Effect of Halocin H4 on Cells of *Halobacterium halobium*

By INMACULADA MESEGUER AND F. RODRIGUEZ-VALERA*

Departamento de Microbiología, Facultad de Medicina, Universidad de Alicante, Alicante, Spain

(Received 4 March 1986; revised 22 May 1986)

The killing of a population of a sensitive strain of *Halobacterium halobium* by halocin H4 followed exponential kinetics, and the percentage survival of sensitive cells exposed to different concentrations of halocin H4 corresponded to single-hit-type kinetics. Morphological changes were observed in treated cells, which showed swollen, spherical shapes. Halocin H4 affected macromolecule synthesis very little, and only late after the start of the treatment, although the transport of 2-aminoisobutyric acid, a non-metabolizable amino acid, was rapidly stopped. Bacteriorhodopsin-mediated H⁺ extrusion worked very efficiently in treated cells, and much larger pH decreases were found in treated than in untreated suspensions after illumination, although ATP synthesis was not markedly affected. These findings suggest that the primary target of halocin H4 may be located in the membrane, producing permeability changes and ionic imbalance, which lead to death and cell lysis.

INTRODUCTION

Antagonistic interactions were found in members of the *Halobacteriaceae* by Rodriguez-Valera *et al.* (1982), and were attributed to the production of bacteriocin-like substances which were named halocins. These organisms are extremely halophilic archaeobacteria, one of the three primary kingdoms of cellular organisms (Woese & Fox, 1977). In spite of the phylogenetic gap which exists between eubacteria and archaeobacteria, halocin H4 was found to have many characteristics in common with some eubacterial bacteriocins. This halocin is a simple protein of M_r about 28000 (Meseguer & Rodriguez-Valera, 1985), and is denatured by heat or exposure to low salt concentrations (Rodriguez-Valera *et al.*, 1982); it adsorbs to the target cell causing death and lysis.

Eubacterial bacteriocins are diverse in their mode of action. Colicins E₂ and E₃ are nucleases acting directly on the DNA or RNA of the target cells (Konisky, 1982). Colicins E₁, A, Ia, Ib and K form permeability channels to ions through the cell membrane (Konisky, 1982; Levisohn *et al.*, 1967). Colicin L acts by inhibiting protein, DNA and RNA synthesis, and colicin M inhibits peptidoglycan biosynthesis (Schaller *et al.*, 1982). Mechanisms such as membrane depolarization or disintegration have also been suggested as the mode of action of some bacteriocins (Ozaki *et al.*, 1966; Yang & Konisky, 1984). In the present work we have studied some effects of halocin H4 on sensitive cells.

METHODS

Bacterial strains. Halocin H4 was obtained from culture supernatants of *Halobacterium mediterranei* ATCC 33500. *Halobacterium halobium* CCM 2090 was used as the sensitive strain for all the experiments except those on light-induced pH changes (see below).

Media and growth conditions. All the media contained a mixture of marine salts referred to as SW (Rodriguez-Valera *et al.*, 1983), at a total concentration of 25% (w/v) unless otherwise indicated, and supplemented with yeast extract (Difco), 0.5% (w/v). The pH was adjusted to 7.2 with 1 M-KOH. All cultures were incubated at 37 °C.

Abbreviations: AIB, 2-aminoisobutyric acid; AU, arbitrary units; DCCD, *N,N'*-dicyclohexylcarbodiimide; SW, marine salts mixture (see Methods).

Cultures were grown in 250 ml Erlenmeyer flasks containing 100 ml medium, with orbital shaking at 150 strokes min^{-1} , or in cylindrical flasks containing 5 l medium aerated by a soft humidified airflow and magnetically stirred. Solid media contained 2% (w/v) Bactoagar (Difco); plates were incubated in sealed plastic bags.

