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Attacin, an Antibacterial Protein from *Hyalophora cecropia*, Inhibits Synthesis of Outer Membrane Proteins in *Escherichia coli* by Interfering with *omp* Gene Transcription

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Attacins are antibacterial proteins synthesized by pupae of the giant silk moth, *Hyalophora cecropia*, in response to a bacterial infection. In this report we show that the previously described, attacin-induced alteration in the structure and the permeability of the outer membrane of *Escherichia coli* is associated with a specific inhibition of the synthesis of several outer membrane proteins, including OmpC, OmpF, OmpA, and LamB. The inhibition is expressed as a reduction in the steady-state mRNA levels and is at least in part the result of a block in transcription of the corresponding genes. Transcription directed by the promoter of *ompR*, the positive regulator of *ompC* and *ompF* expression in response to environmental conditions, is also affected by attacin. The effects on mutant strains show that the primary activity of attacin is not mediated by the *ompR-envZ* regulatory system. Instead our data suggest the existence in *E. coli* of a previously unknown system for the transcriptional regulation of a large set of outer membrane proteins previously not known to be coordinately regulated. We propose that the activity of attacin is directed towards this system.

Bacterial infection of pupae of the giant silk moth *Hyalophora cecropia* will result in the appearance of a very potent antibacterial activity in the hemolymph. This activity results, at least in part, from the de novo synthesis of three different groups of proteins which show antibacterial activity: the cecropins constitute a family of basic amphipatic proteins with a molecular weight of approximately 4,000 (11) and a strong lytic activity against the cytoplasmic membrane of a broad spectrum of bacteria (13, 31); the lysozyme from *H. cecropia* is the chicken type (6), is thus structurally related to lysozymes from other organisms, and is believed to exert its activity towards bacteria by degrading the peptidoglycan layer of the cell wall (13, 28); The third group of antibacterial proteins, the attacins (molecular weight, approximately 20,000), consists of one protein with a basic isoelectric point (pI9) and one with a neutral isoelectric point (pI7) with similar (80%) primary sequences (5, 12, 18).

Our previous studies (4) on the action of attacin on *Escherichia coli* indicated that the main target is the outer membrane. Attacin causes an alteration in the permeability properties of the outer membrane, which results in an improved access of lysozyme and of cecropin to their targets in the cell wall and the cytoplasmic membrane, respectively.

In the present study we have attempted to determine the mechanism by which attacin affects the structure of the outer membrane. The results indicate that attacin specifically inhibits the synthesis of several outer membrane proteins, including OmpA, OmpC, OmpF, and LamB (approximate molecular weights, 30,000, 38,000, 37,000, and 47,000, respectively), and that the inhibition occurs at a pretranslational level.

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MATERIALS AND METHODS

Preparation of attacin. Diapausing pupae of *Hyalophora cecropia* were injected with live *Enterobacter cloacae* β12, and the hemolymph was collected after 7 days as previously described (13). Attacin was purified from freshly collected immune hemolymph as previously described (5). The neutral form (pI7) of attacin was used in the experiments.

Bacterial strains and growth conditions. The bacterial strains used are listed in Table 1. In strains carrying gene fusions all fusions are chromosomally located. Bacteria were grown in L broth (except as indicated) in microtiter plates (Nunc) (250 μl per well). Attacin was added to the medium to a final concentration of 400 μg/ml (except as indicated), and the medium was then inoculated with 5×10^6 cells in mid-log phase. The bacteria were grown at 37°C on a rotary shaker.

Outer membrane preparation. The cells from 1 ml of culture (optical density at 560 nm = 0.5) were pelleted by centrifugation in a Beckman microfuge (13,750 rpm) for 5 min. The pellet was resuspended in 50 μl of 0.2 M Tris-HCl, pH 8.0, on ice. Sucrose (100 μl at 1 M in 0.2 M Tris-HCl, pH 8.0), 10 μl of 10 mM EDTA, and 10 μl of lysozyme (2 mg/ml) were added and mixed, and then 320 μl of H_2O was added. The mixture was incubated at room temperature until spheroplasts were formed. Next, 500 μl of extraction buffer (2% Triton X-100, 50 mM Tris-HCl [pH 8.0], 10 mM $MgCl_2$) and 10 μl of DNase (1 mg/ml) were added and carefully mixed. When the viscosity decreased, the mixture was centrifuged for 30 min in a Beckman microfuge (13,750 rpm). The resulting membrane pellet was washed two to four times with H_2O, with a 20-min centrifugation in-between.

