An Extracellular Blood-anticoagulant Glycopeptide Produced Exclusively during Vegetative Growth by *Myxococcus xanthus* and Other Myxobacteria Is Not Co-regulated with Other Extracellular Macromolecules

By PHILIPPE J. MASSON¹ AND J. F. GUESPIN-MICHEL^{2*}

¹ Laboratoire de Génétique Microbienne, Université de Technologie de Compiègne, BP 233–60206, Compiègne, France

² Laboratoire de Microbiologie, Université des Sciences et Techniques de Rouen, BP 118–76134, Mont-Saint-Aignan Cedex, France

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We have shown that the blood anticoagulant activity (BAA) secreted by *Myxococcus xanthus* (designated myxaline) is a heat-stable molecule; a high-molecular-mass extracellular fraction also with an apparent BAA is a thermolabile protease. This property allowed us to assay the BAA content in crude boiled culture supernatants and to study the conditions under which it is produced. Heat-stable BAA is strictly extracellular and its production is restricted to vegetative growth in *M. xanthus*. Unlike the other extracellular proteins, its production is not affected by mutations that regulate secretion; mutations that modify the extracellular proteolytic activity do not modulate the amount of myxaline produced either. Several other species of *Myxococcus* and one other myxobacterial species produce a heat-stable BAA during vegetative growth.

INTRODUCTION

Myxobacteria are Gram-negative bacteria notable for their ability to undergo a morphogenetic multicellular development upon nutrient starvation (Wireman & Dworkin, 1975). They move by gliding (Kaiser *et al.*, 1979) and produce numerous extracellular molecules.

All species of myxobacteria produce extracellular proteins. Some of these, most notably the proteases (Gnossopelius, 1978; Coletta & Miller, 1986) have been studied in *Myxococcus xanthus* and *M. virescens*. All species also produce slime, the sugar composition of which differs slightly from one species to another and is roughly constant throughout growth and morphogenesis (Sutherland & Thomson, 1975; Sutherland, 1979). In addition numerous strains produce, during the growth phase, antibiotics and other secondary metabolites, the nature of which differs greatly not only between species but also from strain to strain (Gerth *et al.*, 1983; Reichenbach *et al.*, 1987).

A glycopeptide with blood anticoagulant activity (BAA) has recently been purified from the supernatant of growing cells of *M. xanthus* (El Akoum *et al.*, 1987) and designated myxaline. Its biological activity was studied at different stages of coagulation and differed from that of heparin (El Akoum, 1987). In this paper we have investigated further the properties of this BAA.

METHODS

Bacterial strains. These are listed in Table 1.

Media and growth conditions. The bacteria were grown in Erlenmeyer flasks under rotating agitation at 300 r.p.m. in CTT medium [1%, w/v, Casitone (Difco); 10 mM-Tris/HCl; 1 mM-KH₂PO₄/K₂HPO₄; 0.2%, w/v, MgSO₄·7H₂O; pH 7.6] at 30 °C (Bretscher & Kaiser, 1978). Myxospore formation was induced by the technique

Abbreviations: PA, proteolytic activity; BAA, blood-anticoagulant activity; PMSF, phenylmethylsulphonyl fluoride; 2-ME, 2-mercaptoethanol.

Table 1. *Bacterial strains*

Y, yellow; T, tan; Fru, fruiting body formation; Mot, motile; Muc, mucus; Exc, excretion.

Strain	Relevant characteristics	Source or reference
<i>Myxococcus xanthus</i>		
CM001	Derived from DK101 Y Fru ⁺ Mot ⁺ Muc ⁺ Exc ⁺	Hodgkin & Kaiser (1979)
CM005	CM001::Tn5	Nicaud <i>et al.</i> (1984)
CM011	CM001::Tn5	Guespin-Michel <i>et al.</i> (1984)
CM019	CM001::Tn5	Nicaud <i>et al.</i> (1984)
DZ1	T Fru ⁻ Mot ⁻ Muc ⁻ Str ^r Exc ⁻	Zusman <i>et al.</i> (1978)
CM101	DZ1::Tn5	This laboratory
CM102	DZ1::Tn5	
CM103	DZ1::Tn5	
CM2050	DZ1::pME305	
CM2053	CM101::pCM2019	
CM2056	CM101::pCM2019	
CM2057	CM101::pCM2019	
DK1622	Y Fru ⁺ Mot ⁺ Exc ⁺	Hodgkin & Kaiser (1977)
Mxx12	Myxalamid-producing strain	Gerth <i>et al.</i> (1983)
CM500	Mxx12 Str ^r	This laboratory
<i>Myxococcus fulvus</i>		
Mxf16	Myxothiazol-producing strain	Gerth <i>et al.</i> (1980)
CM700	Mxf16 Str ^r	This laboratory
<i>Myxococcus stipatus</i>		
Mxs1		Reichenbach <i>et al.</i> (1987)
CM800	Mxs1 Str ^r	This laboratory
<i>Stigmatella aurantiaca</i>		Kunze <i>et al.</i> (1984)
<i>Myxococcus virescens</i>		Reichenbach <i>et al.</i> (1987)

