Attacin – an insect immune protein – binds LPS and triggers the specific inhibition of bacterial outer-membrane protein synthesis

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Attacin is a 20 kDa antibacterial protein, originally isolated from the immune haemolymph of *Hyalophora cecropia*. It has been demonstrated previously that attacin causes increased permeability of the outer membrane of *Escherichia coli* and inhibition of outer-membrane protein synthesis at the transcriptional level. This is accompanied by inhibition of growth. Here, LPS is shown to serve as the receptor for attacin and evidence is presented that attacin does not need to enter the cell to exert its activity. The increase in outer-membrane permeability precedes any increase in inner-membrane permeability by at least one generation time (~45 min), and the inhibiting effect of attacin on synthesis of outer-membrane proteins is detectable after only 10 min. It is also shown that attacin causes induction of several stress proteins and increased synthesis of LPS within, respectively, 25 and 60 min of treatment. Based on the results presented, it is proposed that attacin has the unique ability to specifically interfere with synthesis of outer-membrane proteins without entering the inner membrane or cytoplasm.

Keywords: attacin, outer-membrane proteins, lipopolysaccharide, stress protein induction, *Escherichia coli*

INTRODUCTION

Attacin is a 20 kDa immune protein produced in pupae of *Hyalophora* spp. (Lepidoptera) in response to bacterial infection (Hultmark *et al.*, 1983). We have previously shown that there are two forms of attacin, one neutral (pI 7) and one basic (pI 9) (Engström *et al.*, 1984a). The primary sequences of the two forms are 80% identical. The difference in pI is explained by a difference in aspartic acid content (Engström *et al.*, 1984a; Kockum *et al.*, 1984). Both forms contain three hydrophobic regions in the amino-terminal part (Engström *et al.*, 1984a). Attacin-like proteins have been described in other insect species, such as *Drosophila melanogaster* (Flyg *et al.*, 1986) and *Sarcophaga peregrina* (Ando *et al.*, 1987). Attacin inhibits growth of Gram-negative bacteria and increases the permeability of the outer membrane (OM) (Engström *et al.*, 1984b) – a structure unique to Gram-negative cells serving as a permeability barrier to the passage of hydrophobic and large (> 800 Da) hydrophilic substances. The synthesis

of several OM proteins (Omps) is inhibited by attacin, and this occurs at least partly at the level of transcription (Carlsson *et al.*, 1991).

In the present study we wanted to investigate further the mechanisms involved in this inhibition of Omp synthesis. Our main questions were: (a) is binding of attacin to the cell surface sufficient for the subsequent effects, or does it have to enter into the cell; (b) are there any other specific effects on protein synthesis; and (c) is LPS synthesis affected?

We show that LPS on the cell surface serves as the receptor for attacin and we suggest that the effects on Omp biosynthesis are achieved by an 'indirect mechanism', i.e. attacin does not need to enter the inner membrane (IM) or cytoplasm to turn off the synthesis of Omps.

METHODS

Preparation of attacin. Diapausing pupae of *Hyalophora gloveri* or *Hyalophora cecropia* were injected with 10⁵ live *Enterobacter cloacae* β12 and the haemolymph was collected after 6–8 d as described by Hultmark *et al.* (1980) and stored at –20 °C. Neutral and basic attacins were purified by salt

Abbreviations: BPI, bactericidal/permeability-increasing protein; IM, inner membrane; OM, outer membrane; Omp, outer-membrane protein.

