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EFFECTS OF COLICIN A AND STAPHYLOCOCCIN 1580 ON AMINO ACID UPTAKE INTO MEMBRANE VESICLES OF *ESCHERICHIA COLI* AND *STAPHYLOCOCCUS AUREUS*

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SUMMARY

Staphylococcin 1580 increased the relative amount of diphosphatidylglycerol and decreased the amount of phosphatidylglycerol in cells of *Staphylococcus aureus*, while the amounts of lysylphosphatidylglycerol, phosphatidic acid and total phospholipid remained constant.

Treatment of cells of *Escherichia coli* and *S. aureus* with colicin A and staphylococcin 1580, respectively, did not affect proton impermeability but subsequent addition of carbonylcyanide-*m*-chlorophenylhydrazone resulted in a rapid influx of protons into the cells.

Bacteriocin-resistant and -tolerant mutants of *E. coli* and *S. aureus* were isolated. The bacteriocins caused leakage of amino acids preaccumulated into membrane vesicles of resistant mutants and had no significant effect on membrane vesicles of tolerant mutants.

The uptake of amino acids into membrane vesicles was inhibited by both bacteriocins, irrespective of the electron donors applied. The bacteriocin inhibition was noncompetitive. The bacteriocins did not affect oxygen consumption and dehydrogenases in membrane vesicles.

Both bacteriocins suppressed the decrease in the fluorescence of 1-anilino-8-naphthalene sulfonate caused by D-lactate or α-glycerol phosphate when added to membrane vesicles.

It is concluded that the bacteriocins uncouple the transport function from the electron transport system.

INTRODUCTION

Bacteriocins are high molecular weight, bacteriocidal antibiotics produced by certain strains of bacteria. Colicin A and staphylococcin 1580 are bacteriocins produced by *Citrobacter freundii* and *Staphylococcus epidermidis*, respectively. The former consists of a single protein molecule with a mol. wt of 60000[1] and the latter of a lipid–carbohydrate–protein complex[2,3].

Abbreviations: DCIP, 2,6-dichlorophenolindophenol; ANS, 1-anilino-8-naphthalene sulfonic acid.

Bacteriocins adsorb to specific receptor sites on the membrane of sensitive bacteria. Mutant strains no longer sensitive to bacteriocins can be distinguished into two classes, namely, bacteriocin-resistant mutants which have lost the ability to adsorb the bacteriocin onto the specific receptor site and bacteriocin-tolerant mutants which still adsorb bacteriocin to the receptor site but which are not killed[4].

Studies on the mode of action revealed quite different specific biochemical effects on sensitive bacteria. Colicin E2[5], megacin C[6] and vibriocin[7] cause degradation of the bacterial DNA. Colicin E3[8] and cloacin DF13[9] inhibit protein synthesis. It seems that colicin E2[10] and E3[11] molecules penetrate somehow into the cytoplasm after adsorption and then affect DNA and ribosomes, respectively. Colicins E1 and K[12], Ia and Ib[13], and A[14,15], and staphylococcin 1580[16,17] inhibit macromolecular synthesis and transport of various compounds and seem to act directly on the membrane. The mechanism of action of these bacteriocins is yet unknown but it has been suggested previously[16,18], that conformational changes, alterations in phospholipid metabolism or in transport might be involved in it. The aim of this study was to evaluate these possibilities. Furthermore, the study on these bacteriocins may be of interest for the elucidation of transport phenomena and the possible role which cations and lipids may play in it.

MATERIALS AND METHODS

Bacterial strains

The colicin A- and staphylococcin 1580-producing strains were *C. freundii* C31 and *S. epidermis* 1580, respectively. The indicator strains used were *Escherichia coli* K12, *Staphylococcus aureus* Oxford 209 P and *Bacillus subtilis* W23, which was kindly donated by W. N. Konings. All strains were maintained on trypticase soy agar. Stable L-forms of *S. aureus* 502 A were maintained on a medium containing 1.5% agar, 3.8% brain heart infusion, 5% NaCl and 20% unheated horse serum.

Production and purification of bacteriocins

Colicin A was obtained by a modified procedure according to Dandeu[1]. An overnight culture of *C. freundii* C31 cells in 3% trypticase soy broth was inoculated 1:100 into the same medium and grown under aeration at 37 °C. When the culture reached an absorbance of 0.2 at 600 nm colicin A induction was achieved by addition of mitomycin C (0.5 mg/l). The culture was incubated further for 4 h and centrifuged for 10 min at $10000 \times g$. Colicin A was partially purified by $(NH_4)_2SO_4$ precipitation and DEAE-cellulose chromatography. Staphylococcin 1580 was produced and purified as described previously[2].

Assay of bacteriocin activity

The bacteriocins were assayed as described previously[2]. The activity was expressed in arbitrary units per ml.