of Dworkin & Gibson (1964). Glycerol (10 M) (Merck) was added to an exponential phase culture to a final concentration of 0.5 M.

Bacteria were also grown in Petri dishes according to Kuner & Kaiser (1982). Exponentially growing *M. xanthus* strain DK1622 was diluted to 10^7 cells ml⁻¹ and dispersed into polystyrene Petri dishes (10 ml CTT) and left standing undisturbed at 30 °C for 24 h. The cells adhered to the bottom of the dish and the growth medium was removed by aspiration. Fruiting body formation was then induced by addition of 10 ml per dish 1 mM-CaCl₂ in 10 mM-MOPS/NaOH (pH 6.8). Fruiting bodies containing myxospores were formed within 72 h.

Analytical methods. (i) *Protease assay.* (Millet, 1970.) Protease activity was assayed using the synthetic substrate azocasein (Sigma) at a concentration of 0.5% (w/v) in 0.1 M-Tris/HCl (pH 8.0). The sample (1 ml) was added to 1 ml of azocasein solution and the mixture was incubated at 37 °C for 30 min. The reaction was stopped by the addition of 10% (w/v) trichloroacetic acid (2 ml). After standing at 4 °C for 30 min the mixture was centrifuged for 20 min at 6000 g and 1 ml 0.5 M-NaOH was added to 1 ml of the supernatant. One unit (UA) of protease activity is defined as the amount of enzyme that hydrolysed 1 mg azocasein in 30 min at 37 °C.

(ii) *Protein assay.* (Sedmak & Grossberg, 1977.) Extracellular protein concentration was measured by the addition of 0.5 ml Coomassie blue G (Sigma) (0.06%, w/v, in 0.6 M-HCl) to 0.5 ml of sample. The ratio A_{620}/A_{465} was used to determine protein concentration; BSA was used as a standard.

(iii) *Carbohydrate assay.* (Haska & Stahl, 1971.) The amount of polysaccharide in the culture supernatant was estimated by the anthrone method with glucose as a standard, and is expressed as glucose equivalents (G_{eq} ; μ g ml⁻¹). To minimize the effect of free sugars and substances other than polysaccharides on the colour reaction, the polysaccharides were precipitated from the culture solution by the addition of 3 vols 95% (v/v) ethanol; after storing at -20 °C overnight the polysaccharides were collected by centrifugation (5000 g for 10 min) and suspended in distilled water to a suitable dilution. The sample (1 ml) was then added to 2 ml of anthrone solution (Sigma) at 0.2% (w/v) in H₂SO₄ at 0 °C. The mixture was heated for 16 min at 90 °C. The absorbance was measured at 625 nm.

(iv) *Anticoagulant activity.* This was assayed by recalcified plasma clotting time, with a KC10 Dade coagulometer, using heparin (Roche) as reference. Plasma (0.1 ml) was incubated with 0.1 ml of sample in Michaelis buffer (25 mM-sodium barbital, 40 mM-sodium acetate, 100 mM-sodium chloride; pH 7.3) for 5 min at 37 °C. Then 0.1 ml 30 mM-CaCl₂ was added. One international unit (UI) is defined as the amount of product that increases the clotting time by as much as one heparin unit. The sample was either ethanol-precipitated (3 vols 95%, v/v, ethanol) material or an ultrafiltrate on Amicon membrane (cut-off as described) of a culture

Fig. 1. Correlation between PA and BAA in ethanol-precipitated culture supernatants of various mutants of *M. xanthus*.

supernatant. Cell-bound anticoagulant activity was assayed on whole cells extracted with phenol (final concentration 25%, w/v), the aqueous phase being precipitated with 95% ethanol.