Table 1. Bacterial strains

Strain	Relevant characteristics	Reference or source*
<i>E. coli</i> K-12		
MC4100	LPS Ra	Casabadan (1976)
D21	<i>rfa</i> , LPS Ra	Boman <i>et al.</i> (1968)
D21e7	<i>rfa</i> , LPS Rc	Eriksson-Grennberg <i>et al.</i> (1971)
D21f1	<i>rfa</i> , LPS Rd	Boman & Monner (1975)
D21f2	<i>rfa</i> , LPS Re	Boman & Monner (1975)
CE1224	<i>ompR</i> , LPS Ra	Tommasen <i>et al.</i> (1983)
CE1448	CE1224 $\Delta(ompT\ degP)$	C. Jansen ¹
JM105	<i>pldA</i> ⁺	Yanisch-Peron <i>et al.</i> (1985)
CE1349	JM105 $\Delta pldA$	R. Brok ¹
KP1229	W3110 $\Delta(ana-tonB-trp)$	Ahmer <i>et al.</i> (1995)
MO/pRZ540	<i>tonB</i> ⁺	Postle & Reznikoff (1979)
PC1989	<i>tolA</i>	Phabagen Collection ¹
PC1990	<i>tolB</i>	Phabagen Collection ¹
AB1157	<i>tol</i> ⁺	Tommasen <i>et al.</i> (1983)
PND2000	MC4100- λ RS88(<i>degP-lacZ</i>)	Danese <i>et al.</i> (1995)
AF633	MC4100 $\lambda\Phi(uspB-lacZ)$	A. Farewell & T. Nyström ²
AF634	MC4100 $\lambda\Phi(usp-lacZ)$	Farewell <i>et al.</i> (1996)
TN 21512	MC4100 $\lambda\Phi(bolA-lacZ)$	A. Farewell & T. Nyström ²
<i>P. mirabilis</i>		
EH193	<i>rfa</i> , LPS Re	Kotelko (1986)

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precipitation (ammonium sulphate) followed by ion exchange chromatography as previously described (Engström *et al.*, 1984a).

Bacterial strains and growth conditions. The bacterial strains used are listed in Table 1. Cells were grown at 37 °C in LB medium or in M9 minimal medium [supplemented with 0.4 % (w/v) glycerol and the amino acids required for the respective strains] as indicated. Growth experiments were performed in microtitre plates, with a culture volume of 200 μ l. Attacin and/or other substances (e.g. $MgCl_2$) to be tested were added to the growth medium and the medium was inoculated with 5×10^6 cells in mid-exponential phase. The cultures were incubated at 37 °C on a rotary shaker and growth was recorded every 30 min by a Multiskan spectrophotometer. Both forms of attacin were used in the experiments and yielded the same type of results, differing only in the degree of activity.

LPS derivatives in binding experiments. The origin of *Escherichia coli* LPS derivatives used for binding experiments was as follows: rough LPS of chemotype Rd, Sigma L6893; monophosphoryl lipid A, Sigma L6638; and diphosphoryl lipid A, Sigma L5399. Stock solutions of LPS were prepared at 1 mg ml⁻¹ in distilled water, heated to 55 °C for 20–30 min, sonicated for 3 min and stored at –20 °C. Prior to use the solutions were heated and sonicated. Stock solutions of lipid A (0.5 mg ml⁻¹) were prepared in 0.025 % (v/v) triethanolamine in distilled water, sonicated for 3 min and stored at 4 °C. Prior to use the solutions were sonicated for 3 min.

Assay of outer- and inner-membrane permeability. Cells were incubated in LB in the presence of IPTG (to induce β -galactosidase), with or without attacin. Samples were withdrawn after various times, centrifuged and the supernatants frozen. When all samples had been collected the supernatants

were thawed and assays of β -lactamase (Lehrer *et al.*, 1988) and β -galactosidase (Miller, 1972) were performed to indicate release of periplasmic and cytoplasmic proteins, respectively. The β -lactamase assay mixture contained 75 μ l supernatant, 125 μ l LB and 15 μ l PADAC (pyridine-2-azo-*p*-dimethylaniline cephalosporin, 0.2 mg ml⁻¹). The β -galactosidase assay mixture contained 75 μ l supernatant, 125 μ l Z-buffer (Miller, 1972) and 40 μ l ONPG (4 mg ml⁻¹). The assay mixtures were incubated at 37 °C. The absorbance was determined at 560 nm for β -lactamase and 420 nm for β -galactosidase, and the optical density at 560 nm for cell cultures using a Multiskan spectrophotometer. The formula $A_{\text{assay}} \times (\text{time} \times OD_{\text{cell}} \times \text{volume})^{-1} \times 1000$ was used to calculate relative enzyme activities. (A_{assay} = absorbance in enzyme assay; OD_{cell} = optical density of cell culture at time of sample withdrawal; time = assay time).