Isolation of mutants

Cells of *S. aureus* Oxford 209 P were irradiated for 15 s with ultraviolet light (900 ergs/cm^2 per s). Staphylococcin-resistant mutants were enriched by growth in the presence of staphylococcin 1580 (2000 arbitrary units/ml) and selected by growth

of suitably diluted amounts on trypticase soy agar containing staphylococcin 1580 (2000 arbitrary units/ml). A similar procedure was followed to isolate staphylococcin-tolerant mutants of stable L-forms of *S. aureus* 502 A. Colicin A-resistant and -tolerant mutants of *E. coli* K12 were isolated as growing colonies after spreading 10^8 cells on trypticase soy agar containing colicin A (5000 arbitrary units/ml). In order to distinguish between resistant and tolerant mutants D-cycloserine- or penicillin-induced spheroplasts[19] were tested for bacteriocin resistance. In the experiments *E. coli* strains K12 R and K12 T were used as colicin-resistant and -tolerant strains, and *S. aureus* strains Oxford 209 PR (V4231) and 502 AT as staphylococcin-resistant and -tolerant strains, respectively.

Extraction and analysis of phospholipids

Cells of *S. aureus* Oxford 209 P were exponentially grown at 37 °C in 500 ml of trypticase soy broth, supplemented with 0.5 mCi $^{32}P_i$. Phosphatidylglycerol, diphosphatidylglycerol, phosphatidic acid and lysylphosphatidylglycerol were extracted according to Bligh and Dyer[20]. The phospholipids were separated by thin-layer chromatography on silica gel with a mixture of chloroform, methanol and water (65:25:4, by vol.) as solvent. Percentages of the different phospholipids were determined by measuring the radioactivity of the different spots.

pH measurement

Cells exponentially growing in trypticase soy broth were gathered on membrane filters (Millipore, 0.45-μm pore size, 5-cm diameter) and washed with 0.3 mM potassium phosphate (pH 6.6) and suspended in the same buffer at a concentration of 10^9 cells per ml. The pH was measured with a Radiometer Digital pH meter (PHM52) and a combined electrode (Radiometer GK 2302C), and monitored with a Servogor recorder (Goerz Electro, Vienna).

Preparation of membrane vesicles

Membrane vesicles were prepared by modified methods according to Kaback[21]. *S. aureus* cells were grown in the medium described by Short and White[22]. *E. coli* was grown in Medium A[21] containing 0.5% sodium succinate and 0.2% casein hydrolysate, and *B. subtilis* in 0.8% Bacto-Tryptone containing 0.5% NaCl. The cells were harvested in the late exponential phase and washed twice with 10 mM Tris–HCl (pH 8.0). Cells of *E. coli* and *B. subtilis* were resuspended in 30 mM Tris–HCl (pH 8.0) containing 20% sucrose. Subsequently EDTA and lysozyme were added to final concentrations of 10 mM and 0.5 μg/ml, respectively. Alternatively, cells of *S. aureus* were resuspended in 50 mM potassium phosphate (pH 7.3) containing 0.2 M NaCl and 20% sucrose, and lysostaphin (50 μg/ml) was added. After 45 min at 30 °C the spheroplasts were centrifuged, resuspended in 0.1 M potassium phosphate (pH 6.8) containing 20% sucrose and 20 mM $MgSO_4$, and lysed at 37 °C by 500-fold dilution in 50 mM potassium phosphate (pH 6.8) containing 10 μg/ml each of deoxyribonuclease and ribonuclease. Subsequently, EDTA (10 mM final concentration) was added, followed by $MgSO_4$ (15 mM final concentration), and after each addition the mixture was incubated for 15 min. The suspension was centrifuged for 20 min at $16000 \times g$. The pellet was resuspended in 50 mM potassium phosphate (pH 6.8)

and after differential centrifugations at $800 \times g$ and $45000 \times g$ the membrane vesicles were stored at -20 °C in the same buffer.

Measurement of transport

The incubation mixtures contained final concentrations of 50 mM potassium phosphate (pH 6.8), 10 mM $MgSO_4$ and 1–2 mg of membrane protein per ml and were preincubated for 15 min at 25 °C. [^{14}C]Glutamic acid (10 μM, final concentration) and electron donors (20 mM final concentration) were rapidly added and the mixtures (0.1 ml final volume) were incubated at 25 °C under special oxygenation[23]. The reaction was terminated by addition of 2 ml 0.1 M LiCl and the mixtures were filtered on membrane filters (Millipore, 0.45-μm pore size). The filters were washed with 2 ml 0.1 M LiCl, dried and the radioactivity measured using a liquid scintillation counter.