Preparation of halocin H4. *H. mediterranei* ATCC 33500 was cultivated in the liquid medium described above and stationary-phase cultures were centrifuged at 9630 *g* for 30 min at 4 °C in a Beckman JA100 refrigerated centrifuge. The supernatant was filtered through 0.45 μm HA Millipore filters, and then concentrated 100-fold by ultrafiltration through CX-10 immersible Millipore ultrafilters. The concentrate was dialysed with 25% SW and again sterilized by filtration. This preparation was assayed for inhibitory activity by the twofold method (Timmis, 1972), diluting the samples with 25% SW and spotting a loopful (0.05 ml) of each dilution on an overlay plate of CCM 2090 indicator strain (10^7 cells). The highest dilution producing visible inhibition was considered as containing one arbitrary unit (AU). The preparation described above contained 1280 AU ml^{-1} .

Killing kinetics. The loss of viability of a population of sensitive cells was measured as a function of time and halocin concentration. In the first case, 0.2 ml volumes of a 10-fold serial dilution (to 10^{-3}) of *H. halobium* exponential cultures (10^8 cells ml^{-1}) were mixed with 1.8 ml of H4 solution (1150 AU ml^{-1} , final concentration) and incubated at 37 °C. As control, 0.2 ml volumes of the same dilutions were mixed and incubated with 1.8 ml 25% SW. Samples of 0.1 ml were taken every 30 min, resuspended in 10 ml sterile 25% SW, and immediately filtered through 0.45 μm HA Millipore filters. After washing with 10 ml 25% SW, the filters were placed on plates of solid 25% SW/yeast extract medium. Colonies were counted after one week of incubation at 37 °C. To determine the effect of halocin concentration, twofold dilutions of the concentrated halocin H4 (1280 AU ml^{-1}) were made in 25% SW, and 0.1 ml volumes were mixed with 0.1 ml of a cell suspension (in 25% SW) containing 5×10^4 cells ml^{-1} . As a control, 0.1 ml volumes of the same cell suspension were mixed with 0.1 ml 25% SW. After 24 h incubation at 37 °C, 0.1 ml of each suspension was filtered as described above, to determine the number of c.f.u.

Electron microscopy. Preparation of samples for electron microscopy, and positive staining, were as described by Stoeckenius & Rowen (1967), except that 25% SW was used as standard salt solution. Three series of samples were prepared: a control without halocin, and samples treated with H4 for 5 h and 24 h.

Negative staining was done on Formvar-coated grids; the samples were treated with 2% (w/v) uranyl acetate in 25% SW for 30 s, the excess liquid was withdrawn with a filter paper and then 2% (v/v) acetic acid was used to eliminate the salts. All observations were made with a Zeiss EM 10 electron microscope.

Effect on macromolecule synthesis. An exponential culture of *H. halobium* was centrifuged at room temperature and resuspended in a medium containing 25% SW salts and 0.2% yeast extract (Difco) (pH 7.2), to a final OD_{520} of 0.70 (corresponding to 10^8 cells ml^{-1}). A 3 ml sample of this suspension was diluted in 27 ml of the same medium, preincubated at 37 °C with orbital shaking (150 strokes min^{-1}) for 30 min, then supplemented with the appropriate labelled compound (glucose, glutamic acid, thymidine or uridine, to measure synthesis of carbohydrate, protein, DNA and RNA, respectively; each at 0.5 μCi ml) and incubated for another 2 h. The cultures were then divided into three fractions. The control received 2 ml concentrated halocin H4, previously denatured for 10 min at 100 °C. The other two flasks received 2 ml of the same concentrate containing 1280 AU (final concentration 116 AU ml^{-1}) and 2560 AU (final concentration 233 AU ml^{-1}), respectively. Duplicate samples of 0.5 ml were taken periodically, added to 2 ml cold 10% (w/v) trichloroacetic acid (TCA) and kept at 4 °C for 30 min. The samples were then filtered through 0.45 μm pore size HA Millipore filters and washed twice with 5 ml 5% TCA. The filters were transferred to scintillation vials to which 5 ml Ready-Solv HP Beckman scintillation liquid was added, and their radioactivity was measured in a Beckman LS-2800 scintillation counter. In the case of [^{14}C]glutamic acid an additional flask was prepared to which anisomycin (Sigma) was added to a final concentration of 500 μg ml^{-1} .