Radioactive pulse-labelling of cellular proteins. Cells were grown in M9 minimal medium. Attacin was added at mid-exponential phase and samples were withdrawn at different times and transferred to tubes containing 30 μ Ci ml⁻¹ (1.1×10^6 Bq ml⁻¹) L-[³⁵S]methionine (> 37 TBq mmol⁻¹; Amersham) and non-radioactive methionine to a final concentration of 65 nM (to ensure linear incorporation during the labelling period). Labelling was stopped after 30 s by the addition of 1 vol. 20 % (w/v) trichloroacetic acid (TCA), or chased after 30 s with non-radioactive methionine (final concn 2 μ M) for 3 min and then stopped by the addition of 1 vol. 20 % TCA.

Radioactive labelling of LPS. Cells were grown in LB medium to mid-exponential phase. Attacin was added and samples were withdrawn at different times and transferred to tubes containing 20 μ Ci ml⁻¹ (7.4×10^5 Bq ml⁻¹) of D-[U-¹⁴C]-galactose (10.9 GBq mmol⁻¹) or N-acetyl-D-[¹⁴C]glucosamine (1.96 GBq mmol⁻¹) or [2-¹⁴C]acetate (2.07 MBq

mmol⁻¹). Labelling was continued for 60 min and then stopped by the addition of 1 vol. 20% TCA.

Gel electrophoresis, autoradiography and fluorography. SDS-PAGE was performed according to Laemmli (1970) with the modifications described by Randall & Hardy (1977). Gels containing protein samples labelled with ³⁵S were dried after staining with Coomassie brilliant blue, overlaid with Fuji film and exposed for 1–3 d at –70 °C for autoradiography.

Gels containing LPS samples labelled with ¹⁴C were stained with silver (Tsai & Frasch, 1982) and incubated for 30 min in 1 M sodium salicylate (Chamberlain, 1979) before being dried and overlaid with Fuji film at –70 °C for fluorography.

Resolution of proteins by two-dimensional gel electrophoresis. Culture samples were processed to produce extracts for resolution by two-dimensional SDS-PAGE as described by O'Farrell (1975) with modifications (VanBogelen & Neidhardt, 1990). Alphanumeric designations were assigned to protein spots after matching them to the reference two-dimensional images of the gene protein database of *E. coli* (VanBogelen *et al.*, 1990).

Coupling of attacin to agarose beads. Attacin was covalently coupled to agarose beads (Affi-Gel10, Bio-Rad) as described by LaPorte *et al.* (1977). Attacin (12 mg) in 0.1 M NaHCO₃, pH 7.5, was coupled to 0.5 ml Affi-Gel. Underivatized agarose beads, i.e. beads treated as above but without attacin, were used as controls.

There are practical problems with this procedure: it requires a large amount of material and uncertainty of coupling efficiency makes it difficult to estimate the effective concentration of coupled attacin. To test LPS-binding ability we incubated agarose-coupled attacin, underivatized agarose and free attacin with solubilized LPS Rd for 30 min at 37 °C. The samples were analysed by SDS-PAGE followed by silver staining for LPS (Tsai & Frasch, 1982). Since the results demonstrated that LPS was present in samples with agarose-coupled attacin as well as in those containing free attacin, whilst samples with underivatized agarose contained no detectable LPS, the protocol for coupling of attacin to agarose beads was judged to be satisfactory.

