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Membrane Permeabilization of *Listeria monocytogenes* and Mitochondria by the Bacteriocin Mesentericin Y105

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Mesentericin Y105, a bacteriocin produced by a *Leuconostoc mesenteroides* strain, dissipates the plasma membrane potential of *Listeria monocytogenes* and inhibits the transport of leucine and glutamic acid. It also induces an efflux of preaccumulated amino acids from cells. In addition, the bacteriocin uncouples mitochondria by increasing state 4 respiration and decreasing state 3 respiration. The bacteriocin inhibits ATP synthase and adenine nucleotide translocase of the organelle while the affinity of ADP for its carrier is not modified. The results suggest that mesentericin Y105 acts by inducing, directly or indirectly, pore formation in the energy-transducing membranes, especially those of its natural target.

Bacteriocins are antibacterial peptides or proteins that are secreted by bacteria and act on target cells by various mechanisms, most of which are, as yet, unclear. In the group of colicins, two classes may be distinguished on the basis of their mode of action. The major class contains colicins (E1, Ia, A, B, K, and N) which kill sensitive cells by forming ion channels in their cytoplasmic membranes (3). The second class contains those colicins displaying a nuclease action (D, E2, E3, M, and V). Nisin is the best known bacteriocin produced by lactic acid bacteria. It has a wide spectrum of activity on gram-positive bacteria (12) and various *Salmonella* species and other gram-negative organisms (23). The bacteriocin alters the permeability of the cytoplasmic membrane, leading to the efflux of amino acids and dissipating the membrane potential and ionic gradients (20). Another bacteriocin, lactostrepcin 5 (Lac 5), which disrupts the integrity of the cell membrane of *Lactococcus lactis* cells, leads to the leakage of ions and ATP (28). Lactococcin A, which acts preferentially on lactococci, increases the permeability of the cytoplasmic membrane of whole cells (26), inhibits amino acid transport into sensitive cells and causes efflux of preaccumulated amino acids.

The bacteriocin mesentericin Y105 (mY105) produced by *Leuconostoc mesenteroides* Y105 exerts a bactericidal effect on *Listeria monocytogenes* E20 (6–8). It is a low-molecular-mass peptide (composed of 36 amino acids) that is highly thermostable and acidostable. Its primary structure closely resembles that of other recently discovered bacteriocins: leucocin A-UAL187 (5), pediocin PA-1 (9), and sakacin P and curvacin A (25).

In this report, we present some biochemical details of the mode of action of bacteriocin mY105. In particular, its effect on *L. monocytogenes* E20 membrane potential was determined by using the fluorescent probe rhodamine 123 (Rh 123) and flow cytometry. Amino acid transport into *Listeria monocytogenes* has also been investigated, in addition to an analysis of the effects of the bacteriocin on various functions of isolated mammalian mitochondria.