Chemicals. Phenylmethylsulphonyl fluoride (PMSF) was purchased from Sigma and 2-mercaptoethanol (2-ME) from Merck.

RESULTS

A heat-stable fraction of total extracellular BAA parallels myxaline production

El Akoum *et al.* (1987) showed that purified myxaline never exceeded 10 to 20% of the total BAA of the culture supernatant, whatever the method used for its purification. In addition the remaining BAA co-eluted with high-molecular-mass and/or low-affinity material during purification of myxaline on a pseudo-affinity column (histidyl-Sepharose 4B) (Vijayalakshmi *et al.*, 1986) from crude concentrated culture supernatant (unpublished results). Thus either a high-molecular-mass precursor or a complex of myxaline is present in the supernatant, or another molecule is able to prevent blood coagulation in our assay. The most obvious candidates for the latter hypothesis are the extracellular proteases produced by *M. xanthus*. In order to test this hypothesis, we checked the correlation between proteolytic activity (PA) and total BAA, when the former was decreased by mutations (Fig. 1), or by inactivating treatments (Table 2). The majority of the BAA was found to be due to high-molecular-mass, heat-labile PA, whereas a small fraction (10%) of BAA was due to a heat-stable low-molecular-mass molecule. The latter activity was inactivated by 2-ME.

The properties of this activity were thus consistent with that investigated by El Akoum *et al.* (1987). Not all the proteolytic enzymes had the same BAA (Table 2), since heat-inactivation of the low-molecular-mass PA or its inactivation by PMSF treatment did not decrease the BAA of this fraction. Moreover, purified myxaline is indeed heat stable. This property is extremely useful for assaying myxaline in boiled crude culture supernatants, after concentration by ethanol precipitation or by ultrafiltration on a 1 kDa cut-off Amicon membrane. Reproducible results can be obtained from these procedures. For example, assays of ethanol-precipitated supernatants of strains CM011 and CM001 gave BAA values of 0.019 ± 0.006 and 0.015 ± 0.004 UI ml⁻¹ respectively. In addition, heat-treated concentrated supernatant was stabilized with regard to BAA as compared with untreated supernatant.

Production of heat-stable BAA is not co-regulated with that of extracellular proteins or polysaccharides

Several mutations impair the secretion of most extracellular proteins in *M. xanthus* (Nicaud *et al.*, 1984; Breton & Guespin-Michel, 1987). We therefore investigated the production of heat-stable BAA and polysaccharides by these Exc⁺⁻ or Exc⁺⁺ mutants. Table 3 shows that

Table 2. *Effects of various treatments on PA and BAA activities from strain CM001*

High-molecular-mass (> 10 kDa) and low-molecular-mass (< 10 kDa) fractions of ethanol-precipitated culture supernatant from *M. xanthus* CM001 were obtained by ultrafiltration on an Amicon membrane. The fractions were treated as shown and assayed for PA and BAA. Values are in units per ml of supernatant. Note that precipitation and ultrafiltration decrease the proteolytic activity of the standard (1 UA ml⁻¹).

	> 10 kDa fraction		< 10 kDa fraction	
	PA (UA ml ⁻¹)	BAA (UI ml ⁻¹)	PA (UA ml ⁻¹)	BAA (UI ml ⁻¹)
No treatment	1	0.18	0.22	0.02
50 °C, 10 min	0.17	0.02	0.1	0.02
50 °C, 20 min	0.12	0.016	0.06	0.02
100 °C, 2 min	0	0	0	0.02
PMSF (1 mM)	0.4	0.063	0.03	0.02
2-ME (1%)	0.04	0.004	0	0

Table 3. *Proteins, proteases, polysaccharides and heat-stable BAA in culture supernatants from various strains and mutants of M. xanthus*

Values are per ml of supernatant.