Assay of induction of stress proteins. Strains containing transcriptional fusions of various stress promoters with *lacZ* were incubated with or without attacin (20 µM) in LB medium. Samples were withdrawn after various times and frozen. β -Galactosidase assays of the thawed culture samples were performed as described for the permeability assay, but using cell culture samples instead of supernatants. In addition to the strains listed in Table 1, we assayed strains from a commercial kit, the Pro-Tox (C) assay from Xenometrix, containing various stress-related gene promoters fused with *lacZ*.

RESULTS

The effect of attacin on OM integrity is separate in time from the effect on IM integrity

We previously concluded (Engström *et al.*, 1984b) that the primary target for interaction with attacin is the OM, and that this interaction causes the subsequent effect on protein synthesis. Possible damage to the IM appears to be a secondary effect, not essential for attacin action. These conclusions are reinforced here by the demonstration that when strain D21 was treated with attacin, leakage of β -lactamase from the periplasm into

Fig. 1. β -Lactamase and β -galactosidase enzyme assays of supernatants from D21 cells incubated with 10 µM attacin or without attacin. Values for relative enzyme activity were calculated as described in Methods.

the culture supernatant occurred early and increased rapidly (approx. 40% had leaked out after 40 min). This damage to the OM barrier precedes the leakage of β -galactosidase from the cytoplasm by at least 40 min (Fig. 1). Less than 10% of the total content of β -galactosidase had leaked out by 2 h. These results confirm our previous conclusion that the primary target of attacin is the OM.

The LPS chemotype of the cell affects its sensitivity to attacin

To determine if the LPS chemotype is important for the effect of attacin on the cell, we compared the growth-inhibiting effect exerted by attacin on four isogenic *E. coli* K-12 strains of different chemotypes, i.e. differing only in the length of the LPS core (Fig. 2). The deep rough, heptose-less strain D21f2 was the most sensitive and there was a step-wise decrease in sensitivity of the strains as the length of the LPS core increased. The effect on growth of strain D21 was very low at the concentration of attacin used (10 µM), but nevertheless there was a significant effect on OM integrity (Fig. 1). These observations suggest that attacin interacts with the inner regions of the LPS moiety, since these parts are more exposed on the surface of cells having short LPS cores.

Binding of attacin to LPS in solution inhibits its antibacterial activity

To test if the activity of attacin is inhibited by free LPS, attacin was incubated with varying amounts of solubilized LPS of chemotype Rd at 37 °C for 30 min,

Fig. 2. Effect of attacin on growth of cells with different LPS chemotypes. Four *E. coli* strains were incubated in LB medium in the absence or presence of 10 μ M attacin. The control curve shown represents D21f2; results obtained with the other three strains were similar. A schematic representation of the polysaccharide chain length of the different LPS chemotypes is included.

whereafter the mixture was added to the cell culture (Fig. 3). Preincubation of attacin with a $5 \times$ molar excess of LPS totally abolished the growth-inhibiting effect of attacin. Using mono- and diphosphoryl lipid A instead of LPS also resulted in a decreased effect of attacin, but only partially. This indicates that an interaction of attacin with the inner core might be necessary for efficient binding.

Polymyxin-resistant cells are sensitive to attacin

Proteus mirabilis and other *Proteus* strains are inherently resistant to antibacterial cationic substances such as the insect immune peptide cecropin (Hultmark *et al.*, 1980) and the lipopeptide polymyxin (Storm *et al.*, 1977). For *P. mirabilis* (Sidorchuk *et al.*, 1983), this resistance is suggested to be due to the high content of phosphate-linked 4-aminoarabinose in the LPS, which interferes with binding of cationic agents by making the LPS moiety less acidic (Vaara & Viljanen, 1985). To investigate further the nature of binding of attacin to LPS, we tested the sensitivity of a deep rough, heptoseless *P. mirabilis* strain (EH193). We found that this strain was very sensitive to the neutral form of attacin, in fact as sensitive as would be expected from its Re chemotype, but was resistant to a 100-fold higher concentration of polymyxin than that which affected an

Fig. 3. Attacin interacts with purified LPS. Attacin (7.5 μ M) was incubated with varying concentrations of purified LPS Rd in LB medium for 30 min at 37 °C. These mixtures were then inoculated with exponential-phase D21f2 cells and growth was monitored at 560 nm.