Gel electrophoresis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in 10 or 13% (wt/vol) slab gels by the method of Laemmli (19) with the modifications described by Engström et al. (4) or in the presence of 8 M urea (29). Dried gels containing radioactive

TABLE 1. *E. coli* strains

Strain	Relevant genotype	Genotype[a]	Reference or source
D11	Wild type	*pro trp his lac*	2
HSK24	*envZ* null mutant	MC4100 Δ(*envZ-bioH*)*3* Φ(*ompC-lacZ*$^+$) *10–25* [λ*p*1(209)] *recA56 srl*::Tn*10*	30
MC4100	Wild type	F$^-$ *araD139* Δ(*argF-lac*)*U169 rpsL150 relA1 flbB5301 ptsF25 deoC1 rbsR*	3
MH150	*ompC* null mutant	MC4100 *ompC12*::Tn*5*	9
MH450	*ompF* null mutant	MH20 *ompF1*::Tn*5*	10
MH225	*ompC-lacZ* transcriptional fusion	MC4100 Φ(*ompC-lacZ*$^+$)*10–25*[λ*p*1(209)]	9
MM294	Wild type *lacZ*	F$^-$ *endA1 hsdR17 supE44 thi-1 gyrA96 pro*	23
PK193	*ompA-lacZ* translational fusion	MC4100 Φ(*ompA-lacZ*)(Hyb1)[λ*p*1(209)]	27
RT302	*ompR-lacZ* transcriptional fusion	MC4100 Φ(*ompR-lacZ*$^+$)*1*[λ*p*1(209)]	Ronald K. Taylor
TK770	*ompR-lacZ* translational fusion	MC4100 Φ(*ompR-lacZ*)(Hyb4)[λ*p*1(209)]	This study
TK786	*ompR-lacZ* translational fusion	MC4100 (λ*p*1TK770)	This study
TK1167	*ompR-lacZ* transcriptional fusion	MC4100 (λ*p*1RT302)	This study
TK1332	*lamB* null mutant	MC4100 Δ(*lamB*)*830 zja*::Tn*10*	20
TK2024	*envZ* null mutant	MC4100 *envZ22*(Am) *zhe*::Tn*10*	This study
TK2030	*envZ* null mutant	MC4100 Δ(*envZ'*)*4 zhe*::Tn*10*	This study

[a] Translational fusions (indicated by Hyb) give rise to hybrid proteins that retain activity of β-galactosidase, whereas transcriptional fusions produce native β-galactosidase.

samples were autoradiographed on Kodak X-Omat AR film for 3 to 5 days at room temperature.

Immunoblotting. Immunoblotting was carried out essentially as described by Towbin et al. (32) with the following modifications. SDS-PAGE was followed by electrophoretic transfer of the proteins onto a nitrocellulose membrane (Schleicher & Schuell BA 85 or PH 79). Transfer was effected at 700 mA for 3 h in 192 mM glycine–25 mM Trizma base in a Bio-Rad Transblot Cell. Residual binding sites were blocked with 3% bovine serum albumin (BSA) in TBS (20 mM Tris, 0.5 mM NaCl, pH 7.5). After washing in TBS with 0.1% BSA–0.05% Tween 20, the membrane was incubated with a 1:1,000 dilution of the appropriate antiserum in TBS with 0.5% BSA–0.05% Tween 20 overnight. After an additional washing, the membrane was incubated for 2 h with a 1:1,000 dilution of horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (Bio-Rad). The membrane was washed and then stained with 4-chloronaphthol (25).

Radioactive labelling of bacteria. Bacteria in mid-log phase were inoculated into M9 minimal medium supplemented with glucose and a mixture of amino acids, except methionine. Attacin was added to a final concentration of 400 μg/ml. Cultures were grown in microtiter plates at 37°C. At different times 5 μCi of [^{35}S]methionine (Amersham) was added and chased after 10 min by the addition of nonradioactive methionine to a final concentration of 1 μM. Cultures were allowed to grow for an additional 20 min and were then precipitated with trichloroacetic acid (10% [wt/vol] final concentration).

