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Sulfolobicins, Specific Proteinaceous Toxins Produced by Strains of the Extremely Thermophilic Archaeal Genus *Sulfolobus*

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Several novel strains of "*Sulfolobus islandicus*" produced proteinaceous toxins, termed sulfolobicins, which killed cells of other strains of the same species, as well as of *Sulfolobus solfataricus* P1 and *Sulfolobus shibatae* B12, but not of the producer strains and of *Sulfolobus acidocaldarius* DSM639. The sulfolobicin purified from the strain HEN2/2 had a molecular mass of about 20 kDa. It was found to be associated with the producer cells as well as with cell-derived S-layer-coated spherical membrane vesicles 90 to 180 nm in diameter and was not released from the cells in soluble form.

It has been shown previously that strains of extremely halophilic archaea of the euryarchaeotal genera *Halobacterium* and *Haloferax* produce toxic bacteriocin-like proteins, termed halocins, possibly for competition with related sensitive strains (5, 8, 9, 13, 15). Here we present evidence for the production of similar specific proteinaceous toxins by strains of the extremely thermophilic crenarchaeote *Sulfolobus*.

Strains and cell growth. The strains of *Sulfolobus* sp. described in this communication were isolated from samples taken from solfataric fields throughout Iceland. The methods for sampling and enrichment were similar to those described previously (16). The minimal medium (4), used either in liquid form or in Gelrite (Kelco, San Diego, Calif.) gels, was supplemented with 2 g of tryptone (Difco, Detroit, Mich.) per liter and adjusted to pH 3.2 with sulfuric acid. More than 400 isolates were obtained from heterotrophic enrichment cultures via single colonies. All these strains belonged to one species provisionally named "*Sulfolobus islandicus*" (16).

Demonstration of sulfolobicin production. The strains were screened for the inhibition of the growth of *Sulfolobus solfataricus* P1 (DSM 1616) by a "spot-on-lawn" procedure. Two microliters each of exponentially growing cultures of 420 different "*S. islandicus*" strains was spotted onto 1.5-ml soft layers of 0.2% Gelrite routinely seeded with about 6×10^7 cells of *S. solfataricus* and laid over 0.8% Gelrite supporting gels, as described by Zillig et al. (16). The spots of 41 cultures were surrounded by sharp-edged, nearly clear zones of growth inhibition (halos) with an area of about 0.8 cm^2 after incubation at 80°C for 48 h. The size of the halo did not depend on the incubation time. The inhibitory agent was not infectious and therefore not a virus. The effect rather appeared to be caused by an inhibitory substance resembling a bacteriocin (1, 6), which we thus called sulfolobicin, according to standard terminology.

The size of the halo was roughly inversely proportional to the initial density of the indicator lawn: a fourfold decrease of the soft-layer inoculum increased the area of the halo about threefold, and a fourfold increase of the inoculum decreased this area about threefold (data not shown).

All 41 sulfolobicin-producing strains inhibited not only the growth of *S. solfataricus* P1 but also that of *Sulfolobus shibatae* B12 (DSM 5389) and of six strains of "*S. islandicus*" which did not produce the toxins. They did not, however, inhibit the growth of each other or of *Sulfolobus acidocaldarius* DSM639. Cross immunity and inhibition of the same strains imply that sulfolobicins produced by different strains share the mode of action. The sulfolobicins of strains HEN2/2 and LAL17/3, which were studied in detail, had the same basic properties. In the following, we will therefore describe the toxin from HEN2/2 as sulfolobicin.

The progeny of each cell produced sulfolobicin. This was demonstrated by comparing the number of CFU in serial dilutions of growing cultures of the producer strain with the number of halos with central colonies produced at the same serial dilution when spread together with a lawn-forming inoculum of *S. solfataricus* P1 as indicator. The counts were essentially equal (data not shown).

Soluble sulfolobicin is not excreted into the culture medium. In cell-free culture supernatants, sulfolobicin activity could be detected only after about a 100-fold concentration, e.g., by precipitation with ammonium sulfate at 30% saturation, or with polyethylene glycol 6000 (105 g/liter) and NaCl (58 g/liter) (overnight at 4°C), or by centrifugation for 5 h at 50,000 rpm in a 55 Ti rotor (Beckman). For estimation of the activity, 2 μl of twofold serial dilutions of samples in 20 mM Tris-acetate, pH 6, was applied to standard lawns of *S. solfataricus*. The highest dilution producing recognizable inhibition was considered to contain 1 arbitrary unit (AU) of sulfolobicin. Maximal extracellular sulfolobicin activity was detected when the cells entered the stationary phase. The total extracellular activity of a 500-ml culture was about 5×10^3 AU. An approximately 30-times-higher amount of the toxin could be purified from the cells of a 500-ml culture following the procedure described below.