Measurement of oxygen consumption

The rates of oxygen uptake were measured by use of a Clark electrode with the Oxygraph (Gilson Medical Electronics, Middleton, Wisc.). The assay mixture (1.0 ml) contained 50 mM potassium phosphate (pH 6.8), 10 mM $MgSO_4$ and 50–500 μg of membrane protein. The reaction was initiated by injecting 20 μl of the substrate (20 mM final concentration, 5 mM for NADH). The measurements were performed at 30 °C.

Assay of dehydrogenase activity

Dehydrogenase activities were assayed by use of 2,6-dichlorophenolindophenol (DCIP) and, in the case of NADH dehydrogenase, potassium ferricyanide as electron acceptor. Incubation mixtures contained 0.2 M potassium phosphate (pH 7.3), 0.1–0.5 mg of membrane protein per ml, 0.002% DCIP or 0.1 M potassium ferricyanide and 20 mM lithium D-lactate, disodium succinate, sodium α-glycerol phosphate or 10 mM NADH. The absorbance at 620 nm (DCIP) or 425 nm (ferricyanide) was followed.

Fluorescence measurements

Fluorescence was measured with an Aminco Bowman spectrofluorimeter. The slit arrangement was 3 mm for Positions 1, 3, 4 and 6, and 2 mm for Positions 2, 5 and 7; the sensitivity was 45. The excitation and emission wavelengths were 285 and 475 nm, respectively. The reaction mixture (2 ml) contained approximately 0.2 mg of membrane protein per ml, 50 mM potassium phosphate (pH 6.8), 10 mM $MgSO_4$ and 50 μM 1-anilino-8-naphthalene sulfonic acid (ANS) and was well aerated. The measurements were performed at 30 °C and the fluorescence monitored.

Chemicals

L-[U-^{14}C]Glutamic acid (260 Ci/mole), L-[U-^{14}C] proline (165 Ci/mole) and $NaH_2{}^{32}PO_4$ (10 Ci/mole) were purchased from the Radiochemical Centre, Amersham. Lysostaphin was purchased from Schwarz-Mann, Orangeburg, N.Y., lysozyme from Boehringer, Mannheim and *N*-methylphenazonium methosulfate from Schuchardt, München. DCIP was obtained from Merck, Darmstadt and ANS from Sigma Chemical Co., St. Louis. Trypticase soy broth was purchased from B.B.L., Cockeysville.

RESULTS

Isolation of mutants

Two kinds of mutants of *E. coli* K12 were isolated, namely, colicin A-resistant strains (K12 R), of which spheroplasts were sensitive to colicin A, and colicin A-tolerant strains (K12 T), of which protoplasts were still resistant to colicin A. The tolerant mutants isolated exhibited a higher sensitivity to deoxycholate and EDTA than the wild-type and resistant strains since the growth of the tolerant mutants was inhibited completely at concentrations of 1.0% deoxycholate or 1 mM EDTA, whereas concentrations ten times higher were required to inhibit the growth of the wild-type and resistant strains. From *S. aureus* Oxford 209 P, staphylococcin 1580-resistant strains (209 PR) could be isolated of which spheroplasts were sensitive to the bacteriocin. Staphylococcin 1580-tolerant L-forms (502 AT) were isolated as mutants of stable L-forms of *S. aureus* 502 A. These tolerant mutants were equally sensitive to deoxycholate as their parent L-forms.

Effect on phospholipid metabolism

Treatment of *E. coli* cells with colicins A, E1 and K caused an increase of the percentages of diphosphatidylglycerol and lysophosphatidylethanolamine and a decrease of phosphatidylglycerol[24]. Like these colicins staphylococcin 1580 caused an increase of the relative amount of diphosphatidylglycerol and a decrease of phosphatidylglycerol in *S. aureus* Oxford 209 P cells but had no significant effect on phosphatidic acid and lysylphosphatidylglycerol (Fig. 1A). The total amount of ^{32}P-labeled phospholipids decreased very slowly and the absolute alterations of diphosphatidylglycerol and phosphatidylglycerol were nearly equal (Fig. 1B).