E. coli strain of the same chemotype. These results indicate that the mechanism for binding of attacin to LPS is different from that of polymyxin and cecropin.

Basic attacin is only half as active as the neutral form, but *P. mirabilis* is clearly also sensitive to the basic form. This is in contrast to the situation in *E. coli*, where basic attacin is approximately twice as active as the neutral form.

Mg²⁺ inhibits the bacteriostatic effect of attacin

Divalent cations stabilize the OM. The addition of 40–50 mM Mg²⁺ has been shown to abolish the antibacterial effects of polymyxin (Newton, 1953) and of the bactericidal/permeability-increasing protein (BPI) from mammalian neutrophils (Weiss *et al.*, 1976). By adding Mg²⁺ at different concentrations together with attacin to growing cells, the effect on growth was inhibited in a dose-to-response fashion. Addition of 80 mM Mg²⁺ completely abolished the effect of attacin. These results strengthen the suggestion that the negative charges in LPS are required for binding to attacin.

Immobilized attacin can affect growing cells

When added to cultures of strain D21f2, underivatized agarose beads had a minor effect on cell growth, whilst the agarose-coupled attacin caused a distinct effect, the growth curve having the shape that is characteristic for

Fig. 4. Effect of immobilized attacin on growth of strain D21f2. Attacin was coupled to agarose beads and then added to growing cells to an approximate concentration of 15 μ M (assuming a 50% coupling efficiency). Beads without coupled protein were used as a control, as was uncoupled attacin (10 μ M).

the effect of unbound attacin on this strain (Fig. 4). Samples were taken after 3 h incubation, centrifuged and supernatants precipitated with TCA (10% final concn) and analysed by SDS-PAGE to reveal a possible presence of cellular proteins as a sign of lysis/leakage. Large quantities of cellular proteins were present in the culture supernatants from cells incubated with either agarose-coupled or free attacin, whereas the supernatant from cells grown in the presence of underivatized beads did not contain more cellular protein than did the supernatant from untreated cells. These results indicate that attacin can affect growth and permeability without entering the cytoplasm.

Attacin activity is not dependent on *ton* or *tol*

To rule out two possible ways for attacin to enter the cell, we tested its effect on *tonB*, *tolA* and *tolB* mutant strains. TonB, an IM/periplasmic protein, is part of the Ton system, which is involved in uptake of large molecules, such as vitamin B₁₂, ferric siderophores and group B colicins (Braun, 1995). TolA and TolB are part of the Tol system, which is also involved in uptake of macromolecules, such as group A colicins (Webster, 1991). In addition, they are believed to be important for structural integrity of the cell. A *tonB* strain treated with attacin displayed the same effects on growth and on OM synthesis as a wild-type strain. The finding that growth of strains lacking either of the Tol proteins was more inhibited as compared to wild-type cells when incubated with attacin is likely to be due to changes in OM integrity. Thus, attacin does not depend on *ton* or *tol* for its activity.

Attacin specifically inhibits the synthesis of Omps

Our previous conclusion (Carlsson *et al.*, 1991) that attacin inhibits the synthesis of OmpF, OmpC and OmpA was confirmed here by two-dimensional SDS-PAGE analysis of the protein content of attacin-treated cells (Fig. 5, Table 2). In addition, there was a decrease in two other Omps: FepA, a receptor for ferric enterochelin (Fiss *et al.*, 1982) and CirA, a colicin I receptor and iron transporter (Konisky, 1979; Nikaido & Rosenberg, 1990). The synthesis of several other, as yet identified proteins was also inhibited. All identified proteins that decreased in relative quantity were found to be Omps, which suggests a specific effect on their synthesis. No cytoplasmic precursors of Omps were detected on the two-dimensional gels, which indicates that attacin treatment does not lead to accumulation of export-defective proteins.