β-Galactosidase assay. The activity of β-galactosidase was assayed by the method of Miller (24). The enzyme activity per cell is given in units as defined by Miller (24).

Northern (RNA) blot. Total bacterial RNA was isolated essentially as described by Johnson (16). Gel electrophoresis of RNA was run in agarose gels after denaturation of the RNA with glyoxal and dimethyl sulfoxide (22). Transfer to GeneScreen Hybridization Transfer Membrane (Du Pont) was carried out by following the manufacturer's procedure for capillary blot. The DNA probe for hybridization was labelled by nick translation with a biotinylated nucleotide (dUTP) and the Nick Translation System from Bethesda Research Laboratories. Hybridization (utilizing dextran sulfate) was carried out by following the GeneScreen manufacturer's (Du Pont) procedure with the modifications necessary for using a biotin-labelled probe. Bands were detected with the DNA Detection System from Bethesda Research Laboratories.

RESULTS

Attacin inhibits the synthesis of outer membrane proteins. We have previously shown that attacin has a drastic effect on the permeability properties of the outer membrane of gram-negative bacteria (4). An analysis of the protein composition of attacin-treated bacterial cells revealed that this alteration in outer membrane permeability is associated with the specific loss of several proteins, as detected by SDS-PAGE of whole-cell extracts. On the basis of the absence or presence of two of these proteins in mutant strains and by the use of specific antisera, we identified them as the outer membrane-localized products of the *ompC*, *ompF*, and *ompA* genes (Fig. 1a). By using a specific antiserum we identified the maltose-inducible outer membrane protein LamB as a fourth protein affected by attacin. The decrease in the induced levels of LamB was more than fivefold (Fig. 1b).

The loss of these outer membrane proteins could result from an attacin-induced degradation of the proteins or, alternatively, from a failure of the attacin-treated cells to synthesize or assemble the proteins into the outer membrane. To discriminate between these possibilities we pulse-labelled bacteria with [^{35}S]methionine after incubation in the absence or presence of attacin. The results show that attacin had no effect on the rate of total protein synthesis of the bacteria within 2 to 3 h after addition. However, the synthesis of the *ompC*, *ompF*, and *ompA* products, as well as of a small number of additional, unidentified proteins, was specifically inhibited by the attacin treatment (Fig. 1c). The reduction in the rate of synthesis of these proteins was obvious after 30 min of incubation with attacin and already detectable after 10 min of incubation (data not shown).

Attacin affects mRNA levels and transcriptional activity of outer membrane protein genes. To analyze whether the attacin effect was on translation and/or protein assembly into the outer membrane or alternatively directed towards earlier stages in *omp* gene expression, we studied the impact of attacin on the steady-state levels of mRNA corresponding to