Effect on proton permeability

The pH of a cell suspension of *E. coli* K12 was lowered from pH 6.6 to 6.2 by

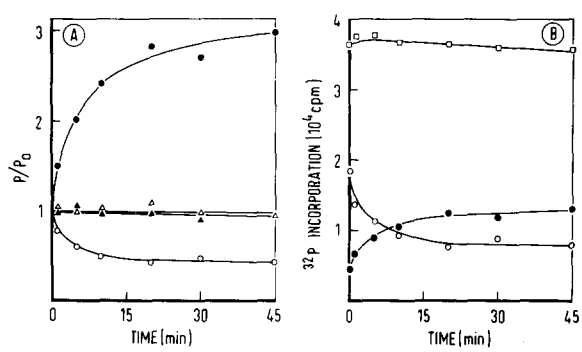

Fig. 1. Effect of staphylococcin 1580 on the phospholipid composition of *S. aureus* Oxford 209 P cells. Cells were grown exponentially in a medium containing $^{32}P_i$. When the culture reached the absorbance of 0.5 at 600 nm, staphylococcin 1580 (1000 arbitrary units/ml) was added. At various time intervals samples (100 ml) were removed and the ^{32}P contents of the various phospholipids were determined. (A) Ratio between the percentages of ^{32}P of various phospholipids in treated (P) and untreated (P_0) bacteria as a function of time. (B) Absolute amount of ^{32}P in phospholipids from 100-ml sample of treated cells as a function of time. ●, Diphosphatidylglycerol; ○, phosphatidylglycerol; △, phosphatidic acid; ▲, lysylphosphatidylglycerol; □, total phospholipid.

addition of HCl. Subsequent addition of carbonylcyanide-*m*-chlorophenylhydrazone caused a rather slow and gradual increase of pH (Fig. 2A). A similar behavior was observed with colicin A-pretreated and untreated cells of *E. coli* K12 R and K12 T. Upon addition of HCl and carbonylcyanide-*m*-chlorophenylhydrazone to a suspension of colicin A-pretreated cells of *E. coli* K12, the pH rose very sharply (Fig. 2B). Addition of colicin A after the pH was lowered and subsequent incubation with carbonylcyanide-*m*-chlorophenylhydrazone caused a sharp rise of pH (Fig. 2C). Comparable results were obtained in tests with *S. aureus* Oxford 209 P and Oxford 209 PR cells and staphylococcin 1580, although the effect of carbonylcyanide-*m*-chlorophenylhydrazone was somewhat less (Figs 3A–3C). *E. coli* K12 and *S. aureus* Oxford 209 P cells pretreated for more than 30 min with colicin A and staphylococcin 1580, respec-

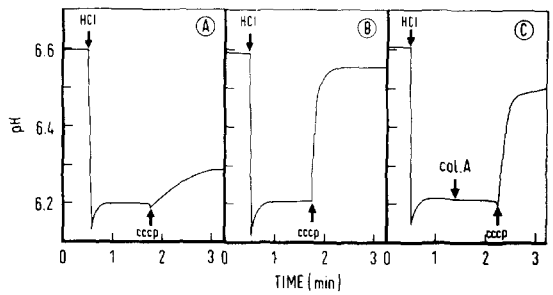

Fig. 2. Effect of colicin A (col. A) on proton permeability of *E. coli* K12 cells. The cell suspension (10^9 cells/ml) was prepared as described in Materials and Methods. (A) About 40 μl of 0.01 M HCl was added to a 10-ml suspension of untreated cells, followed by addition of carbonylcyanide-*m*-chlorophenylhydrazone (cccp) (2 μM final concentration). (B) About 40 μl of 0.01 M HCl were added to a 10-ml suspension of colicin A-pretreated (1000 arbitrary units/ml for 10 min) cells, followed by addition of carbonylcyanide-*m*-chlorophenylhydrazone. (C) About 40 μl of 0.01 M HCl were added to a 10-ml suspension of untreated cells, followed by addition of colicin A (5000 arbitrary units/ml) and carbonylcyanide-*m*-chlorophenylhydrazone.

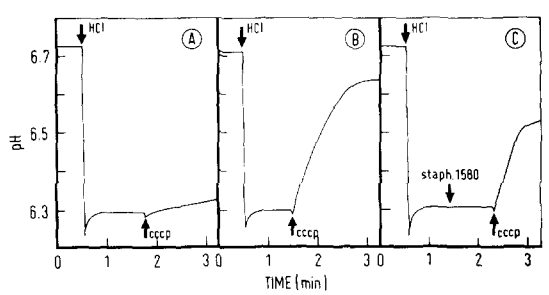

Fig. 3. Effect of staphylococcin 1580 (staph. 1580) on proton permeability of *S. aureus* Oxford 209 P cells. The cell suspensions (10^9 cells/ml) were prepared as described in Materials and Methods. (A) About 40 μl of 0.01 M HCl were added to a 10-ml suspension of untreated cells followed by addition of carbonylcyanide-*m*-chlorophenylhydrazone (cccp) (2 μM final concentration). (B) About 40 μl of 0.01 M HCl were added to a 10-ml suspension of staphylococcin-pretreated (2000 arbitrary units/ml for 15 min) cells, followed by addition of carbonylcyanide-*m*-chlorophenylhydrazone. (C) 40 μl of 0.01 M HCl were added to a 10-ml suspension of untreated cells, followed by addition of staphylococcin 1580 (4000 arbitrary units/ml) and carbonylcyanide-*m*-chlorophenylhydrazone.

tively, required more HCl to lower the pH to 6.2 and the pH rise upon carbonyl-cyanide-*m*-chlorophenylhydrazone addition became less drastic. The results described here were not influenced by the presence of 50 mM KCl in the cell suspension.

