Bacteriocins have traditionally been defined as proteinaceous compounds produced by bacteria that inhibit or kill closely related bacteria (16). A special group of bacteriocins are high-molecular-weight particles, which can be sedimented by ultracentrifugation and are resolved by electron microscopy as phage tail-like particles (8, 13). These particles have been regarded as defective bacteriophages, which might have arisen from temperate phages by several successive mutations (8, 13). Bacteriocins of this type have been found in cultures of several gram-negative bacteria, including members of the families Enterobacteriaceae, Vibrionaceae, and Pseudomonadaceae (8, 10).

The most thoroughly studied phage tail-like bacteriocins are the F-type and R-type pyocins produced by Pseudomonas aeruginosa. The F-type pyocins resemble flexible but noncontractile tail structures of bacteriophages, whereas the R-type pyocins are similar to contractile but nonflexible tails (26). The bactericidal activity of the R-type pyocins is caused by the depolarization of the cytoplasmic membranes of sensitive bacteria (33). The pyocin biosynthesis genes were chromosomally located, and the gene organization clearly demonstrated that pyocins possess an ancestral origin common with bacteriophages. It was suggested that the pyocins have evolutionarily specialized as phage tails, rather than being just simple defective phages (26).

As the increase in bacteria resistant to a wide range of antibiotics has become a major public health problem, alternative strategies are being looked for to counter bacterial infections. In recent years the approach of using bacteriocins for the treatment of bacterial infections has come into focus again (2, 5, 23), as the advantage of phages as therapeutic agents is the high specificity for their target organisms, which would enable the selective elimination of phase-susceptible bacteria from a bacterial community (20). Bacteriocins are comparable to bacteriophages in terms of specificity for target bacteria, and given the morphological similarity, the use of phage tail-like bacteriocins for therapeutic use is conceivable, although bacteriocins lack the ability of phages to multiply during infection of target bacteria.

In the genus Yersinia, the production of phage tail-like particles by strains of Y. kristensenii, Y. frederiksenii, and Y. intermedia has been reported (9). These particles were used as diagnostic tools for typing Yersinia strains. Another early report about a phage tail produced by a Y. enterocolitica strain consisted mainly of morphological data (18). By studying a number of Yersinia isolates (19, 25) we found a food-borne strain of Y. enterocolitica which produced a bacteriocin-like substance with an inhibitory activity against serogroups O:3, O:5,27 and O:9 of Y. enterocolitica, which are the dominating pathogenic serogroups for humans (7, 21). The aim of our study was to characterize structural and functional features of this bacteriocin, which was designated enterocoliticin according to the usual nomenclature of bacteriocins (16), by studying its inhibitory activity against a pathogenic Y. enterocolitica O:3 strain.

### MATERIALS AND METHODS

**Bacterial strains and growth medium.** Y. enterocolitica 29930 (serogroup O:7,8, biogroup 1A), a food-borne isolate (19), was the strain producing enterocoliticin. Y. enterocolitica 13169 (serogroup O:3, biogroup 4) (25) and its derivative 13169-2, which had been cured of the Yop virulon plasmid, were used as sensitive indicator strains for most experiments. Y. enterocolitica 29807 (serogroup O:5, biogroup 1A) (30) was used as the nonsusceptible control strain. Y. enterocolitica Frederiksen P413 (biogroup 1A, serogroup O:7,8), a guinea pig isolate, was obtained from the Institute Pasteur, Paris, France.

Yersinia strains used for the determination of the inhibitory activity of enterocoliticin (Table 1) were taken from the strain collection of the Robert Koch Institute and include several strains initially obtained from the Institute Pasteur. The Enterobacteriaceae strains used (see Table 1) were obtained from L. Beutin and H. Steinrück, Robert Koch Institute. Bacterial strains were grown in Luria-

### CHARACTERIZATION OF ENTEROCOLITICIN, A PHAGE TAIL-LIKE BACTERIOCIN, AND ITS EFFECT ON PATHOGENIC YERSINIA ENTEROCOLITICA STRAINS

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**Yersinia enterocolitica** 29930 (biogroup 1A; serogroup O:7,8) produces a bacteriocin, designated enterocoliticin, that shows inhibitory activity against enteropathogenic strains of *Y. enterocolitica* belonging to serogroups O:3, O:5,27 and O:9. Enterocoliticin was purified, and electron micrographs of enterocoliticin preparations revealed the presence of phage tail-like particles. The particles did not contain nucleic acids and showed contraction upon contact with susceptible bacteria. Enterocoliticin addition to logarithmic-phase cultures of susceptible bacterial strains led to a rapid dose-dependent reduction in CFU. Calorimetric measurements of the heat output of cultures of sensitive bacteria showed a complete loss of cellular metabolic activity immediately upon addition of enterocoliticin. Furthermore, a dose-dependent eﬄux of K⁺ ions into the medium was determined, indicating that enterocoliticin has channel-forming activity.