Attacin induces synthesis of stress proteins

We have previously observed induced synthesis of several unidentified proteins within 30 min of incubation with attacin and we suspected that they represent stress proteins. Use of two-dimensional SDS-PAGE analysis made it possible to identify two of the induced proteins as UspA and SspA (Fig. 5, Table 2). The UspA (universal stress protein A) is induced by growth arrest (Nyström & Neidhardt, 1992). Expression of the SspA (stringent starvation protein A) is growth-regulated and induced by starvation for glucose, nitrogen, phosphate or amino acids (Williams *et al.*, 1994).

We have also tested whether attacin induces β -galactosidase activity in *E. coli* strains containing transcriptional fusions of the promoters of *bolA*, *uspA* or *uspB* with *lacZ*. BolA is growth-rate- and σ^S -dependent and required for the expression of the penicillin-binding protein PBP6, which plays a role in cell wall synthesis at the septum (Aldea *et al.*, 1989). UspB is also regulated by σ^S and is induced by all starvation conditions so far tested (A. Farewell & T. Nyström, unpublished results). All these gene fusions were induced as a result of the attacin treatment. In addition, we observed a clear induction of β -galactosidase activity in one of the Pro-Tox (C) kit strains, containing an *osmY* fusion. *osmY* is a σ^S -dependent gene encoding a protein that is induced during changes in medium osmolarity and also by growth rate (Lange & Hengge-Aronis, 1991; Yim & Villarejo, 1992). Thus, attacin promotes induced synthesis of proteins that are normally induced in response to slow growth, starvation and during transition to stationary phase.

DegP is induced by attacin, but is not necessary for the effects on Omp synthesis

If attacin disturbs the structure of the OM it might cause newly synthesized Omps to accumulate in the periplasm. This could result in an extracytoplasmic stress signal causing the induction of the periplasmic protease DegP (Lipinska *et al.*, 1990; Meccas *et al.*, 1993; Danese *et al.*,

Fig. 5. Autoradiograms after two-dimensional PAGE of strain MC4100. Exponential-phase cells were incubated with 20 μ M attacin for 20 min, then labelled with [35 S]methionine and processed for two-dimensional PAGE as described in Methods. The names of the proteins in the marked spots are listed in Table 2. (a) Untreated cells, (b) attacin-treated cells.

1995) Therefore, we treated a *degP-lacZ* fusion strain (PND2000) with attacin and assayed culture samples for β -galactosidase activity after various times of incubation. The results showed that there was indeed an induction of DegP, so that after 1 h the β -galactosidase activity was 1.5-fold higher and after 3 h it was 5.3-fold higher than the enzyme activity in untreated cells. This is late when compared to the time required to inhibit Omp synthesis (10–30 min). To test if DegP induction is a necessary step in the effect of attacin on protein synthesis, we used an *E. coli* mutant (CE1448) lacking DegP and also OmpT. OmpT is an OM protease (Baneyx & Georgiou, 1990), possibly also involved in turnover of accumulated proteins. When CE1448 was incubated with attacin, it displayed an even stronger inhibition of growth as compared to the parent strain CE1224. The inhibitory effect on synthesis of OmpA, analysed by SDS-PAGE, was the same in both strains. Thus, though DegP is induced by attacin, it is not required for the effect on Omp synthesis. Neither is OmpT essential for this effect.

Attacin affects the synthesis of LPS

Porins depend on intact LPS for assembly in the OM. Cells that produce LPS with a defective core have a reduced synthesis of porins, due to feedback inhibition (Ried *et al.*, 1990a). The same is true when lipid synthesis

has been blocked with cerulenin, an antibiotic that inhibits fatty acid synthesis (Bocquet-Pages *et al.*, 1981). This connection between Omps and LPS led us to investigate the effect of attacin on LPS synthesis. We labelled cells with [14 C]galactose after incubation with attacin and analysed the LPS content by SDS-PAGE. The results show a clear increase in LPS synthesis after 1 h incubation with attacin (Fig. 6). The same results were obtained using [14 C]acetate and *N*-acetyl- 14 Cglucosamine. Thus, the effect of attacin on synthesis of Omps is likely to be a consequence of a perturbed membrane function/structure rather than a decrease in LPS synthesis.