Action of the bacteriocin mY105 on the membrane potential of *L. monocytogenes* E20. Since mY105 is a small peptide, it may act like several bacteriocins by permeabilizing the cytoplasmic membrane. To investigate this hypothesis, the effect of mY105 on the membrane potential of *L. monocytogenes* E20 was measured by flow cytometric analysis. This was assessed by the incorporation of the fluorescent potential probe Rh 123, which occurs only in cells bearing intact membranes (13). To ascertain the efficiency of the treatment, we employed ionophores known to depolarize or to hyperpolarize plasma membranes. Their influence on the plasma membrane potential of *Listeria* cells was determined and compared with that of mY105 under the same conditions (Table 1). Nigericin carries out the antiport of K^+ for H^+ and therefore hyperpolarizes the membrane. The addition of 5 μM nigericin to *Listeria* cells increases Rh 123 uptake by 31%. The K^+ ionophore valinomycin and the protonophore carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) are known to collapse the transmembrane potential. In the presence of 5 μM valinomycin or 5 μM CCCP, Rh 123 incorporation decreased by 26 or 43%, respectively, compared with that in the control (Table 1). Bruno et al. (2) reported that it is possible to decrease completely the membrane potential in the presence of valinomycin by increasing the K^+ concentration in the incubation medium to 200 mM. With the protonophore CCCP, these investigators (2) and others (13) reported only a 36% decrease in $\Delta\Psi$. According to these data, we could predict that the decrease in the membrane potential, in our experiments, would not be greater than 40%. As expected, mY105 decreases the incorporation of the fluorescent potential probe by *L. monocytogenes* E20 to some extent with valinomycin or CCCP (Table 1). Indeed, increasing the amount of bacteriocin up to 600 activity units (AU) leads to a 45% decrease in the membrane potential (data not shown). When mY105 (64 AU) is employed in conjunction with the ionophore CCCP (5 μM) or valinomycin (5 μM), there is no complete inhibition of the incorporation of Rh 123 (Table 1). Moreover, its action, at the effective concentrations, is very rapid since it occurs within 10 min. Finally, it may be suggested that the decrease in the membrane potential is induced by the permeabilization of the target cells, which results in the free exchange of ions across the membrane.

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TABLE 1. Mesentericin Y105 action on transmembrane potential of *L. monocytogenes* E20[a]

Supplement (concn)	Rh 123 relative fluorescence (mean ± SD)
None (control)	100
mY105 (0.064 AU/ml)	81.0 ± 2.0
mY105 (0.64 AU/ml)	73.5 ± 3.5
mY105 (6.4 AU/ml)	67.0 ± 5.0
mY105 (64 AU/ml)	58.8 ± 2.0
mY105 (64 AU/ml) + CCCP (5 μM)	60.7 ± 5.0
mY105 (64 AU/ml) + valinomycin (5 μM)	62.6 ± 6.0
mY105 (64 AU/ml) + nigericin (5 μM)	74.7 ± 5.0
CCCP (5 μM)	57.0 ± 3.0
Valinomycin (5 μM)	74.5 ± 9.0
Nigericin (5 μM)	131.0 ± 7.0

[a] Bacteria were grown for 16 h in tryptic soy medium at 30°C. mY105 and/or CCCP, valinomycin, or nigericin was added to the inoculum and incubated with 1 μM Rh 123 at 30°C for 5 min. The bacteria were centrifuged (5 min, 6,000 × *g*) and then resuspended in phosphate-buffered saline supplemented with 5 g of glucose per liter. They were finally analyzed by flow cytometry. Experiments were performed in triplicate.

Action of mY105 on amino acid transport by *L. monocytogenes* E20. To further investigate the last conclusion, mY105 action on amino acid transport was assessed according to the method of van Belkum et al. (26). The study was carried out with whole cells in the presence of 0.4% (wt/vol) glucose. We started by measuring the transport of leucine, which, as has been demonstrated with lactococci (14) and is presumably true for *Listeria* spp., appears to be taken up by a symport with one proton and driven by the proton motive force. In the absence of mY105, the initial velocity of leucine transport is close to 0.35 pmol/min (Fig. 1). When bacteria are preincubated with 100 AU of mY105 per ml, leucine uptake is strongly inhibited and the initial velocity of amino acid incorporation is drastically reduced (Fig. 1). Moreover, the addition of the bacteriocin (100 AU/ml) to bacteria which had previously accumulated 9.5 pmol of leucine provokes an