The release of sulfolobicin from exponentially growing producer cells could not be induced by UV irradiation (7), cold shock effected by cooling the culture from 80 to 25°C, or pH shock effected by changing the pH value of the culture from 3 to 7. In all three cases, normal growth conditions were then restored for a further 10 h before measuring the extracellular activity.

To check the possibility that the signal for the induction of sulfolobicin release could be the presence of the sensitive cells,

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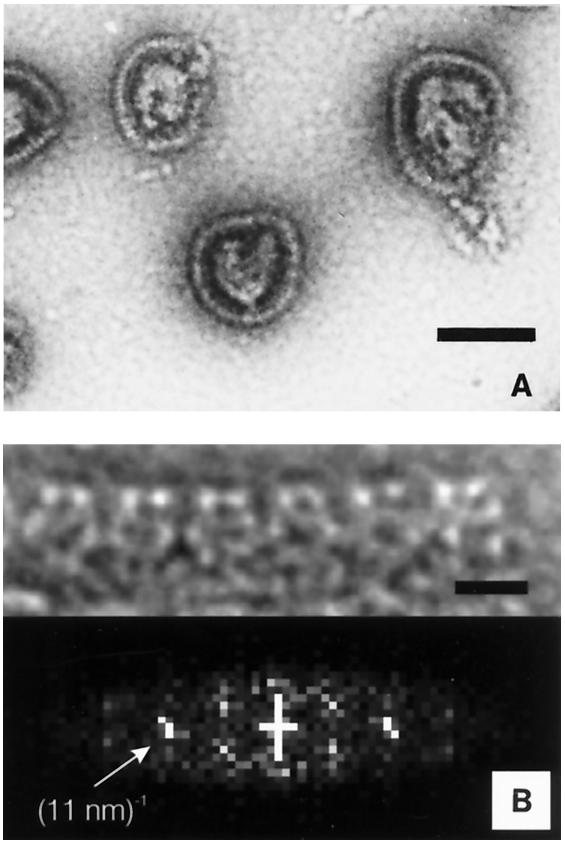

FIG. 1. (A) Electron micrograph of cell-derived vesicles with which extracellular sulfolobicin activity was associated. Vesicles were negatively stained with 2% uranyl acetate. Bar, 150 nm. (B) Electron micrograph of a fragment of the surface layer of a vesicle, stained with 2% uranyl acetate, and its diffraction pattern showing a clear reflex of the second order at (11 nm)$^{-1}$. Bar, 20 nm.

exponentially growing cultures of "*S. islandicus*" HEN2/2 and *S. solfataricus* P1 were mixed (1:1) and the extracellular sulfolobicin activity was measured 3, 10, 14, and 48 h later. Again, no increase of extracellular activity was observed.

The sulfolobicin released by the cells into liquid medium was found to be associated with spherical particles 90 to 180 nm in diameter, also formed by different *Sulfolobus* strains which do not produce sulfolobicin (Fig. 1A). Low numbers of these vesicles were formed by growing cells, mostly in the early stationary growth phase, where about one particle per 100 cells was observed. The number of the vesicles did not increase in the course of cell lysis in the stationary phase. We concentrated the vesicles from cell-free culture supernatants as described above and purified them by equilibrium density centrifugation in a CsCl gradient following the protocol developed for the purification of *Sulfolobus* viruses (16). In the CsCl gradient, the vesicles formed a sharp, white opalescent band with a buoyant density of about 1.29 g per ml. An inner core and a surrounding layer were visible on electron micrographs of the vesicles (Fig. 1A). The diffraction pattern of a fragment of the surrounding layer, obtained as described in reference 10, shows a periodicity of 22 nm (Fig. 1B), which corresponds to the lattice constant of the S layer of *Sulfolobus* cells (12).

We do not exclude the possibility that some freely diffusing sulfolobicin is released, e.g., by leakage, from cells or membrane vesicles into culture supernatants which we were not able to detect due to its low concentration. A much higher concentration of freely diffusing toxin around producer spots than in liquid culture could be a reason causing large zones of inhibition on Gelrite plates. The situation with the sulfolobicin resembles that with some cell-bound bacteriocins where release could be detected only in the course of growth on solid media (1).