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The combined pellets were resuspended in 8 ml of water and applied to a CsCl density gradient (1.3 to 1.7 g/ml) and centrifuged at 28,000 rpm (141,000 × g). The combined supernatants were dialyzed against water and applied to the column, and protein elution from the column was monitored by absorbance at 260 nm. Dialyzed aliquots of CsCl-purified enterocoliticin preparations were made, and 20 μl of each preparation was spotted on LB plates which had been overlaid with LB soft agar containing 790 μl of the same medium were inoculated with 10 μl of the reference strain Y. enterocolitica 13169. All other bacteria were tested twice. The MIC of enterocoliticin was determined in LB medium following a protocol described for enterococcal bacteriocins. After the bacteriocin suspension had dried, negative staining was performed as described above.

Physical and chemical stability of enterocoliticin was tested by incubating different concentrations of enterocoliticin (highest concentration was 1.3 × 10^8 AU ml⁻¹) for 24 h at 37°C. Enterocoliticin dilutions in 50 mM Tris-HCl, pH 7.5 were analyzed for activity by spot tests on Y. enterocolitica 13169. Enterococcal bacteriocin suspension (20 μl) was used to coat Formvar-carbon-coated copper grids which had been treated with 0.1% phosphotungstic acid, pH 6.8. Enterobacter cloacae RKI 10 was used as a control strain. After the bacteriocin suspension had dried, negative staining was performed as described above. Enterococcal bacteriocin suspension (20 μl) was used to coat Formvar-carbon-coated copper grids which had been treated with 0.1% phosphotungstic acid, pH 6.8. Enterobacter cloacae RKI 10 was used as a control strain.
other bacteriocins (6). Cultures of susceptible strains 13169 and 13169-2 were grown at 25 and 37°C to an optical density at 588 nm (OD588) of 0.55. Aliquots of the cultures were diluted (at a ratio of 1:9) in a volume of 200 μL of LB containing a twofold serial dilution of enterocoliticin in 96-well microtiter plates. The plates were incubated at either 25 or 37°C. The OD588 was read after 6 h by a microtiter reader (Easyreader EAR 400 AT; SLT-LabInstruments, Grödig, Austria), and the results were confirmed by visual control. The experiments were repeated three times for each temperature. The MIC corresponds to the lowest concentration of enterocoliticin that suppressed growth of the indicator strain completely.

Determination of CFU after addition of enterocoliticin was done as follows. A culture of 90 ml was grown at 37°C to an OD588 of 0.55 and divided into 15-ml aliquots. Enterocoliticin was added to give final concentrations of 2.2 × 107, 2.2 × 106, 2.2 × 105, and 2.2 × 104 AU ml−1 and portions of the treated cultures were removed after 1, 3, 5, 10, 20, 30, and 60 min. The bacteria were immediately washed once with 0.9% (wt/vol) NaCl, and CFU were determined on LB plates. Growth inhibition of cultures after addition of enterocoliticin was also determined by measuring the OD588.

Microcalorimetry. Calorimetric measurements were performed on a flow microcalorimeter LKB 2107 (LKB, Bromma, Sweden) as described previously (34). A flow rate of 40 ml h−1 was maintained by a peristaltic pump. The incubation flask (100 ml) containing 60 ml of LB medium was inoculated with about 1 mL of the bacterial culture grown overnight, shaken (175 rpm) at 37°C, and connected to the flow microcalorimeter. The bacterial suspension was pumped through the measuring coil (0.7 ml) of the microcalorimeter, and the outflow was returned to the incubation flask. The heat output (microwatts) of the culture was recorded continuously and included a short thermal equilibration period (about 10 min). The experiment was maintained throughout the experiment by a microtiter reader (Easyrider EAR 400 AT; SLT-LabInstruments, Großdig, Austria), and the results were confirmed by visual control. The experiments were repeated three times for each temperature. The MIC corresponds to the lowest concentration of enterocoliticin that suppressed growth of the indicator strain completely.