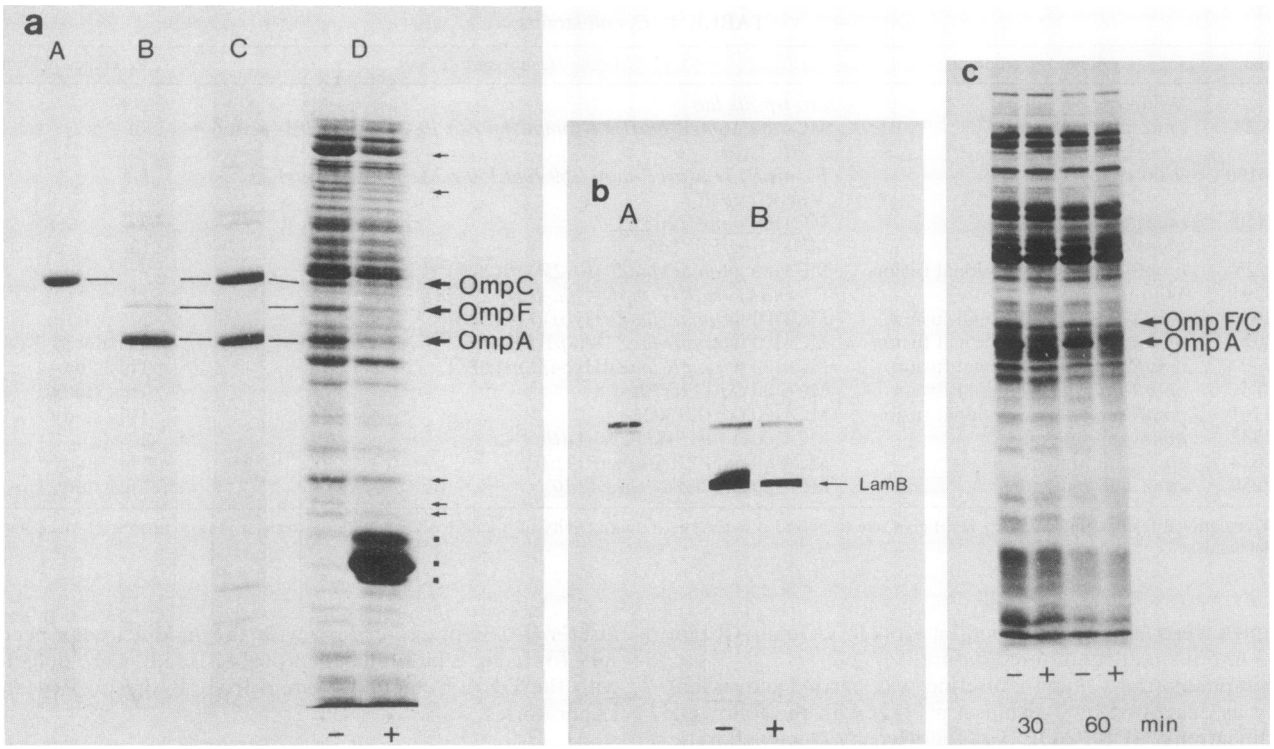

FIG. 1. Effects of attacin on the amount and synthesis of outer membrane proteins. Bacteria were grown in LB medium in the absence (−) or presence (+) of attacin at a concentration of 400 μg/ml for the time indicated. (a) Whole-cell proteins (strain TK1332) analyzed on SDS–13% (wt/vol) PAGE in the presence of 8 M urea to allow the separation of the OmpF and OmpC proteins. Arrows indicate proteins that are affected after treatment with attacin. Filled squares indicate attacin. Lanes: A, control, purified OmpC protein; B, control, outer membrane preparation from an *ompC* mutant strain (MH150); C, control, outer membrane preparation from an ompF mutant strain (MH450); D, TK1332. (b) Amount of LamB as detected by immunoblots with antiserum directed against LamB. Strain MM294 was grown in M9 minimal medium supplemented with amino acids. Maltose was used to induce the *lamB* gene. Lanes: A, control, *lamB* mutant strain (TK1332); B, wild-type *lamB* (MM294). (c) Synthesis of outer membrane proteins in strain D11. The cells were pulse-labelled with [^{35}S]methionine, and the whole-cell protein extract was analyzed on SDS-13% (wt/vol) PAGE.

ompC in Northern blot experiments. The results show a reduction in *ompC* message which was dependent on the concentration of attacin and of a magnitude similar to that in the rate of protein synthesis (Fig. 2).

The reduction in mRNA levels could result from an attacin-induced increase in the turnover of RNA or a reduction in the rate of transcription of the corresponding genes. To distinguish between these possibilities, we studied the impact of attacin on strains harboring *omp* gene promoter fusions to the reporter gene *lacZ*. In these strains the synthesis of the *lacZ* product, β-galactosidase, is under the genetic control of the respective *omp* promoter, and thus the transcriptional activity of the *omp* promoter can be assessed by monitoring the change in the activity of β-galactosidase. If the effect of attacin were on transcription we would expect to observe a change in β-galactosidase activity of the strains similar to that in the synthesis of the respective outer membrane protein in the wild type. Assuming that the enzyme is very stable and its rate of turnover is low in relation to the generation time of the cultures, whereas the turnover rate of the *lacZ* mRNA is very high, a complete and immediate block of transcription of the *omp-lacZ* fusion would result in a reduction in enzyme activity per cell by approximately one-half at each cell division. The results from experiments with an *ompC-lacZ* strain, MH225, are shown in Fig. 3A. In nontreated, logarithmically growing cultures the β-galactosidase activity increased at an approximately constant rate, by a factor of 1.5 over a 3-h incubation. In parallel cultures treated with attacin, the level of β-galactosidase activity decreased to approximately 75% of the initial activity within 165 min after addition of attacin. Since the generation time of the attacin-treated culture was approximately 100 min, a complete and immediate block of transcription would be expected to result in a reduction of enzyme activity to 35 to 40% of the initial value. The result consequently indicates that the attacin treatment caused a reduction in the rate of transcription by an average of approximately 50% over the incubation period.