Strain	Proteins (µg ml ⁻¹)	Proteases (UA ml ⁻¹)	Polysaccharides (µg Glc eq. ml ⁻¹)	Heat-stable BAA (UI ml ⁻¹)
CM001	50 ± 10	3.2 ± 0.9	31 ± 5	0.015 ± 0.004
CM011	46 ± 12	3.2 ± 1	35 ± 5	0.019 ± 0.006
DK1622	32 ± 4	5.3 ± 0.1	15 ± 2	0.020 ± 0.005
CM005	35 ± 8	1.5 ± 0.8	22 ± 4	0.018 ± 0.007
CM019	39 ± 11	1.6 ± 0.4	23 ± 8	0.015 ± 0.003
DZ1	33 ± 8	1.3 ± 0.5	18 ± 5	0.016 ± 0.005
CM103	41 ± 7	2.5 ± 0.6	27 ± 10	0.014 ± 0.003
CM2050	54 ± 12	3.8 ± 0	30 ± 10	0.013
CM2053	37 ± 0	1.4 ± 0	26 ± 4	0.012
CM2056	33 ± 4	0.9 ± 0.3	24 ± 2	0.012
CM2057	36 ± 4	0.6 ± 0	26 ± 0.5	0.016
CM101	40 ± 9	0.6 ± 0.2	23 ± 6	0.017 ± 0.009
CM102	36 ± 6	0.8 ± 0.2	25 ± 8	0.022 ± 0.009

polysaccharide production was affected by these mutations, but no significant correlation with myxaline production could be seen. Thus, myxaline is not controlled by the genes that regulate protein secretion.

In repeated assays we could not detect any cell-bound BAA; in addition, Table 3 shows that mutants that produce less or more proteases are not altered in their heat-stable BAA production, which indicates that at least those proteases are not involved in the production of this activity.

Myxaline production is strictly restricted to vegetatively growing cells

The lack of regulation of myxaline secretion by the genes that control protein secretion during growth of *M. xanthus* prompted us to check whether its synthesis could be developmentally regulated. Glycerol-induction of myxospores from strains CM001 and CM011 did not increase the amount of BAA in the medium. Strain DK1622 (which is the only suitable strain for morphogenesis induction in submerged culture) produces heat-stable BAA during growth in Petri dishes, but no activity was detected after morphogenesis induction at any stage of the developmental cycle. In agreement with these results Fru⁻ mutants are not affected in their production of BAA (Table 3). Since Mot⁺, Mot⁺⁻ and Mot⁻ strains were assayed, motility is not required for the production of myxaline.

A heat-stable BAA is produced by several myxobacterial species

Since all the strains we have assayed are derived from the same wild-type ancestor, we wondered whether myxaline could be a secondary metabolite such as those produced by many myxobacteria during vegetative growth (Reichenbach *et al.*, 1987). Such secondary metabolites differ, not only from species to species, but even from strain to strain within the same species.

A heat-stable BAA was found in the culture supernatant of growing cells of another strain of *M. xanthus* as well as from strains of several other species of myxobacteria. The activities (UI ml⁻¹) were 0.007 (*M. xanthus* strain CM500), 0.009 (*M. virescens* Mxv48), 0.011 (*M. stipitatus* CM800), 0.012 (*M. fulvus* CM750) and 0.013 (*Stigmatella aurantiaca* DW4).

DISCUSSION

A heat-stable extracellular BAA is produced by growing cells of several species of myxobacterales (genera *Myxococcus* and *Stigmatella*). In *M. xanthus* this activity has been purified and shown to be a glycopeptide, termed myxaline (El Akoum *et al.*, 1987), whose production is not controlled by the genes that regulate the secretion of most extracellular proteins. Since these genes also regulate the production of extracellular polysaccharides, myxaline is unlikely to be a precursor of the slime, nor a product of proteolytic or glycosidic cleavage of this molecule. Whatever the nature of its production, it seems to be strictly restricted to the vegetative growth phase. Its production is proportional to the cell dry weight as shown by El Akoum (1987).

At least two surface glycoproteins are known in *M. xanthus* (Dobson & McCurdy, 1979; Maeba, 1986). The composition of myxaline (El Akoum *et al.*, 1987) differs from both these molecules. However, it is still possible that myxaline is derived from an as yet unidentified glycoprotein precursor.

The production of myxaline by strains from several different species of myxobacteria is not consistent with it being a secondary metabolite. As myxaline production is apparently not regulated by the mechanisms that control the secretion of most extracellular macromolecules and as activity is present in several strains and species of myxobacteria, myxaline may have a specific and important role during vegetative growth of these bacteria.

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