Effect on amino acid transport

Colicin A caused a rapid leakage of [^{14}C]glutamate accumulated in membrane vesicles of *E. coli* K12 and K12 R, but no significant effect was observed with membrane vesicles of *E. coli* K12 T (Fig. 4). Also staphylococcin 1580 caused a rapid leakage of [^{14}C]glutamate from membrane vesicles of *S. aureus* Oxford 209 P and its resistant mutant 209 PR (Fig. 5A). Membrane vesicles of *S. aureus* 502 A behaved in a similar way, while little effect was observed on membrane vesicles of its tolerant mutant strain 502 AT (Fig. 5B).

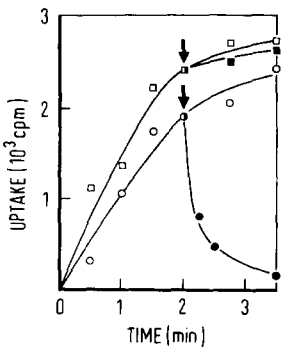

Fig. 4. Effect of colicin A on [^{14}C]glutamate accumulation by membrane vesicles of *E. coli* K12 and K12 R (circles) and K12 T (squares). The uptake was measured in the presence of ascorbate (20 mM) and phenazine methosulfate (100 μM). Open symbols: control. Closed symbols: colicin A (2000 arbitrary units/ml) added after 2 min.

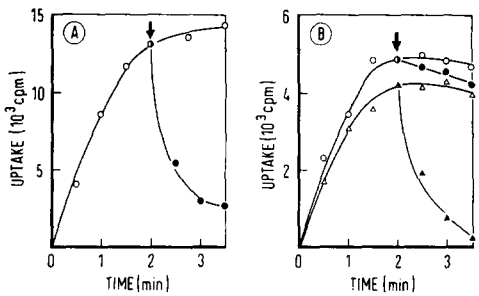

Fig. 5. Effect of staphylococcin 1580 on [^{14}C]glutamate accumulation by membrane vesicles of *S. aureus*. The uptake was measured in the presence of ascorbate (20 mM) and phenazine methosulfate (100 μM). Open symbols: control. Closed symbols: staphylococcin 1580 (2500 arbitrary units/ml) added after 2 min. (A) Membrane vesicles of *S. aureus* Oxford 209 P and 209 PR. (B) Membrane vesicles of *S. aureus* 502 A (triangles) and 502 AT (circles).

The amino acid uptake by membrane vesicles prepared from *E. coli* cells can be stimulated by the following energy donors: ascorbate *plus* phenazine methosulfate, D-lactate, succinate and NADH, given here in order of decreasing effectiveness[25,26]. Irrespective of the electron donor applied, the uptake of glutamate was inhibited 85–95% by 7500 arbitrary units of colicin A per ml (Figs 6A and 6B). Parallel experiments with heat-inactivated (30 min at 120 °C) colicin A or with staphylococcin 1580 (5000 arbitrary units/ml) did not show any inhibition. The transport of amino acids in membrane vesicles of *S. aureus* could be stimulated by α-glycerol phosphate and by ascorbate *plus* phenazine methosulfate[27] and was 85–95% inhibited in both cases by 7500 arbitrary units of staphylococcin 1580 per ml (Fig. 7). The uptake of amino acids in membrane vesicles of *B. subtilis* W23 was sensitive to staphylococcin 1580. Colicin A (7500 arbitrary units/ml) and heat-inactivated (30 min at 120 °C) staphylococcin 1580 did not affect the uptake in membrane vesicles of *B. subtilis* W23 or