In experiments using an *ompA-lacZ* fusion strain (PK193) control cultures had approximately constant levels of β-galactosidase activity. The addition of attacin to this strain resulted in a decrease in activity to approximately 80% of the initial activity within 180 min of incubation (data not shown). In this strain the generation time of the attacin-treated culture was 115 min, and consequently the attacin-induced reduction in transcription rate was approximately 50%.

The possibility that this effect was the result of a direct effect of attacin on the activity of the enzyme or on the synthesis of the enzyme at a stage later than transcription was ruled out by the finding that the β-galactosidase activity was not affected by attacin in strain MM294 which harbored a wild-type *lacZ*, in which the synthesis of β-galactosidase is driven by the *lacZ* promoter (Fig. 3B). Further, immunoblot experiments using an antiserum against β-galactosidase

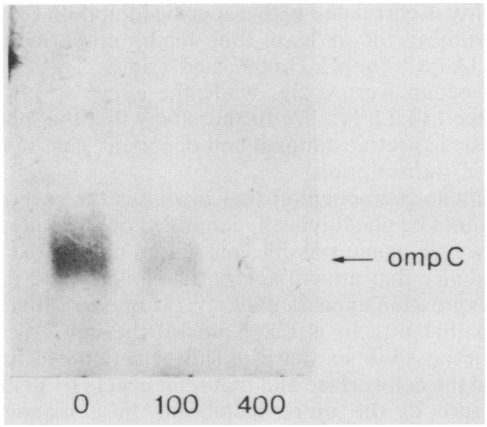

FIG. 2. Effects of attacin on the amount of *ompC* mRNA. Northern blot of total RNA from the wild-type strain MC4100 hybridized with a probe constructed from the plasmid pHSK21 containing the *ompC* gene is shown. Cells were grown as described in the legend to Fig. 1. The concentrations (in micrograms per milliliter) of attacin are indicated below each lane.

showed that the effect of attacin on enzyme activity in the *ompC-lacZ* fusion strain MH225 was parallelled by a reduction in the amount of enzyme present in the cells (Fig. 3D) and consequently was not a result of a reduction in its specific activity caused by attacin.

We conclude that attacin inhibits the de novo synthesis of β-galactosidase in strains MH225 and PK193 by interfering with synthesis at a pretranslational level. The suppression of transcription of the *omp* genes caused by attacin is distinct but apparently partial. This suggests that an increase in the rate of turnover of *omp* mRNAs may contribute to the effect. Alternatively, the block of transcription may not occur immediately after the addition of attacin to the culture but instead may occur gradually and increase with time after addition, resulting in an average inhibition of less than 100% over the incubation time. At present we cannot distinguish between these possibilities.

The regulatory locus *ompB* is not a target of attacin. Transcription of the *ompC* and *ompF* genes is under the genetic control of the regulatory locus *ompB*, composed by *ompR*, encoding a cytoplasmic DNA-binding protein, and *envZ*, encoding a cytoplasmic membrane-localized environmental sensor protein (8, 10, 15, 21). To test whether attacin exerts its effect by interfering with the *ompB* regulatory system, we first analyzed the effect of attacin on transcription of *ompR*. Attacin treatment of two strains carrying *ompR-lacZ* fusions (the transcriptional fusion strain RT302 and the translational fusion strain TK770) resulted in a distinct increase in β-galactosidase activity, indicating an increased rate of transcription from the *ompR* promoter compared with that in the nontreated control cultures which showed approximately constant levels of transcription throughout the experiments (Fig. 3C). As was the case with *ompC-lacZ*, the change in enzyme activity was parallelled by changes in the amount of enzyme present in the cells (Fig. 3F). In both strains (TK770 and RT302) the gene fusion is the result of an insertion into the *ompR* gene, and they thus carry *ompR* null mutations. In contrast to the results obtained with these strains, transcription of *ompR-lacZ* fusions carried by a lambda transducing phage lysogenized into an *ompR* wild-type background (strains TK786 and TK1167) was unaffected by attacin. This was shown in immunoblot

