3). Secretion of all other extracellular FAPs was unchanged.

Inhibition studies on extracellular aminopeptidases

Inhibition tests were performed on gel slabs after native agarose-PAGE using L-Leu MNA or L-Pro MNA as substrates. The results (Table 2) show that L-Leucine chloromethylketone and *o*-phenanthroline were general inhibitors of all extracellular FAPs. No inhibitor could be found to differentiate FAP 1.BR from FAP 2.BR, but both were insensitive to 5 μ M-amastatine and 1 mM-*p*-chloromercuriphenylsulphonate (*p*-CMS). Also FAP

4.BR and FAP 6.BR could not be differentiated with the inhibitors tested. FAP 3.BR, specific for amino-terminal proline residues, was the only FAP which was not inhibited by EDTA. Furthermore, 2 μ g leupeptin ml⁻¹ or 1 mM-PMSF did not inhibit any of the FAPs (not shown). These results indicated that the sum of FAP 1.BR and FAP 2.BR activities could be measured in crude extracts using L-Leu *p*-nitroanilide as substrate in the presence of 0.1 mM-*p*-CMS or 5 μ M-amastatin. Also FAP 3.BR could be selectively measured in crude extracts by using L-proline-*p*-nitroanilide as substrate and 0.5 mM-EDTA or 2 μ M-amastatin as inhibitors. The concentration of inhibitors needed for assays in a liquid system was lower than those in the gel-liquid system (Table 2). This was probably due to the diffusion barrier

Table 2. Minimal concentration of inhibitors for total inhibition (in the gel) of extracellular *Frankia* aminopeptidases after native agarose-PAGE

Gel slabs were pre-treated with inhibitors for 30 min before testing for protease activity – see Methods for details. –, without effect; * a very weak band was seen at the concentrations indicated; NT, not tested.

Inhibitor	Minimal inhibitory concentration for:			
	FAP 1-2.BR†	FAP 3.BR‡	FAP 5.BR†	FAP 4-6.BR†
EDTA	5 mM	—	5 mM	5 mM
<i>o</i> -Phenanthroline	3 mM	3 mM	3 mM	3 mM
Bestatine	5 μ M	—	—	—
Amastatine	—	—	5 μ M	7.5 μ M*
Captopril	NT	10 mM*	10 mM	NT
Leu-chloromethylketone	5 mM	5 mM*	1 mM	1 mM
<i>p</i> -Chloromercuriphenylsulphonate	—	0.5 mM*	0.5 mM	1 mM*

† Using 1 mM-L-Leu-4-methoxy- β -naphthylamide as substrate.

‡ Using 1 mM-L-Pro-4-methoxy- β -naphthylamide as substrate.

Fig. 4. Variation of extracellular FAP 3.BR activity as a function of the age of the culture in BAP medium (●) and BAP-PCM medium (○). L-Pro-*p*-nitroanilide was used as substrate and 0.5 mM-EDTA was added as a specific inhibitor for all the other aminopeptidase activities (see Table 2). 100% FAP3.BR specific activity corresponds to 5.3×10^{-6} units mg^{-1} .

present in the gel-liquid system. Finally, the selective determination of FAP 5.BR could be achieved by using glutamic acid *p*-nitroanilide as substrate. From the data obtained, no conditions could be found for the selective determination of FAP 4.BR and/or FAP 6.BR in crude extracellular concentrates.

Based on these results we were able to specifically measure the variations in FAP 3.BR activity in crude extracts. As shown in Fig. 4 a similar rapid decrease in extracellular FAP 3.BR specific activity after day 3 was observed in both BAP medium and BAP-PCM medium.

Taken together, our findings suggest that all *Frankia* aminopeptidases (except perhaps FAP 3.BR) are metalloenzymes. Furthermore, they can be classified into two groups according to their sensitivity to inhibitors. The first group, including FAP 1-2.BR, is sensitive to bestatine which inhibits aminopeptidase B (EC 3.4.11.6) and leucine aminopeptidase (EC

Fig. 5. Extracellular gelatinolytic activity in stirred BAP-PCM medium.

3.4.11.1) (Umezawa *et al.*, 1976). The second group, comprising FAPs 4-5-6.BR, is sensitive to amastatin which inhibits aminopeptidase A (EC 3.4.11.7) and leucine aminopeptidase (Aoyagi *et al.*, 1978).

Variation of whole extracellular gelatinolytic activity as a function of the age of the culture

Overall extracellular proteolytic activity could be measured using [¹⁴C]gelatin as substrate in the presence of 0.1% Triton X-100. [¹⁴C]Casein was only marginally degraded under similar conditions. As shown in Fig. 5, global extracellular gelatinolytic activity showed a peak at day 4 in BAP-PCM medium in stirred conditions. This corresponded to both maximal growth in BAP-PCM medium and arrest of secretory activity (Fig. 1*b, d*). These results are in good agreement with the variations in band activities obtained by electrophoretic analysis of the extracellular proteolytic activities discussed below. Cells grown in BAP medium, but in static conditions also

Fig. 6. SDS-gelatin-PAGE analysis of *Frankia* proteinases. Bands of extracellular activities are arrowed to the left. For comparison, bands of intracellular activities are arrowed to the right. Encircled numbers indicate bands of proteolytic activities present both extracellularly and intracellularly.

secreted proteolytic activities. Maximal secretion and gelatinolytic activity were also coincidental with growth arrest (results not shown).