TABLE 1. Purification of sulfolobicin from 5 g (wet weight) of "*S. islandicus*" HEN2/2

Fraction	OD_{280}[a]	Sulfolobicin activity (total AU)	Activity recovered (%)
30% $(NH_4)_2SO_4$ precipitate	98.7	1.2×10^6	100
Microsep 100K filtrate	14.8	4.2×10^5	35
Superose 6 eluate	3.1	2.2×10^5	18

[a] OD_{280}, optical density at 280 nm.

Purification procedure. For the extraction and purification of sulfolobicin, cultures of the producer cells were grown to the late stationary phase. The cells were collected, suspended in buffer A (20 mM Tris-acetate, pH 6), and disrupted by sonication (Branson sonifier fitted with a macro tip; 7 min). Residual unbroken cells were removed by centrifugation at 3,000 rpm in a Minifuge 2 (Heraeus). The cell ghosts were collected by high-speed centrifugation (30 min at 39,000 rpm in a Beckman SW41 rotor). No sulfolobicin activity was present in the supernatant. The ghosts were washed twice in buffer A and then subjected to extraction with either 6 M urea, 1 M NaCl, 0.1% Triton X-100 (all in buffer A), diethylether, or trichlormethan or the mixture trichlormethan-methanol-water (65:25:4) or *n*-butanol–acetic acid–water (80:20:20). Only Triton X-100 extraction was able to release the sulfolobicin from the ghosts.

The sulfolobicin was precipitated from the Triton extract by addition of ammonium sulfate to 30% saturation. The precipitate was collected by centrifugation, washed twice with 30% ammonium sulfate in buffer A to remove all Triton X-100, and dissolved in buffer B (buffer A containing 6 M urea). Further purification steps included ultrafiltration through a 100-kDa-cutoff membrane (Filtron) and chromatography on a Superose 6 preparation-grade (Pharmacia) column in buffer B. The fractions containing sulfolobicin activity eluted in the range of proteins with molecular masses of 30 to 40 kDa (data not shown). They were combined, concentrated, and extensively dialyzed against buffer A. The last steps of the purification of the sulfolobicin from 5 g (wet weight) of cells of "*S. islandicus*" HEN2/2 are summarized in Table 1.

In the course of ultrafiltration, the presence of 6 M urea in the buffer was essential. In its absence, no detectable sulfolobicin passed through the concentrator membranes. Cellophane membranes with dilated pores and PLMK cellulose membranes (Millipore) with molecular mass cutoffs between 100 and 300 kDa were also impermeable for the sulfolobicin in the absence of urea. Considering that the molecular mass of purified sulfolobicin estimated by electrophoresis in denaturing conditions (see below) is only 20 kDa, the results indicate aggregation and/or adsorption to the membranes, which are reduced in the presence of 6 M urea.

The purified sulfolobicin had the same inhibitory specificity as the producer strain. It had no effect on the growth of *Halobacterium salinarum* R1 (DSM671) or *Escherichia coli*. No loss of the activity (750 AU/ml) was detected after 6 months at 4°C or after 5 days at 85°C, pH 3.5 to 6.5.

Chemical nature. To elucidate the chemical nature of the sulfolobicin, the purified preparation was treated with α-amy-

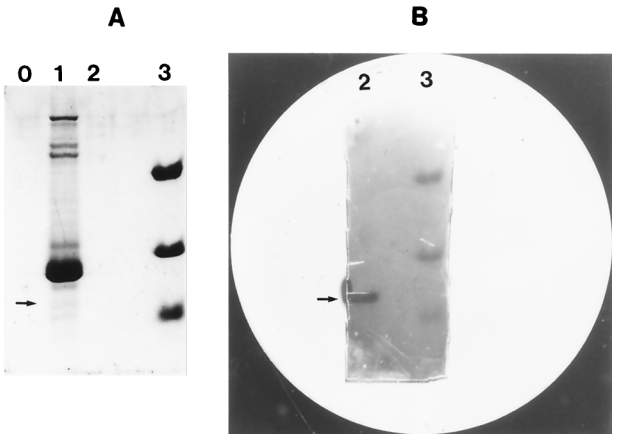

FIG. 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of partially purified sulfolobicin. (A) Coomassie blue-stained gel. (B) A portion of the Coomassie blue-stained gel containing lanes 2 and 3 laid onto an indicator lawn. Lane 1, 10^5 AU of sulfolobicin; lanes 2, 100 AU of sulfolobicin; lanes 3, protein markers with molecular masses of 39.2, 26.6, and 20.1 kDa. The arrows indicate the clearing of the lawn at the position of sulfolobicin (B) and a Coomassie blue-stained protein band with the same mobility (A).