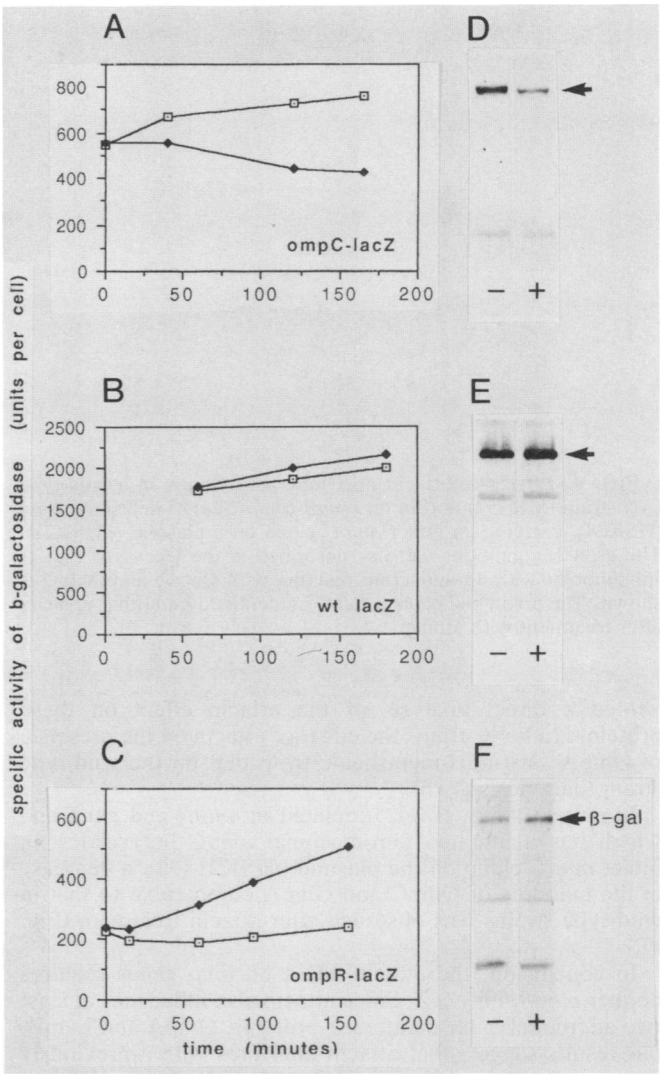

FIG. 3. (A through C) Effects of attacin on the activity of β-galactosidase in strains containing different gene fusions. Open squares represent controls without attacin; filled squares represent cells treated with attacin (400 μg/ml). (D through F) Effects of attacin on the amount of β-galactosidase as detected by immunoblots with antiserum directed against β-galactosidase. Arrows indicate β-galactosidase. Cells were incubated in the absence (−) or presence (+) of attacin (400 μg/ml) for 2 h in M9 minimal medium supplemented with glucose and amino acids. For strain MM294 lactose instead of glucose was used as a carbon source to induce *lacZ*. (The antiserum cross-reacts to *E. coli* proteins of unknown origin with molecular weights both higher and lower than that of β-galactosidase.) (A and D) *ompC-lacZ* (MH225); (B and E) control, wild-type *lacZ* (MM294); (C and F) *ompR-lacZ* (TK770 and RT302).

experiments in which the level of β-galactosidase in attacin-treated cells was indistinguishable from the enzyme level in nontreated control cells (data not shown). Thus, attacin treatment had an effect on the transcription of *ompR*, but in the presence of the wild-type OmpR protein this effect is not seen, which may indicate a negative autoregulation of the transcription of *ompR* by the *ompR* product.

Second, the potential function of the *envZ* product as a target for attacin was examined by using two *envZ* null mutant strains, TK2024 and TK2030. However, the low level of OmpF and the absence of OmpC in these strains pre-

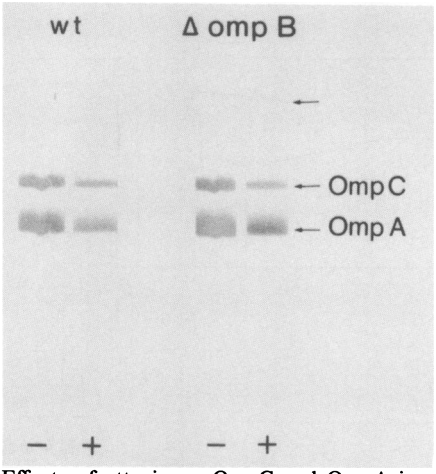

FIG. 4. Effects of attacin on OmpC and OmpA in a wild-type (wt) strain (MM294) and in an *ompB* (*ompR-envZ*)-deficient strain (HSK24), carrying an intact *ompC* clone on a plasmid (pHSK21). The growth conditions were as described in the legend to Fig. 1. Immunoblot with an antiserum reacting with OmpA and OmpC is shown. The arrow indicates a faint, unidentified band that appears after treatment with attacin.

vented a direct analysis of the attacin effect on these proteins. In both strains the effect of attacin on the presence of OmpA was indistinguishable from that on the wild-type strain (data not shown).