Electrophoretic analysis of extracellular proteolytic activities

Up to 15 proteinases were secreted when *Frankia* BR cells were grown in BAP-PCM medium under stirred conditions (Fig. 6). Proteinases were visualized after treatment of extracellular extracts (100 µg protein) with 2.5% SDS, 1% sucrose, 0.5% bromophenol blue at 37 °C for 15 min and analysis by SDS-gelatin-PAGE. The presence of 1 mM-DTT was essential for good results. In Fig. 6, *Frankia* intracellular and extracellular proteinases (PF) were compared for electrophoretic mobility and classified from the lowest migration rate (PF-1) to the highest (PF-22). Only five of the secreted proteinases were exclusively extracellular, while seven were exclusively intracellular, and 10 secreted activities were found

Table 3. Localization, molecular mass and nomenclature of *Frankia* proteinases

These data were obtained from Fig. 6.

Extracellular			Both		Intracellular			
No.	kDa	Designation	No.	kDa	Designation	No.	kDa	Designation
2.	39.5	PF-39.5	1.	40	PF-40	4.	38	PF-38
3.	38.5	PF-38.5	5.	37.5	PF-37.5	9.	32	PF-32
6.	36.5	PF-36.5	7.	35	PF-35	10.	30.5	PF-30.5
15.	25.5	PF-25.5	8.	33	PF-33	12.	28.5	PF-28.5
20.	20.5	PF-20.5	11.	30	PF-30	14.	26	PF-26
			13.	27.5	PF-27.5	18.	22	PF-22
			16.	24	PF-24	19.	21	PF-21
			17.	22.5	PF-22.5			
			21.	19.5	PF-19.5			
			22.	19	PF-19			

to be both. The molecular mass of each proteinase and their localization are indicated in Table 3.

Analysis of the electrophoretic pattern of extracellular

proteinases as a function of age in BAP-PCM medium indicated that for some of them (i.e. PF-8, -11, -15, -16, -17, -20, -21 and -22) there was a sharp and coordinate increase at day 4 that coincided with growth arrest under these conditions (Fig. 1*a*). Slight decreases of activity were also observed after day 5 for PF-3, -5, -8, -11 and -20. This may explain the variations observed when measuring global gelatinolytic activities as a function of the age of the culture under the same conditions (Fig. 5).

Discussion

In this paper we describe changes in extracellular proteins, particularly proteinases and aminopeptidases, of *Frankia* BR as a function of age and different growth conditions. *Frankia* BR cells secreted a variety of proteins during exponential growth under stirred conditions. Protein secretion also occurred during unbalanced growth under batch conditions, indicating that it is an intrinsic property of *Frankia* cells and is independent of culture conditions.

Cells grown under stirred conditions in BAP-PCM medium secreted proteins at a higher rate than in stirred BAP medium alone, indicating that both phosphatidylcholine and MES buffer present in BAP medium may play a role in the secretion process. Phosphatidylcholines are ubiquitous membrane constituents and may influence membrane fluidity and integrity. On the other hand, cells grown under stirred conditions, in the presence of phosphatidylcholine and MES buffer, show a characteristic 'open mesh' type mycelium (Schwencke, 1991), and this may enhance the elimination of waste products as well as secretion. Furthermore, several authors (Chahal & Nada, 1976; Fairbairn & Law, 1987; Strasser de Saad *et al.*, 1987) have found that a pH around 6.8–7.0 is optimal for secretion of some proteins. This condition was obtained by addition of MES buffer to the *Frankia* culture which otherwise became alkaline during growth.

Secretion of proteins increased just before growth arrest in exponentially growing cells and ceased at growth arrest. The slight increase observed thereafter is most probably due to the release of intracellular protein after cell lysis. Similar behaviour was observed for cells growing in unbalanced static conditions. We also found a number of qualitative and quantitative changes in the pattern of secreted proteins depending on the age of the culture as well as on the nature of the medium. This suggests that *Frankia* has a considerable ability to adapt to varying physiological conditions. The SDS-PAGE patterns of these proteins, which have been proposed as a valuable approach to the identification of *Frankia* strains and other actinomycetes species (Benson & Hanna, 1983; Gardes & Lalonde, 1987; Dietz, 1988), should

therefore be interpreted with caution. In contrast, intracellular aminopeptidase patterns were found to be remarkably stable in cultures of different ages, confirming the suggestion that they may be useful for strain classification (Gardes *et al.*, 1987; Benoist & Schwencke, 1990). Furthermore, extracellular aminopeptidases did not show any age-dependent changes, with the exception of FAP 3.BR, specific for proline, which showed a significant decrease after cell growth arrest, although the intracellular enzyme activity remained unchanged. Whether loss of extracellular activity is due to arrest of secretion or proteolytic degradation or both remains an open question.

Extracellular and intracellular aminopeptidases shared the same substrate specificity and electrophoretic migration values. However, they are not necessarily identical as minor changes in molecular mass and biochemical properties may be undetectable by the methods employed here. The same can be said for intracellular and extracellular proteinases having similar electrophoretic migration characteristics.

Although gelatinolytic activity was previously reported in some strains of *Frankia* (Horrière, 1984), this paper is the first report of secretion of proteinases by *Frankia*. The ability of *Frankia* to secrete proteinases is not surprising, as proteinase secretion is well-documented for the genus actinomycete *Streptomyces* [Renko *et al.*, 1981; Peczyńska-Czok & Mordarsky, 1988 (review); Aretz *et al.*, 1989]. The fact that several extracellular proteinases showed a coordinate rise in activity at growth arrest is intriguing. One possible explanation is increased secretion of high-molecular-mass proteinase complexes (proteasomes). Current work (unpublished) from our laboratory supports this hypothesis. It is also interesting to note that periplasmic proteasomes have been proposed to exist in *E. coli* (Vaithilingam & Cook, 1989).

Because studies of *Frankia* proteinases are very scanty, their physiological role is not clearly understood. Proteinases have been proposed to play a role in a diversity of processes (Bond & Butler, 1987). Further work will be required to define their function(s) in *Frankia*.

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