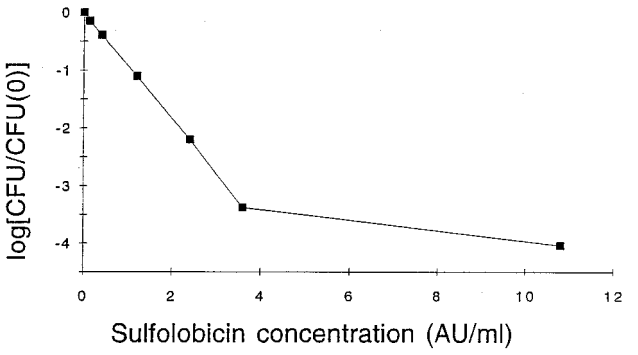

FIG. 3. Survival of *S. solfataricus* in the presence of sulfolobicin. Different amounts of sulfolobicin were added to 25 ml of growing cultures of *S. solfataricus* containing about 5×10^6 cells/ml. After 24 h of growth, the samples were plated for the detection of CFU. CFU(0) was determined before addition of sulfolobicin.

lase, α- and β-glucosidases, lipase, phospholipase C, lipoprotein lipase, pronase E, proteinase K, and trypsin (all from Sigma and used as recommended by the manufacturer). The assay mixtures containing 0.1 mg of the enzyme tested per ml and 20 AU of the sulfolobicin per μl were incubated for 3 h at 37°C. The activity was determined by the spot-on-lawn test in comparison with a corresponding control without enzyme. No decrease of sulfolobicin activity was detected after treatment with glycolytic or lipolytic enzymes. Incubation with all proteolytic enzymes tested led to the complete loss of sulfolobicin activity, indicating that an intact protein is required for activity.

Molecular mass. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 0.7-mm gels as described by Schägger and von Jagow (14) was used to estimate the molecular mass of the sulfolobicin. Since no protein band was visible on a Coomassie blue-stained gel with 100 AU of sulfolobicin purified as described above (Fig. 2A, lane 2), the sulfolobicin band was detected via its activity. A Coomassie blue-stained gel with 100 AU of sulfolobicin and molecular mass standards was washed in distilled water for 6 h and laid over a soft layer seeded with *S. solfataricus*. A zone of growth inhibition was observed after development of the lawn at 80°C for 48 h (Fig. 2B, lane 2). The molecular mass of the sulfolobicin was estimated from its mobility to be approximately 20 kDa. To directly visualize the sulfolobicin band by Coomassie blue staining, we had to apply about 10^5 AU of the toxin (Fig. 2A, lane 1). The sulfolobicin from isolated S-layer-coated membrane vesicles had the same molecular mass as that solubilized and purified from cell membranes.

Concentration dependence of archaeocidal effect. Addition of sulfolobicin (100 AU/ml) to an *S. solfataricus* culture at an optical density at 600 nm of 0.25 caused a decrease in the number of CFU to about 50% in 20 min, whereas the optical density remained constant (data not shown). Thus, the effect of the toxin is archaeocidal rather than archaeolytic. The decrease of the fraction of viable cells as a function of the sulfolobicin concentration is shown in Fig. 3.

Plasmids of sulfolobicin-producing strains. Some of the sulfolobicin-producing strains of "*S. islandicus*," e.g., HEN2/2, contained conjugative plasmids (11). The production of and the resistance to sulfolobicin were, however, not transferred to transcipients by the DNAs of these plasmids (D. Prangishvili and W. Zillig, unpublished results). The results indicate that the genes for sulfolobicin production and immunity might be located on the chromosomes of the producer cells.

Perspectives. Although sulfolobicin shares key characteristics of bacteriocins, such as the proteinaceous nature, the killing mode of action, and the narrow range of activity directed primarily against closely related strains (6), it is in some respects different. In contrast to many bacteriocins, sulfolobicin is apparently not released from the producer cells in soluble form in liquid medium but remains bound to the membranes of the cells or of cell-derived S-layer-coated membrane vesicles. These vesicles resemble recently described enzyme-containing killer vesicles produced by different gram-negative bacteria (2).

The genes encoding sulfolobicin synthesis and resistance should be useful candidates for genetic markers, which are still scarce in *Sulfolobus*.

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