Electron micrographs of enterocoliticin preparations revealed the presence of phage tail-like particles without contamination with other particle structures (Fig. 1A). Typical structural elements of phage tails were identified, such as extended tails, contracted tails, sheath, and core structures (Fig. 1B). In some particles a base plate with spikes was visible (Fig. 1B, inset). The length of the extended particles was 80 ± 5 nm and the diameter was 15 ± 1 nm. The contracted structures were 35 ± 2 nm in longitudinal length and 20 ± 2 nm in diameter. Core particles had a diameter of 5 ± 1 nm.

In Fig. 2 adsorption of enterocoliticin particles to a sensitive bacterium is shown, revealing contraction of the particles upon contact with the surface of the bacterium. Electron micrographs were taken from appropriate dilutions of enterocoliticin preparations spotted on grids with a defined mesh size. After determining the numbers of particles, it was concluded that an activity of 1.3 × 107 AU ml−1 corresponds to 2 × 1012 ± 0.4 × 1012 particles ml−1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of enterocoliticin preparations revealed the presence of approximately 15 proteins of between 10 and 60 kDa (data not shown).

An aliquot of a CsCl-purified enterocoliticin preparation was subsequently purified on a size exclusion column to demonstrate that the inhibitory activity copurified with the phage tails (Fig. 3). More than 90% of the activity was eluted in fractions with a molecular mass over 669 kDa (Fig. 3). Electron micrographs taken from peak fractions also revealed the presence of phage tails.

Nucleic acid content and chemical stability of enterocoliticin. Enterocoliticin preparations containing about 1012 particles (1.3 × 107 AU ml−1) were examined for nucleic acid content following standard protocols for phage DNA isolation. However, DNA was never detected in such preparations (estimated detection limit for DNA was 0.5 ng). To further determine the chemical stability, enterocoliticin preparations were incubated for 10 min at different temperatures. No loss of activity was detected at incubation temperatures of up to 50°C; in contrast, more than 90% of the activity was lost at 55°C, and no activity was retained after incubation at 60°C. Incubation in the presence of trypsin for 1 h did not affect enterocoliticin activity; however, proteinase K treatment reduced enterocoliticin activity to less than 1%.

Comparison of enterocoliticin with a phage tail particle produced by Y. enterocolitica Frederiksen P413. Hamon et al. (18) described a bacteriocin-like substance from a Y. enterocol-
FIG. 1. Electron micrographs of enterocoliticin particles after negative staining. (A) Extended and contracted phage tail-like structures. (B) Higher magnification of enterocoliticin particles with different structural elements, showing the similarity to the architecture of phage tails (ex, extended particles; c, contracted particles; sh, sheath; co, core). (Inset) Particles with base plate (bp) and spikes (s).
strain (Frederiksen P413) that was active against a number of Y. enterocolitica strains. The substance was reported to consist of particles resembling phage tails with a length of 80 nm, and in a contracted form a length of 40 nm and a diameter of 23 nm. The diameter of the central core was given as 6 nm. The strain Frederiksen P413 was kindly provided by E. Carniel, Institute Pasteur, Paris, who characterized the strain as serogroup O:7,8, biogroup 1A.

Electron micrographs of the phage tail-like particles that we isolated from the supernatant of strain Frederiksen P413 revealed a close resemblance to enterocoliticin particles (data not shown). Additionally, by testing a number of Yersinia strains, we did not discover a difference in the inhibition spectrum between enterocoliticin and the bacteriocin-like substance of strain Y. enterocolitica Frederiksen P413 (see below). Although both strains belong to the same bio- and serogroup as the enterocoliticin-producing strain, our strain 29330 harbored plasmids, while strain Frederiksen P413 did not.

**MIC determination and inhibitory action of enterocoliticin.** The MIC of enterocoliticin was determined at 25 and 37°C for the susceptible strains Y. enterocolitica 13169 and its derivative 13169-2, which had been cured of the virulence plasmid. The MIC determined for the two strains was $8.2 \times 10^3 \pm 0.4 \times 10^3$ AU ml$^{-1}$ at both growth temperatures, indicating that none of the genes located on the virulence plasmid influenced susceptibility.

The bactericidal activity of enterocoliticin was demonstrated by adding enterocoliticin to logarithmic-phase cultures of the susceptible O:3 indicator strain Y. enterocolitica 13169. The highest concentration used ($2.2 \times 10^7$ AU ml$^{-1}$), corresponding to approximately 25 times the MIC, reduced the CFU by nearly 10,000-fold within 60 min, while an enterocoliticin con-
centration of 2.5 times the MIC reduced the CFU titer by 1,000-fold (more than 99% killing) (Fig. 4).