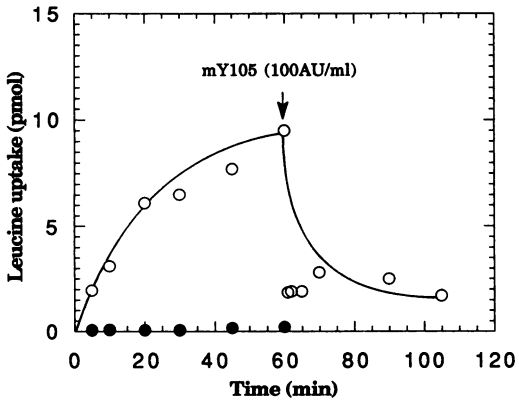

FIG. 1. Action of mY105 on leucine uptake by *L. monocytogenes* E20. The experiment was started by the addition of 8 μM of radiolabeled leucine to the incubation mixture containing bacteria in the absence (○) or presence (●) of mY105 (100 AU/ml). In this experiment, mY105 was added at the time indicated by the arrow. The reaction was stopped by the addition of cold 0.1 M LiCl. Unincorporated amino acid was eliminated by filtration of bacteria through a 0.45-μm-pore-size filter. Leucine uptake was determined by the measurement of radioactivity retained by the filter.

TABLE 2. Mesentericin Y105 action on leucine uptake by *L. monocytogenes* E20 and *L. mesenteroides* Y30 (Bac^-) and L Y105 (Bac^+)[a]

mY105 concn (AU/ml)	Leucine uptake (%) by:		
	E20	Y30 (Bac^-)	Y105 (Bac^+)
0	100	100	100
16	32	39	80
32	18	33	78
64	5	25	82
128	2	22	83

[a] Bacteria were preincubated for 5 min in a medium containing 50 mM phosphate buffer (pH 6.0), 50 mM KCl, 2 mM $MgSO_4$, and various concentrations of mY105. For each mY105 concentration, the experiment was started by the addition of 8 μM [^{14}C]leucine to the incubation mixture and stopped 5 min later by 20-fold dilution of the mixture in cold 0.1 M LiCl. Leucine uptake was determined by the radioactivity retained by the filters after two washes in 0.1 M LiCl; it is expressed as a percentage of the control.

efflux of the amino acid. The release is very fast and involves more than 80% of the amino acid content. This would suggest that the release of leucine is mediated not by a carrier but probably by a simple leakage through the membrane. To confirm this result, glutamic acid was chosen for similar transport experiments, since its uptake, at least by streptococci, is unidirectional and is not driven by the proton motive force (18). As expected, an efflux of glutamic acid is also observed when 100 AU of mY105 per ml was added to cells which had first incorporated 3.3 pmol of the amino acid (data not shown). As with leucine, mY105 does not induce a total efflux of preincorporated glutamic acid. Nevertheless, 65% of the glutamic acid was rapidly released into the incubation medium. This would suggest that a small fraction of the amino acids transported into the cells is incorporated into proteins during the incubation time (nearly 1 h).

Similar transport inhibitions have also been observed with alanine amino-isobutyric acid (a nonmetabolizable analog of alanine) and lysine (data not shown). Since mY105 affects the transport of all the amino acids tested similarly, it is suggested that the bacteriocin could modify the structure of the target membrane. The nonaccumulation and then the efflux of amino acids from sensitive cells agree well with the data presented above indicating that mY105 dissipates the membrane potential of target bacteria. Both types of results could be explained by membrane permeabilization. Thus, the earliest events of mY105 action may be the formation of pores in the plasma membrane followed by the depolarization and the efflux of low-molecular-mass cytoplasmic compounds.

The bacteriocin has been also assessed in leucine transport by *L. mesenteroides* Y105 and its mutant *L. mesenteroides* Y30 (Table 2). It slightly affects the transport by the strain Y105 (Bac^+) while its effect is more pronounced on the mutant Y30 (Bac^-) which does not produce bacteriocin *L. monocytogenes* E20 cells are, nevertheless, the most sensitive to mY105 action (Table 2).