Transport of 2-aminoisobutyric acid. A sample (21 ml) of *H. halobium* in 25% SW (5×10^7 cells ml^{-1}) was preincubated as described above, and after 30 min 2-amino[^{14}C]isobutyric acid ([^{14}C]AIB) was added to a final concentration of 0.5 μCi ml^{-1} . After 20 min the culture was divided into three fractions and halocin with similar activities as above was added. Duplicate samples of 0.5 ml were taken periodically, filtered through 0.45 μm HA Millipore filters and washed twice with 5 ml 25% SW. The radioactivity of the filters was measured as described above.

To measure the ability of treated and untreated cells to retain AIB, a culture was incubated for 2 h with [^{14}C]AIB as above, and then centrifuged at room temperature, washed twice with fresh medium and finally resuspended in 20 ml medium. This suspension was then divided into two flasks, one treated with halocin (233 AU ml^{-1}) and one untreated. Samples (0.5 ml) taken periodically were processed as described above.

ATP determination. The method described by Hubbard *et al.* (1976) was used, slightly modified. A 2 ml sample of *H. halobium* (10^8 cells ml^{-1}) was centrifuged and resuspended in 21 ml of the 25% SW/0.2% yeast extract medium. After incubation at 37 °C for 1 h with orbital shaking the culture was divided into two flasks. One received 2 ml concentrated halocin H4, previously denatured (10 min at 100 °C), and the other 2 ml of the same concentrate containing 2560 AU halocin H4 (final concentration 233 AU ml^{-1}). Samples of 1 ml were taken

periodically, filtered through 0.45 μm HA Millipore filters and washed twice with 5 ml 25% SW. The filters were immediately placed in tubes containing 3 ml 0.025 M-Tris/HCl pH 7.75 and kept in a boiling water bath for 5 min. ATP in the samples was quantified by the ATP-bioluminescence CLS assay (Boehringer Mannheim).

Effect on pH changes induced by light. For this experiment we used a mutant of *H. halobium* CCM 2090 which constitutively overproduces bacteriorhodopsin (Juez & Rodriguez-Valera, 1984); this mutant was as sensitive to halocin H4 as the wild-type strain. Measurement of the pH changes induced by light on suspensions of living cells was as described by Juez & Rodriguez-Valera (1984). To prepare a thick cell suspension, 100 ml of an exponential culture was centrifuged and resuspended in 20 ml 25% SW. This suspension was divided into two equal parts, one of which received 10 ml of the inactivated halocin H4 concentrate, and the other 10 ml of the halocin H4 concentrate containing 1280 AU (final concentration 640 AU ml⁻¹). The pH of the suspensions was adjusted to 7.5 with 0.05 M-HCl. Both suspensions were submitted to alternating 8 min periods of light and darkness. The pH variations were registered by introducing a pH meter electrode at the start of the experiment, and after 150 min and 7, 15 and 24 h.

Chemicals. D-[6-³H]Glucose (40 Ci mmol⁻¹), L-[G-³H]glutamic acid (45 Ci mmol⁻¹), [methyl-³H]thymidine (25 Ci mmol⁻¹), [5-³H]uridine (27 Ci mmol⁻¹) and 2-amino[1-¹⁴C]isobutyric acid (58 mCi mmol⁻¹) (1 Ci = 37 GBq) were purchased from Amersham. All other chemicals were obtained from standard commercial sources and were used without further purification.

RESULTS

Kinetics of killing effect

The death and lysis of *H. halobium* caused by halocin H4 were described by Rodriguez-Valera *et al.* (1982). However, the kinetics of these effects are also important, since for many bacteriocins they are very characteristic. Fig. 1 shows the viability curves of the sensitive strain CCM 2090 as a function of time and concentration. Both curves are very similar to those described for several eubacterial bacteriocins (Sahl & Brandis, 1982). Viability decreased exponentially with time, reaching a minimum after 5 h (Fig. 1a). A semilogarithmic plot of percentage survival against halocin concentration gave a linear correlation in the range of 10–320 AU ml⁻¹, indicating single-hit kinetics. No viable cells could be detected at concentrations above 320 AU ml⁻¹, and in the range 1–5 AU ml⁻¹ no significant decrease in the number of c.f.u. was observed (Fig. 1b).