The lysis of the susceptible strain was determined by following the decrease in the OD588 upon addition of various concentrations of the enterocoliticin to a logarithmic culture. A significant decrease in optical density started 30 to 60 min after addition of enterocoliticin at a concentration above the MIC (Fig. 5). The highest concentration of enterocoliticin chosen was 2.2 x 10^8 AU ml^(-1), which killed more than 99% of the cells. Concentrations of enterocoliticin below the MIC (5.5 x 10^7 AU ml^(-1) and below) lysed the culture only partially, while the lowest concentration used in this test delayed the growth of the culture for 90 min (0.3 x 10^8 AU ml^(-1)). Based on these data, the following calculation was performed: Enterocoliticin preparations with an activity of 1.3 x 10^6 AU ml^(-1) contain 2 x 10^{12} ± 0.4 x 10^{12} particles ml^(-1), which means that a final concentration of enterocoliticin of 5.5 x 10^7 AU ml^(-1) corresponds to ca. 8 x 10^9 ± 1.6 x 10^9 particles ml^(-1). A drop in OD588 from 0.55 to 0.2 corresponds to a decrease from 5.5 x 10^8 ± 0.5 x 10^8 CFU ml^(-1) to 2 x 10^8 ± 0.5 x 10^8 CFU ml^(-1). The calculation indicates that very few and probably even a single enterocoliticin particle is sufficient to kill a bacterial cell.

The experiment described above was repeated with enteropathogenic Y. enterocolitica strains belonging to serogroups O:8, O:9, and O:5,27. The addition of serial dilutions of enterocoliticin to O:9 and O:5,27 strains resulted in a decrease in the OD588 to the same extent as shown for the O:3 strain in Fig. 5, whereas growth of the O:8 strain was not affected (see also below).

**Action of enterocoliticin on growing cultures determined by microcalorimetric investigation.** The effect of enterocoliticin on the metabolic activity of the sensitive indicator strain Y. enterocolitica 13169 was determined by measuring the heat output of a growing culture after addition of enterocoliticin. The nonsusceptible Y. enterocolitica strain 29807 was used as the control. The thermograms of the untreated bacterial cultures are shown in Fig. 6A and 6C. After an initial equilibration phase of 10 min, the heat output, which reflects metabolic activity, increased with the growth of the cultures and decreased when they entered the stationary phase. The thermograms of cultures of the same strains which were treated with enterocoliticin (5.5 times the MIC) are shown in Fig. 6B and 6D. The heat output of the culture of the sensitive strain 13169 declined rapidly after addition of enterocoliticin, while the metabolic activity of the nonsusceptible strain 29807 was not affected. The immediate cessation of metabolic activity in the sensitive strain is characteristic of a bactericidal effect.

**Efflux of K^+ ions after addition of enterocoliticin.** The experiments described above indicated that the immediate
breakdown of the metabolic activity of the sensitive strain *Y. enterocolitica* 13169 after addition of enterocoliticin might be the result of the formation of channels across the bacterial cell envelope. Such channels might be induced by the adsorption and contraction of enterocoliticin particles to susceptible cells and might cause a rapid efflux of metabolites. To test this hypothesis, we measured the efflux of $K^+$ ions after addition of various concentrations of enterocoliticin (Fig. 7). Within a few minutes, a dose-dependent and rapid increase in $K^+$ ions in the extracellular medium was observed, supporting this hypothesis.

**Inhibitory spectrum of enterocoliticin.** The inhibitory spectrum of enterocoliticin was determined for a number of *Yersinia* strains and related bacteria of the family *Enterobacteriaceae*. Furthermore, some other gram-negative and gram-positive bacteria were included in the test (Table 1). The inhibitory spectrum was determined by spotting a twofold serial dilution of enterocoliticin on agar seeded with bacteria (see Materials and Methods).

The main focus of the determination of the inhibitory spectrum was the enteropathogenic *Yersinia* strains. While *Y. pseudotuberculosis* and strains of the pathogenic *Y. enterocolitica* serogroup O:8 were not sensitive to enterocoliticin, all investigated strains of the pathogenic *Y. enterocolitica* serogroups O:3, O:5,27, and O:9 were susceptible. Among the nonpathogenic *Yersinia* strains (*Y. enterocolitica* biogroup 1A, *Y. kristensenii*, *Y. frederiksenii*, and *Y. intermedia*), some strains were also susceptible. Remarkably, almost all *Yersinia* strains were inhibited up to the same final concentration of enterocoliticin in the serial dilution spot test, indicating that all sensitive *Yersinia* strains were susceptible to the same degree as the control strain *Y. enterocolitica* 13169. Only one *Y. frederiksenii* strain exhibited an intermediate sensitivity and was not investigated further. None of the remaining bacterial strains investigated which do not belong to the genus *Yersinia* were susceptible to enterocoliticin.