Action of the bacteriocin on rat liver mitochondria respiration. Isolated rat liver mitochondria were prepared by differential centrifugation (10). Mitochondria were used to investigate some functions which depend on membrane integrity. Oxygen consumption was measured polarographically with a Clark-type oxygen electrode calibrated with sodium dithionite as described by Rickwood et al. (19). State 4 (oxygen consumption in the presence of a respiratory substrate) and

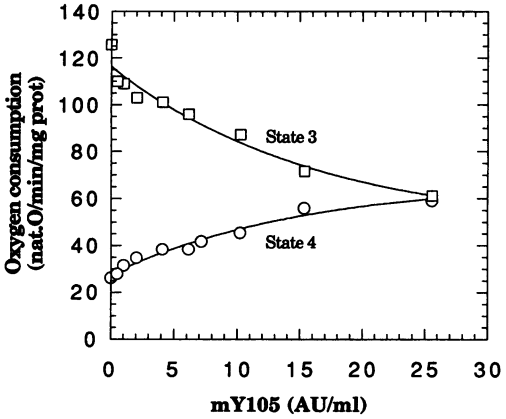

FIG. 2. Measurement of oxygen consumption by isolated mitochondria in the presence of mY105. Oxygen consumption of isolated mitochondria was measured as described by Rickwood et al. (19). The effects of mY105 were assessed before (state 4 respiration) and after (state 3 respiration) addition of 200 μM ADP. nat., nanoatoms.

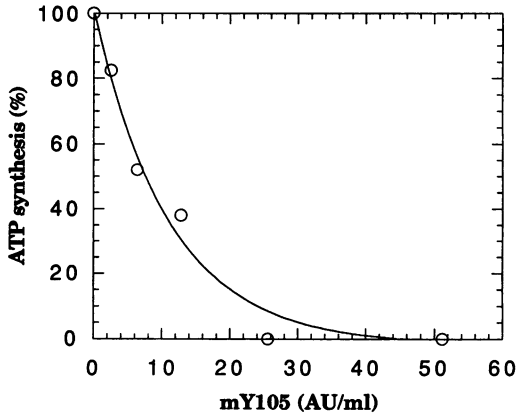

FIG. 3. Effect of mY105 on ATP synthesis by mitochondria. ATP synthesis is expressed as a percentage of that of the control in the absence of the bacteriocin (23).

state 3 (oxygen consumption in the presence of a respiratory substrate and ADP) respirations were recorded by using succinate as a respiratory chain substrate. Oxygen consumption in the presence of 10 mM succinate alone (state 4 respiration) reached 26 nat. of O per min/mg of protein. The respiration rate increased to 126 nat. of O per min/mg of protein following the addition of 200 μM ADP (state 3). Varying mY105 concentrations from 0 to 26 AU/ml increased state 4 respiration up to 2.5 fold compared with its initial value, while state 3 respiration was inhibited by 50% (Fig. 2). For the two respiration states, modifications of oxygen consumption by mitochondria, upon addition of bacteriocin, were immediately observed. Similar results were obtained by using rat liver mitochondria oxidizing glutamate instead of succinate (data not shown). The inhibition of ADP-stimulated respiration by bacteriocin was due to the uncoupling of mitochondria.

Inhibition of mitochondrial ATP synthesis and adenine nucleotide translocase. In order to determine more precisely how the bacteriocin inhibits ADP-stimulated respiration, the effects of mY105 on ATP synthesis by intact mitochondria were assessed by a colorimetric assay (24) (Fig. 3). The bacteriocin strongly decreases ATP production in well-coupled rat liver mitochondria. At a concentration of 25.6 AU of bacteriocin per ml, ATP synthesis is completely inhibited, a result which is in good agreement with the above-reported results indicating a decrease of state 3 respiration following bacteriocin addition. This inhibition may be explained by the dissipation of the membrane potential, a component of the proton motive force needed for ATP synthesis.