DISCUSSION

The results presented in this paper suggest that LPS serves as a receptor for attacin. Accordingly, attacin that has been allowed to react with purified LPS does not inhibit growth of cells. Attacin shows the highest activity against deep rough, heptose-less cells, which lack long polysaccharide chains. This allows closer contact of attacin with the cell surface. Attacin is also inhibited by divalent cations that may compete for negatively charged phosphate groups on the inner parts of LPS that act as binding sites. Thus, electrostatic binding is likely to be important in the interaction of attacin with the bacterial cell. This would explain why basic attacin is more active than the neutral form on *E. coli*, and why

Table 2. Proteins whose synthesis are affected by attacin

Numbers correspond to the marked spots in Fig. 5.

No.	Protein name	Rate of synthesis relative to untreated control*
1	OmpA	0.19
2	OmpA	0.19
3	OmpF	0.14
4	OmpC	0.05
5	CirA	0.08
6	FepA	0.14
7	Not identified	–
8	Not identified	–
9	Not identified	–
10	Not identified	–
11	Not identified	–
12	Not identified	–
13	Not identified	–
14	DnaX	4.11
15	SspA	5.40
16	UspA	1.78
17	Not identified	+
18	Not identified	+
19	Not identified	+
20	Not identified	+

* Untreated control = 1; + and – indicate increase and decrease in synthesis, respectively, rates not determined. SD < 10%

Fig. 6. Effect of attacin on the synthesis of LPS in strain MC4100. Autoradiogram after 18% SDS-PAGE of exponential-phase cells incubated with 20 μ M attacin for 1 h and then labelled with [14 C]galactose for 30 min (in the continued presence of attacin) as described in Methods. Lanes: 1, untreated cells; 2, attacin-treated cells. Only the relevant part of the gel is shown. The LPS nature of the bands was confirmed by silver staining of the gel containing a sample of purified LPS Rd (non-radioactive).

basic attacin is less active on *Proteus*, which has fewer negatively charged groups on its LPS.

In addition, the hydrophobic regions of attacin may interact with the acyl chains of lipid A and possibly also with phospholipids present in the OM. This theory is

supported by the increased sensitivity of strains that have a more hydrophobic surface. The *envA1* mutant strain D22 (Normark *et al.*, 1969) displays an increased sensitivity to attacin compared to other strains with LPS of chemotype Ra (Hultmark *et al.*, 1983). This strain has a 25–30% lower content of LPS, which is suggested to increase the exposure of the lipid bilayer (Grundström *et al.*, 1980). The strain CE1224 used in this study also shows an increased sensitivity to attacin and to SDS (unpublished observation). CE1224 lacks the OmpF and OmpC porins due to an *ompR::Tn10* mutation, and thus has a higher content of phospholipids in the OM. Deep rough strains have a lower amount of Omps but a higher content of phospholipids, which could add to the increased sensitivity due to the lack of polysaccharide chains on the surface (Nikaido & Vaara, 1985).

Immobilized attacin is still able to inhibit cell growth. We cannot rule out the possibility that attacin, though tethered to the agarose beads, is able to partially translocate across the OM. However, we consider it unlikely that immobilized attacin would be able to interact with the transcription machinery in the cytoplasm. Our results suggest that an interaction of attacin with LPS receptor sites in the OM is sufficient to initiate the signalling for the subsequent shutdown of Omp synthesis.