Morphological changes induced by halocin H4

During the first 3 h of treatment no significant differences from control cells (Fig. 2a) were seen by either optical or electron microscopy observation. After 5 h, some cells started to show swollen spherical shapes (Fig. 2b). The proportion of cells showing this effect increased

Fig. 1. (a) Survival of *H. halobium* in the presence of halocin H4 (1150 AU ml⁻¹) as a function of time. (b) Killing action of different concentrations of halocin H4 in cultures containing 2.5×10^4 cells ml⁻¹.

Fig. 2. Electron micrographs of thin sections of *H. halobium* cultures (stained with uranyl acetate and lead acetate). (a) Untreated cells; (b) cells after 5 h exposure to halocin H4; (c, d) cells after 24 h exposure. Bars, 0.5 μm .

progressively in direct relation to the time of exposure. The affected cells gradually acquired the appearance shown in Fig. 2(c), where the cytoplasm appeared fairly empty with unevenly distributed clumps of electron-dense material. After 24 h, a large proportion of the cells were lysed (Fig. 2d) and the remainder showed evidence of advanced deterioration, close to the point of lysis.

Inhibition of macromolecule synthesis

The inhibition of macromolecule synthesis by halocin H4 was slow in all cases, becoming significant only after several hours (Fig. 3). The incorporation of glucose and uridine were the most rapidly inhibited. Glutamic acid incorporation was rapidly inhibited by anisomycin, a well-known inhibitor of protein synthesis in archaebacteria; by contrast halocin H4 produced a less marked effect (Fig. 3d).

Fig. 3. Influence of halocin H4 on macromolecule synthesis. Suspensions (10^7 cells ml^{-1}) in 25% SW supplemented with 0.2% yeast extract (pH 7.2) were preincubated at 37 °C for 30 min, then labelled compounds ($0.5 \mu\text{Ci ml}^{-1}$) were added and the culture was incubated for another 2 h. The culture was then divided into fractions and the following additions were made: 233 AU halocin H4 ml^{-1} ($\blacktriangle$); 116 AU halocin H4 ml^{-1} ($\circ$); heat-denatured halocin H4 ($\bullet$); 500 μg anisomycin ml^{-1} ($\triangle$). Radioactivity in TCA-treated samples was measured as described in Methods. (a) Carbohydrate, (b) RNA, (c) DNA, and (d) protein synthesis.

AIB transport

To check if the membrane permeability was altered, the effect of halocin H4 on the uptake of the non-metabolizable amino acid AIB was studied. The uptake of AIB was inhibited after only 10 min of treatment (Fig. 4a), and its efflux was stimulated (Fig. 4b).

Effect on bacteriorhodopsin-dependent H^+ extrusion

Fig. 5 shows the effect of halocin H4 on light-induced changes in the pH of the medium in suspensions of the bacteriorhodopsin-constitutive mutant of *H. halobium* CCM 2090 (see Methods). Not only was the H^+ gradient generated after illumination, but the effect became much stronger after a relatively short time (Fig. 5a–c). Furthermore, halocin H4 caused a progressive accumulation of H^+ in the medium (Fig. 5b–f).

ATP synthesis

Since one possible return channel for the H^+ ions extruded by bacteriorhodopsin or the respiratory chain is the membrane ATPase, one explanation of the above results could be the inactivation of this enzyme. Furthermore, the results shown in Fig. 5 are similar to the response to certain ATPase inactivators such as DCCD (Danon & Stoekenius, 1974). Therefore, ATP

Fig. 4. Effect of halocin H4 on $[^{14}\text{C}]\text{AIB}$ transport (a), and on the efflux of this compound from the cells (b). Culture conditions and preincubation were as in Fig. 3; then $0.5 \mu\text{Ci } [^{14}\text{C}]\text{AIB ml}^{-1}$ was added. (a) After 20 min incubation the culture was divided into fractions and 233 (▲), 116 (○) or no (●) AU halocin H4 ml^{-1} were added. Duplicate samples (0.5 ml) were filtered, washed and their radioactivity measured as described in Methods. (b) After 2 h incubation with $[^{14}\text{C}]\text{AIB}$ the culture was centrifuged, washed and resuspended in the same volume of medium without $[^{14}\text{C}]\text{AIB}$. This culture was divided into two and 233 (○) or no (●) AU halocin H4 ml^{-1} were added. The samples were processed as in (a).