The bacteriocin-like substance of *Y. enterocolitica* Frederiksen P413 was also tested on *Yersinia* strains by spot tests. It was found that the inhibitory activity of this bacteriocin was identical to that of enterocoliticin, indicating that the substances are identical or closely related.
Most phage tail-like bacteriocins were reported to be resistant to trypsin and to be labile to heat treatment above 50°C (3, 13), which was also confirmed for enterocoliticin. Attempts to find DNA in enterocoliticin particles were not successful. Most of the described phage tail-like bacteriocins do not contain DNA (8, 13). However, a recent publication reported for the first time that in the R pyocins of P. aeruginosa strain C, two small DNA molecules, whose function remained unclear, could be isolated from the particles (24).

The enteropathogenic Y. enterocolitica O:3 strain 13169 was used to investigate the inhibitory activity of enterocoliticin, as this serogroup is a common cause of yersinial infection in humans worldwide (7) and seems to be the most abundant in swine (21, 28), which serve as a major reservoir for the human-pathogenic Y. enterocolitica strains (7). Following protocols for determining the MICs of bacteriocins (6), the MICs of enterocoliticin were determined for wild-type strain 13169 and its derivative 13169-2, which had been cured of the virulence plasmid. The MICs were identical, indicating that traits encoded by the Yop virulon did not play a role in enterocoliticin susceptibility. Presumably, the MICs of all sensitive strains are identical or very similar, as the serial dilution test performed with enterocoliticin preparations of defined activity always showed inhibition to the same extent as the reference strain 13169. Only one Y. frederiksenii strain showed a lower susceptibility in the serial dilution test; we did not investigate this strain further.

To gain more insight into the function of enterocoliticin, we performed cell viability assays by measuring the heat output of cultures after addition of enterocoliticin. The metabolic heat production measured by flow microcalorimetry has been proven to be a very sensitive indicator of metabolic disturbances after addition of antibiotics (22, 34). We showed that treatment of the sensitive strain immediately resulted in a complete loss of metabolic activity. The very rapid action of enterocoliticin might indicate that channels are formed through the bacterial surface, as described for other phage tail-like bacteriocins.

In the case of the pyocins, the mechanism of killing is by pore formation in the membrane causing disruption of the membrane potential (33). The vibriocins of Vibrio cholerae were also shown to form pores and led to an increased efflux of K⁺ ions (10). If bacteriocins with a phage tail-like structure are to be considered defective bacteriophages, it is conceivable to compare adsorption to sensitive cells with the infection process of bacteriophages. For bacteriophages, it is strongly believed that the phage DNA is transported via channels into the bacterial cells induced by the action of the tails. A short transient efflux of K⁺ ions after adsorption to sensitive cells was measured and was remarkably prolonged in defective headless phage ghosts (for a review, see reference 14). The rapid increase in potassium ions in the extracellular medium after addition of enterocoliticin to sensitive bacteria (Fig. 7) might be mechanistically similar to the effects reported for bacteriophages and thus supports the hypothesis of channel formation.

The increase in bacteria resistant to a large number of antibiotics has renewed the scientific discussion about the use of...
bacteriophages to counter bacterial infections. The specific activity of enterocolitcin against the most common pathogenic serogroups of *Y. enterocolitica* makes it conceivable to test phage tails as a possible therapeutic agent. Enteropathogenic *Y. enterocolitica* strains should be an appropriate model pathogen for therapeutic application of phage tail-like particles, since anatomopathological examinations have shown that the pathogen remains mainly extracellular in eukaryotic hosts and forms microcolonies in the gastrointestinal tract and the associated mesenteric lymphatic tissue (12). Thus, it should be accessible to the phage tail particles.

The efficacy of enterocolitcin as an antimicrobial agent will be tested in future experiments in a mouse infection model. The infection of mice with *Y. enterocolitica* belonging to the so-called low-pathogenicity strains of serogroups O:3, O:5,27, and O:9 is not fatal for the animals, but results in prolonged colonization (4). It resembles the infection of swine, which makes it conceivable to test strains belonging to the *Y. enterocolitica* group 1A strains hybridizing to the *Y. enterocolitica* virulence plasmid. Such a strain may serve as an appropriate model pathogen for *Y. enterocolitica* strains.

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