The ADP-ATP translocase has been quantified in beef heart mitochondria (22) by using radiolabeled atractyloside, a specific inhibitor of the adenine nucleotide translocase which interacts with regions exposed on the cytoplasmic side of the inner membrane (27). While ATP synthesis has been found to be completely inhibited at 25.6 AU of mY105 per ml (Fig. 3), the association of ADP to its translocator, estimated by the amount of [^{3}H]atractyloside bound to mitochondria, decreases by only 18% (data not shown). Since atractyloside is a competitive inhibitor of ADP, it may be suggested that bacteriocin, at relatively low concentrations, does not strongly prevent the binding of ADP to its carrier. Consequently, ATP synthesis inhibition cannot be entirely explained by the modification of the ADP binding to the protein. However, adenine nucleotide translocase activity measured by the rate of ADP translocated across inner mitochondria membrane appears to be more sensitive to the bacteriocin. With increasing bacteriocin concentrations (5.1 to 76.8 AU/ml), the rate of ADP transport declines (Fig. 4). At mY105 concentrations higher than 25.6 AU/ml, ADP transport is almost completely inhibited, since the same kinetics are obtained in the presence of 20 μM carboxyatractyloside, a known inhibitor of ADP transport. Hence, the adenine nucleotide translocase activity is affected more than the affinity of ADP to its carrier. Consequently, the action of mY105 on ADP transport could be another inhibiting factor of ATP synthesis.

Apparently, mY105 action on *L. monocytogenes* E20 could be due to its ability to produce pore formation in target membranes. Its mode of action may be similar to that of other small pore-forming peptide antibiotics (2, 4, 16, 21). For some bacteria (11), a membrane-associated protein that acts as a receptor for bacteriocins has been described. Such

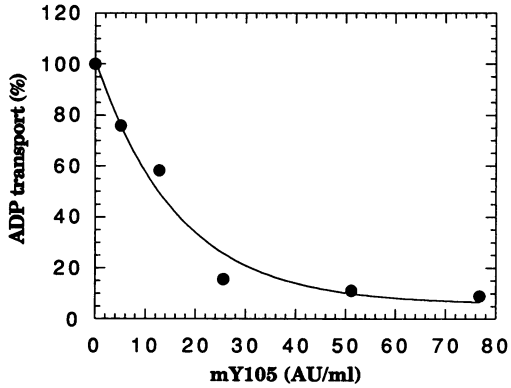

FIG. 4. Modification of the ADP-ATP translocase activity by mY105. ADP or ATP translocase activity was measured by the forward exchange method (27). The kinetics of ADP transport were recorded in the absence or presence of various concentrations of mY105. A 5-min preincubation with mY105 was done before starting of the transport. The translocase activity was then measured 30 s following the beginning of the reaction.

a mechanism has also been shown for channel-forming colicins which first bind to specific receptors located at the surface of susceptible cells (15). Since mammalian mitochondria are not natural targets of mY105, the finding that the bacteriocin also affects the membrane permeability of the inner membrane suggests that *Listeria*-specific receptors are not required for the disruption of target cell membranes. Thus, we propose that the formation of pores by mY105 might be due to the accessibility of regions in target membranes harboring particular phospholipid and/or protein organization. These molecules, accessible for mY105 into the plasma membrane of *Listeria* cells and the inner mitochondrial membrane, may be missing or hidden in insensitive cells.

The bacteriocin action on leucine transport has been observed in *L. monocytogenes* E20 and *L. mesenteroides* Y30 (Bac^-) but only slightly in *L. mesenteroides* Y105 (Bac^+). This poses the problem of how the cells are protected against the bacteriocin they produce. One explanation may lie in the mechanism of recognition of target cells previously discussed. This hypothesis assumes that the organization of insensitive cell membranes does not permit their recognition. Nevertheless, it is not excluded that some membrane proteins of insensitive bacteria may act as immunity-conferring proteins against mY105. Such a mechanism would resemble that which protects some bacteria from colicins (1).

From the known size of mY105 (36 amino acids), it appears that such pores cannot be formed by only one molecule; it must rather be assumed that peptide molecules aggregate or act in conjunction with other membrane molecules to establish a functional pore, as has been previously described for most pore-forming peptides (17).

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