Fig. 5. pH changes in the medium induced by alternate exposure to light and darkness of a suspension ($5 \times 10^8 \text{ cells ml}^{-1}$) of a bacteriorhodopsin-constitutive mutant of *H. halobium* sensitive to halocin H4. (a) no halocin added; (b) immediately after adding halocin H4 at a final concentration of 640 AU ml^{-1} ; (c) after 150 min treatment with halocin H4; (d) after 7 h; (e) after 15 h; (f) after 24 h.

levels were measured after exposure to halocin H4. Although a certain decrease in ATP levels was found it was far from what would be expected if the ATPase were the primary target of halocin H4. The differences between the treated and the control cells never exceeded 20%, and even after 3 h the values were fairly similar (results not shown).

DISCUSSION

Some characteristics of halocin H4, the first archaeobacterial bacteriocin described, are very similar to those of some bacteriocins of eubacteria. Most bacteriocins induce an exponential decrease of the viability of sensitive strains (Reeves, 1965) and this was also the case for halocin H4, although the time required was very long. However, some bacteriocins such as fulvocin C also induce a slow viability decrease (Hirsch *et al.*, 1978).

Halocin H4 induced deformation of the target cell, although not as fast as would be expected if the cell wall were the primary target. Colicin M, which acts on the cell wall of *Escherichia coli* induces very rapid morphological alterations, and bacitracin, which is an inhibitor of cell wall synthesis (Mescher & Strominger, 1978), induces spheroplast formation very rapidly. On the other hand, cytoplasmic alterations appeared simultaneously with, or even before, the changes in cell shape, and therefore it seems that the cell wall is not the primary target of halocin H4. When sensitive cells were placed in contact with halocin in a test tube, they sedimented after a few hours, and turbidity almost completely disappeared.

Neither macromolecule synthesis, nor the creation of channels unrestricted to small molecules, nor energy metabolism, are primary targets of halocin H4, since the effects detected on these processes were too slow. They could be due to a general decrease of metabolic activity produced by gradual cell death and lysis. The primary target is usually identified by the first alterations detected in the normal behaviour (Reeves, 1965). Halocin H4 produced a very rapid enhancement of the light-induced decrease in the pH of the medium (Fig. 5), although the increase continued over a long period. These results indicate that in some way halocin H4 alters the permeability of the membrane towards H^+ ions, impeding their normal return to the intracellular medium. The uptake of H^+ from the medium is mainly attributed to two mechanisms located in the membrane, ATPase and the Na^+/H^+ antiporter. The membrane ATPase does not appear to be the primary target of halocin H4, since the intracellular ATP contents were not significantly different between treated and control cells, even after 3 h treatment. It has been suggested that the Na^+/H^+ antiporter and the AIB permease have a common subunit in some organisms (Krulwich, 1983). If this applies to halobacteria, and if this subunit were partly or totally the target of halocin H4, AIB transport would be affected very soon after exposure to the halocin, as was observed here.

The results of the pH experiment (Fig. 5) exclude the possibility of H4 producing permeable channels in the cell membrane, since such pH gradients could not be possible if channels had been formed. The rapid efflux of AIB from the treated cells is difficult to explain. Ionic changes occurring inside the cell, or the alterations of permeability of the membrane, may be involved.

Although our results appear to indicate that the principal effect of halocin H4 occurs at membrane level, there is as yet no direct evidence that the primary target of this halocin is the Na^+/H^+ antiporter. However, since the halobacteria live in conditions which are toxic to most of their intracellular mechanisms, and keep osmotic balance with their environment by accumulating K^+ ions as compatible solute, by virtue of their ability to maintain large ion gradients across their cell membrane, this equilibrium would be an excellent target for an agent such as halocin H4. It has been postulated that bacteriocin receptors usually have very important functions, otherwise they would be eliminated by natural selection (Tagg *et al.*, 1976). This would be a good example of this principle.

We wish to thank S. Pereira and K. Hernandez for technical assistance.