The effect of attacin is restricted, only affecting synthesis of specific proteins. There are other reports of specific inhibition of Omp synthesis. The overexpression of an Omp or an aberrant Omp structure will inhibit the synthesis of other major Omps (Click & Schnaitman, 1989; Catron & Schnaitman, 1987; Ried *et al.*, 1990b). Also, expression of an *Enterobacter* Omp in *E. coli* causes reduction of synthesis of endogenous Omps (Stoorvogel *et al.*, 1991). Irrespective of the level on which these feedback mechanisms operate (transcription, mRNA stability, translation), these results indicate the existence of a common regulatory mechanism for (a subset of) Omps. However, what makes attacin unique is that it causes a disturbance from the outside of the cell, whereas overexpressed or mutated Omps provide a disturbance coming from the inside.

How is the effect on transcription accomplished? There are examples of systems for signalling from the outside or surface of the cell to the interior, such as the Pup system in *Pseudomonas putida* (Koster *et al.*, 1994) and the ferric citrate system in *E. coli* (Härle *et al.*, 1995). TonB is involved in both of these signalling processes. Here we show that TonB is not involved in the effect of attacin on Omp synthesis. However, there might be other similar systems, involving hitherto unidentified signal transducers.

We have also ruled out the involvement of DegP in the effect of attacin on protein synthesis. The observed induction of DegP might be required to degrade Omps that are prevented from assembling in the OM, to relieve the extracytoplasmic stress signal(s) induced by attacin. This proposal is supported by the findings that a *degP*

strain is even more sensitive to attacin as it has less ability to reduce the effects of stress. Thus, the induction of DegP is concluded to represent a secondary consequence of the binding of attacin to the OM.

A comparison of attacin with polymyxin and BPI is interesting. Although all three substances bind to LPS/lipid A and will affect the integrity of the OM, there are obvious and important differences. Qi *et al.* (1995) have performed two-dimensional PAGE analyses on *Salmonella typhimurium* cells treated with BPI or polymyxin. The majority of the affected proteins are involved in key metabolic processes. BPI and polymyxin cause different cellular responses, which in turn differ from the response caused by attacin on *E. coli*.

The small polymyxin molecule (1.2 kDa) is assumed to enter the cell by self-promoted uptake (Hancock & Bell, 1988) and interact with the IM (Storm *et al.*, 1977), but as shown by LaPorte *et al.* (1977), it can also affect IM functions when it is immobilized by coupling to agarose beads. The mechanism for the action of BPI has not been completely elucidated. As is the case with attacin, deep rough mutants are more sensitive to BPI than cells carrying LPS molecules with longer polysaccharide chains (Weiss *et al.*, 1980). BPI is believed to affect the IM and it has been speculated that it may enter the cell in a colicin-like fashion, i.e. it unfolds and enters through the OM to interact directly with the IM (Qi *et al.*, 1995). BPI has been assumed to be inactive on *P. mirabilis*, but Capodici *et al.* (1994) have shown that a deep rough mutant of *P. mirabilis* is in fact sensitive to BPI. Though similar results are obtained with attacin, two-dimensional analyses demonstrate that there are differences in the range of proteins whose synthesis is affected by the respective proteins.

Conclusion

Our hypothesis for the interaction of attacin with Gram-negative bacteria is as follows. LPS in the bacterial envelope serves as a receptor for attacin. Electrostatic binding of positive charges on the attacin molecule to negative charges on the inner core- and lipid-A-regions of LPS, followed by hydrophobic interactions with lipid A (and possibly also phospholipids in the OM), result in partial integration of attacin into the OM. This external blockage of presumably physiological reaction sites for Omps elicits signal(s) that mimic those occurring *in vivo* in conjunction with OM assembly to regulate Omp synthesis. Accordingly, the presence in the periplasm of any Omps being prevented from integrating into the OM is likely to be an essential part of the signalling events that lead to inhibition of Omp synthesis at the transcriptional level. The observed increase in LPS synthesis is interpreted as an attempt by the cell to restore the integrity of the OM. In contrast to the early and specific effects of attacin on Omp expression, the later events, such as induced synthesis of stress proteins, represent secondary effects related to a perturbed membrane, decreased growth rate and/or starvation of the cell.

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