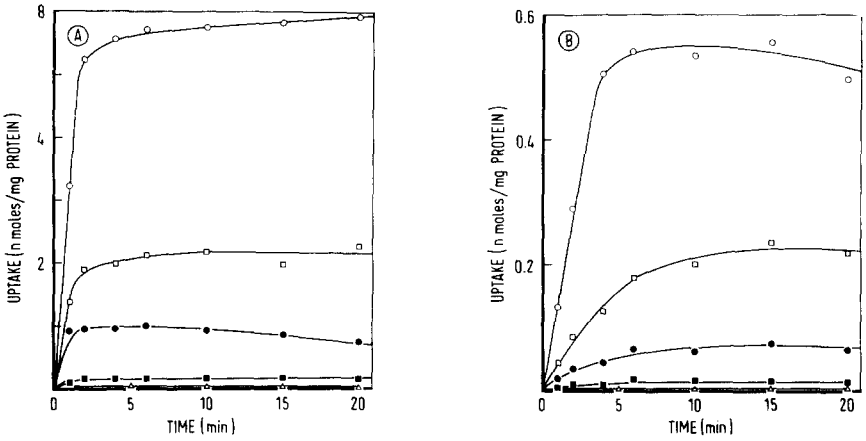

Fig. 6. Effect of colicin A on [^{14}C]glutamate uptake into membrane vesicles of *E. coli* K12. The uptake was measured in the absence (open symbols) or presence of colicin A (7500 arbitrary units/ml, closed symbols). Electron donors applied: (A) △, none; ○, ascorbate (20 mM) *plus* phenazine methosulfate (100 μM); □, D-lactate (20 mM). (B) △, none; ○, succinate (20 mM); □, NADH (20 mM).

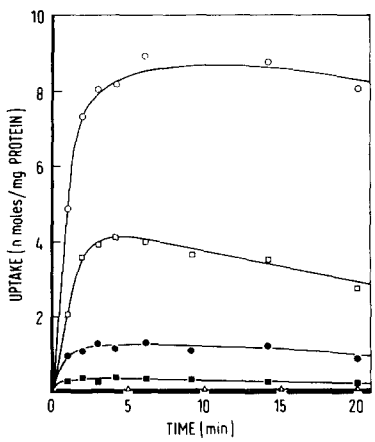

Fig. 7. Effect of staphylococcin 1580 on [^{14}C]glutamate uptake into membrane vesicles of *S. aureus* Oxford 209 P. The uptake was measured in the absence (open symbols) or presence of staphylococcin 1580 (7500 arbitrary units/ml, closed symbols). Electron donors applied: △, none; ○, ascorbate (20 mM) *plus* phenazine methosulfate (100 μM); □, α-glycerol phosphate (20 mM).

S. aureus Oxford 209 P. The inhibiting effect of both bacteriocins was not restricted to glutamate uptake but affected proline uptake as well.

Both bacteriocins inhibited noncompetitively glutamate uptake energized by ascorbate *plus* phenazine methosulfate (Figs 8 and 9). The K_m and V values of glutamate uptake in *E. coli* K12 (*S. aureus* Oxford 209 P) membrane vesicles were 4.4 μM (40 μM) and 5.8 nmoles/mg per min (25 nmoles/mg per min), respectively. The K_i values were in the range of 10 nM and 100 nM for colicin A and staphylococcin 1580, respectively. It was assumed that the molecular weights of staphylococcin

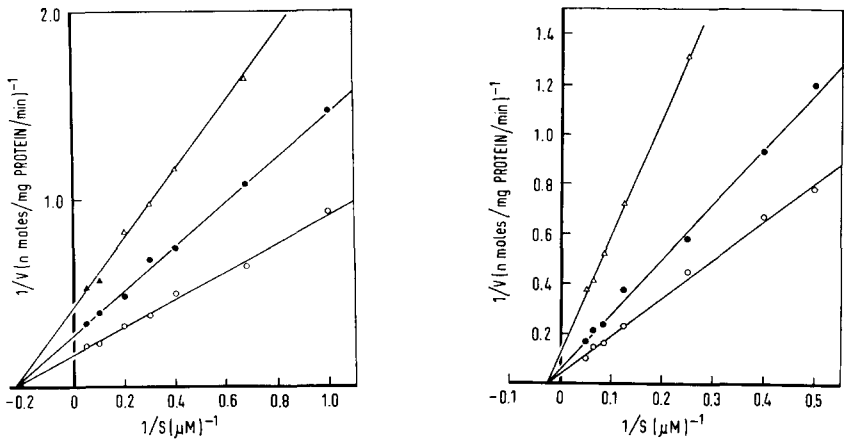

Fig. 8. Colicin A as a noncompetitive inhibitor of [^{14}C]glutamate uptake into membrane vesicles of *E. coli* K12. The amount taken up in 1 min at 25 °C in the presence of 20 mM ascorbate *plus* 100 μM phenazine methosulfate was measured. ○, control; ●, 1000 arbitrary units of colicin A per ml; △, 2000 arbitrary units/ml.

Fig. 9. Staphylococcin 1580 as a noncompetitive inhibitor of [^{14}C]glutamate uptake into membrane vesicles of *S. aureus* Oxford 209 P. The amount taken up in 1 min at 25 °C in the presence of 20 mM ascorbate *plus* 100 μM phenazine methosulfate was measured. ○, control; ●, 750 arbitrary units of staphylococcin 1580 per ml; △, 3500 arbitrary units/ml.

1580 and colicin A were 250000 and 60000, respectively, and that 1 arbitrary unit of staphylococcin = 820 pmoles and 1 arbitrary unit of colicin A = 840 pmoles.