In mutant strain HSK24, deleted in *ompR* and *envZ* and which has a mutated chromosomal *ompC* but carries an intact *ompC* clone on the plasmid pHSK21 (30), a decrease in the amounts of OmpC and OmpA comparable to that in wild-type strains was observed after attacin treatment (Fig. 4).

In conclusion, the attacin effect on *omp* genes requires neither *ompR* nor *envZ*. Since attacin also influences at least two additional outer membrane proteins, OmpA and LamB, our results suggest that attacin interferes with a previously unknown system for the regulation of synthesis of a large set of outer membrane proteins previously not known to be coordinately regulated.

DISCUSSION

Antibacterial proteins with molecular weights similar to that of attacin have been reported to be present in several insects other than *H. cecropia*: the flesh fly *Sarcophaga peregrina* (1), the tsetse fly *Glossina morsitans morsitans* (17), *Drosophila melanogaster* (7), and the tobacco horn worm *Manduca sexta* (14). The flesh fly protein sarcotoxin IIA, like attacin, affects only gram-negative bacteria, alters the morphology of bacterial cells, and has little effect on nongrowing bacteria. The mechanisms by which these proteins affect the growth and survival of bacteria are poorly understood. We have previously shown that treatment of gram-negative bacteria with attacin results in an increase in the permeability of the outer membrane, which renders the cells susceptible to antibiotic activity directed towards the cell wall and the cytoplasmic membrane, most notably to the two other major components of the insect defense system, lysozyme and cecropin (4). In this respect attacin may be similar to the cationic bactericidal/permeability increasing protein (BPI) from human polymorphonuclear leukocytes (26). In this report we show that this increase in membrane permeability is correlated with a specific inhibition by attacin of the synthesis of at least four major outer membrane proteins: OmpC, OmpF, OmpA, and LamB. This effect on synthesis occurs very early, while the effect on growth is visible after 1 to 2 h (4). We further show that the inhibition of synthesis is pretranslational and occurs at least in part at the level of transcription.

The cellular component(s) that mediates the response to attacin cannot be unequivocally identified on the basis of our data. However, some possible mediators can be excluded. The possibility that attacin acts intracellularly and directly on gene expression appears unlikely. At present, there is no indication that attacin is taken up by the cell. We favor instead the possibilities that the site of action of attacin is located on the cell surface and that attacin acts by disturbing the structure of the outer membrane in a manner that interferes with the mechanisms by which the cell controls the overall surface structure. One potential mediator of the response to the attacin effect, the regulatory locus *ompB*, which controls the expression of *ompC* and *ompF* in relation to the environmental changes of the medium, does not control the expression of *ompA* or *lamB*. This argues against the possibility that the attacin activity is directed towards *ompB* and suggests that attacin instead affects a regulatory system common to all four genes. This suggestion was supported by our results from experiments using *ompR* promoter fusions and *envZ* mutant strains, which showed that neither *ompR* nor *envZ* is likely to be the target for attacin. In conclusion, our results show that attacin affects the synthesis of a wide range of outer membrane proteins and suggest the existence of a novel regulatory system for outer membrane assembly.

These results demonstrate the usefulness of attacin as a probe in the study of regulation of outer membrane assembly in *E. coli*. A further example is given by the effects of attacin on the transcription of the *ompR* gene. At present we do not understand the basis for the stimulation by attacin of transcription from the *ompR* promoter. The result indicates, however, that transcription of *ompR* is influenced by events in the outer membrane, by mechanisms that are unlikely to involve *envZ*. Further, the fact that the stimulation of *ompR* transcription was counteracted by the presence in the cell of a wild-type *ompR* locus suggests that *ompR* transcription may be negatively regulated by the *ompR* product.

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