REFERENCES

- DANON, A. & STOECKENIUS, W. (1974). Photophosphorylation in *Halobacterium halobium*. *Proceedings of the National Academy of Sciences of the United States of America* **71**, 1234–1238.
- HIRSCH, H. J., TSAI, H. & GEFFERS, I. (1978). Purification and effects of fulvocin C, a bacteriocin from *Myxococcus fulvus* Mxfl6. *Archives of Microbiology* **119**, 279–286.

- HUBBARD, J. S., RINEHART, C. A. & BAKER, R. A. (1976). Energy coupling in the active transport of amino acids by bacteriorhodopsin-containing cells of *Halobacterium halobium*. *Journal of Bacteriology* **125**, 181–190.
- JUEZ, G. & RODRIGUEZ-VALERA, F. (1984). A mutant of *Halobacterium halobium* with constitutive production of bacteriorhodopsin. *FEMS Microbiology Letters* **23**, 167–170.
- KONISKY, J. (1982). Colicins and other bacteriocins with established modes of action. *Annual Review of Microbiology* **36**, 125–144.
- KRULWICH, T. A. (1983). Na⁺/H⁺ antiporters. *Biochimica et biophysica acta* **726**, 245–264.
- LEVISOHN, R., KONISKY, J. & NOMURA, M. (1967). Interaction of colicins with bacterial cells. IV. Immunity breakdown studied with colicins Ia and Ib. *Journal of Bacteriology* **96**, 811–821.
- MESCHER, M. F. & STROMINGER, J. L. (1978). The cell surface glycoprotein of *Halobacterium salinarum*. In *Energetics and Structure of Halophilic Micro-organisms*, pp. 503–514. Edited by S. R. Kaplan & H. Ginzburg. Amsterdam & New York: Elsevier/North Holland Biomedical Press.
- MESEGUER, I. & RODRIGUEZ-VALERA, F. (1985). Production and purification of halocin H4. *FEMS Microbiology Letters* **28**, 177–182.
- OZAKI, M., HIGASHI, Y., SAITO, H., AN, T. & AMANO, T. (1966). Identity of megacin A with phospholipase A. *Biken Journal* **9**, 201–213.
- REEVES, P. (1965). The bacteriocins. *Bacteriological Reviews* **29**, 24–45.
- RODRIGUEZ-VALERA, F., JUEZ, G. & KUSHNER, D. J. (1982). Halocins: salt-dependent bacteriocins produced by extremely halophilic rods. *Canadian Journal of Microbiology* **28**, 151–154.
- RODRIGUEZ-VALERA, F., JUEZ, G. & KUSHNER, D. J. (1983). *Halobacterium mediterranei* spec. nov., a new carbohydrate-utilizing extreme halophile. *Systematic and Applied Microbiology* **4**, 369–381.
- SAHL, H. G. & BRANDIS, H. (1982). Mode of action of the staphylococcin-like peptide Pep-5 and culture conditions affecting its activity. *Zentralblatt für Bakteriologie und Hygiene (Abteilung originale A)* **252**, 166–175.
- SCHALLER, K., HÖLTJE, J. V. & BRAUN, V. (1982). Colicin M is an inhibitor of murein biosynthesis. *Journal of Bacteriology* **152**, 994–1000.
- STOECKENIUS, W. & ROWEN, R. (1967). A morphological study of *Halobacterium halobium* and its lysis in media of low salt concentrations. *Journal of Cell Biology* **34**, 365–393.
- TAGG, J. R., DAJANI, A. S. & WANNAMAKER, L. W. (1976). Bacteriocins of Gram-positive bacteria. *Bacteriological Reviews* **40**, 722–756.
- TIMMIS, K. (1972). Purification and characterization of colicin D. *Journal of Bacteriology* **109**, 19–20.
- WOESE, C. R. & FOX, G. E. (1977). Phylogenetic structure of the prokaryotic domain: the primary kingdoms. *Proceedings of the National Academy of Sciences of the United States of America* **74**, 5088–5090.
- YANG, C. C. & KONISKY, J. (1984). Colicin V-treated *Escherichia coli* does not generate membrane potential. *Journal of Bacteriology* **158**, 757–759.