Effect on oxygen consumption and dehydrogenase activities

To determine whether the inhibition of amino acid uptake was caused by an inhibition of the dehydrogenases or of the electron transport chain, the effect of the bacteriocins on oxygen consumption was determined. Table I shows that both bacteriocins have no or little effect on the oxygen consumption with various electron donors. The inhibition by staphylococcin 1580 in the presence of NADH could not be enhanced further by higher staphylococcin concentrations. For comparison also the effects of KCN and 2,4-dinitrophenol were determined. Furthermore, both bacteriocins had no effect on the NADH, D-lactate, α-glycerol phosphate or succinate dehydrogenase activity in membrane vesicles with DCIP or ferricyanide as electron acceptor.

ANS fluorescence

Changes in the fluorescence of ANS show a striking correlation with the effects exerted on the dehydrogenase-coupled transport system in membrane vesicles and seem to be associated with structural transitions in components of the membrane[28] or/and alterations of the membrane potential[29,30]. Addition of D-lactate to membrane vesicles of *E. coli* K12, K12 R and K12 T in the presence of ANS resulted in a rapid decrease of the fluorescence (Fig. 10A). A similar effect was observed for membrane vesicles of *E. coli* K12 T pretreated with colicin A, but not for colicin A-pretreated vesicles of *E. coli* K12 and K12 R cells (Fig. 10B). Colicin A reversed the decrease

TABLE I

EFFECT OF COLICIN A, STAPHYLOCOCCIN 1580, KCN AND 2,4-DINITROPHENOL ON THE OXIDATION SYSTEM OF MEMBRANE VESICLES

Membrane vesicles were pretreated with bacteriocins, KCN or 2,4-dinitrophenol for 10 min. Then the substrate was added and oxygen consumption was measured as described in Materials and Methods.

Membrane vesicles	*Electron donor (20 mM)*	*Relative rate of oxygen consumption (%)*				
		Control	*Staphylococcin 1580 (5000 arbitrary units/ml)*	*Colicin A (5000 arbitrary units/ml)*	*KCN (2 mM)*	*2,4-Dinitrophenol (1 mM)*
E. coli K12	Ascorbate–phenazine methosulfate	100	101	98	11	100
	D-Lactate	100	95	101	9	98
	Succinate	100	99	97	3	97
	NADH	100	102	105	1	95
S. aureus Oxford 209 P	Ascorbate–phenazine methosulfate	100	103	101	9	101
	α-Glycerol phosphate	100	96	97	6	97
	NADH	100	81	102	2	96

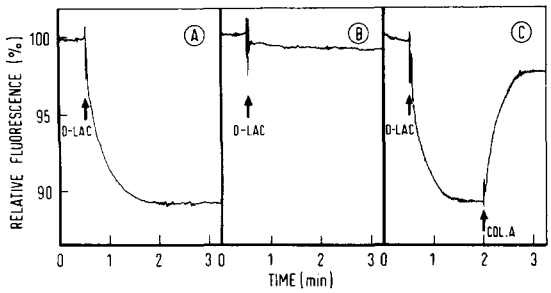

Fig. 10. Effect of colicin A (COL. A) on D-lactate-induced fluorescence changes. The experiments were carried out with *E. coli* K12 membrane vesicles as described in Materials and Methods. (A) D-lactate (D-LAC, 20 mM) was added to untreated membrane vesicles. (B) D-lactate (20 mM) was added to membrane vesicles pretreated with 6000 arbitrary units of colicin A per ml for 10 min. (C) D-lactate (20 mM) was added to untreated vesicles, followed by addition of colicin A (6000 arbitrary units/ml).

of fluorescence when added after D-lactate (Fig. 10C). A similar result was obtained with KCN and amino acids, in accordance with the results of Reeves *et al.*[28]. The ANS fluorescence also decreased when α-glycerol phosphate was added to membrane vesicles of *S. aureus* Oxford 209 P and 209 PR and staphylococcin pretreated vesicles of strain 502 AT (Fig. 11A). This decrease was prevented by staphylococcin 1580 added before α-glycerol phosphate and was abolished when it was added after α-glycerol phosphate (Figs 11B and 11C). Both bacteriocins caused a small increase (1–2%) of fluorescence when added to membrane vesicles in the presence of ANS.

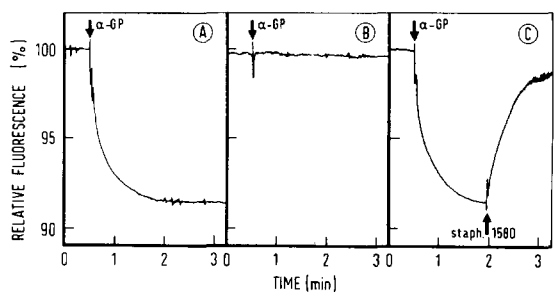

Fig. 11. Effect of staphylococcin 1580 (staph. 1580) on α-glycerol phosphate(α-GP)-induced fluorescence changes in membrane vesicles of *S. aureus* Oxford 209 P. The experiments were carried out as described in Materials and Methods. (A) α-Glycerol phosphate (20 mM) was added to untreated membrane vesicles. (B) α-Glycerol phosphate (20 mM) was added to vesicles pretreated with 7000 arbitrary units of staphylococcin per ml for 15 min. (C) α-Glycerol phosphate (20 mM) was added to untreated vesicles followed by addition of staphylococcin (7000 arbitrary units/ml).

DISCUSSION

Two classes of colicin A-insensitive mutants were obtained from *E. coli* K12, namely, colicin A-resistant mutants, whose spheroplasts were sensitive to colicin A, and colicin A-tolerant mutants, whose spheroplasts were still resistant to colicin A. Staphylococcin 1580-resistant and -tolerant mutants were isolated from *S. aureus*

Oxford 209 P and stable L-forms of *S. aureus* 502 A, respectively. The cell wall of the resistant mutants might be altered in such a way that the bacteriocins could no longer attach to the receptor site, whereas in tolerant mutants the biochemical target appeared resistant to bacteriocin action[31]. The higher sensitivity of colicin A-tolerant mutants to deoxycholate and EDTA indicates that the membrane of the mutants may be altered.

Amino acid accumulation into membrane vesicles of wild-type and resistant strains of *E. coli* K12 and *S. aureus* was sensitive to the respective bacteriocins, while the vesicles of tolerant strains were unaffected by them. Similar results were obtained for colicin E1[31]. These results show that this group of bacteriocins affect primarily a biochemical process in the membrane or the membrane itself.

The inhibition of amino acid uptake into membrane vesicles of *E. coli* K12 and *S. aureus* Oxford 209 P by the respective bacteriocins was noncompetitive and the extent did not depend on the amino acid or electron donor applied. According to Kaback and Barnes[32,33] amino acid transport is exclusively coupled *via* one or more dehydrogenases and transport carriers to the electron transport chain. The results presented here reveal that inhibition of amino acid uptake was not caused by a blockade in the electron transport chain or dehydrogenase activities, as exerted by cyanide and oxamate, respectively. Hence, the transport carriers appear not to be obligatory electron transfer intermediates as was already concluded from the action of uncoupling agents.

A proton gradient and electrical potential was suggested to provide the immediate driving force for the active transport of a variety of compounds in bacterial cells[34,35]. The proton gradient may be generated by an oxidative input under aerobic conditions and a phosphorylative input under anaerobic conditions in intact cells, but in membrane vesicles only the first action is operative[36,37], possibly because of the loss of coupling factors involved in the phosphorylative input[38]. Transport inhibition could result from the development of proton permeability causing a collapse of the proton gradient; the action of dinitrophenol and carbonylcyanide-*m*-chlorophenylhydrazone may be explained in this way[39]. Colicin A and staphylococcin 1580 did not promote proton permeability. However, addition of carbonylcyanide-*m*-chlorophenylhydrazone to colicin- or staphylococcin-treated cells resulted in a rapid loss of the proton gradient. Since previous experiments[16] showed that staphylococcin 1580 caused a rapid leakage of Rb^+ out of the cell, counter-ion movement is probably required to manifest the effect of carbonylcyanide-*m*-chlorophenylhydrazone maximally. A similar interpretation was offered for the action of carbonylcyanide-*m*-chlorophenylhydrazone in the presence of colicin E1 or valinomycin *plus* K^+ (ref. 40).

A decrease of ANS fluorescence was observed when membrane vesicles of *E. coli* K12 or *S. aureus* Oxford 209P were energized by D-lactate or α-glycerol phosphate, respectively, and may result from a decreased binding of ANS caused either by conformational changes[28] of the membrane or by an alteration of the potential[29,30,38] across it. The effects of colicin A and staphylococcin 1580 on ANS fluorescence may be explained to be due to a reduction of the energized state of the membrane.

Colicin A and staphylococcin 1580 inhibit the uptake of amino acids both in intact cells[14,16] and in membrane vesicles. The mechanism by which the uptake is coupled to the energized state of the membrane is unknown. The results presented

here may be interpreted as an uncoupling of transport by these bacteriocins either by dissipation of the energized state or by an interaction with proteins or phospholipids involved in the coupling.

The mechanism by which these group of bacteriocins affect this coupling is not yet clear. Colicin A^{23} and staphylococcin 1580 cause an increase of diphosphatidylglycerol and a decrease of phosphatidylglycerol. These changes may induce conformational changes and affect transport, otherwise, they are a consequence of transport inhibition.

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