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Butyricin 7423 and the Membrane H^+-ATPase of *Clostridium pasteurianum*

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Abstract. The bacteriocin butyricin 7423 inhibited the activity of the membrane H^+-ATPase (BF_oF_1) of vegetative cells of *Clostridium pasteurianum* but not that of its soluble BF_1 component. In vitro studies with the H^+-ATPases of mutant strains selected for diminished sensitivity (a) to butyricin 7423 and (b) to dicyclohexylcarbodi-imide, confirmed that butyricin 7423 interacts with the BF_o component of this enzyme complex. Even so, certain other mutant strains displaying decreased sensitivity to butyricin 7423 possessed H^+-ATPases which in vitro showed undiminished sensitivity to inhibition by the bacteriocin. Furthermore, from the changes in intracellular ATP concentration and in the rates and net extent of efflux of intracellular $^{86}Rb^+$ ions that were provoked by exposure of the parent and several of the mutant strains to butyricin 7423, it was concluded that its primary bactericidal action was not attributable to stoichiometric inhibition of the membrane H^+-ATPase. High extracellular concentrations of K^+ ions enabled *Cl. pasteurianum* to survive exposure to low concentrations of this membrane-active bacteriocin.

Key words: Bacteriocin – Butyricin 7423 – *Clostridium pasteurianum* – Membrane H^+-ATPase – Adenosine triphosphatase

Butyricin 7423 is a bacteriocin produced by *Clostridium butyricum* NCIB 7423 which is lethal to several other species of *Clostridium* including *Cl. pasteurianum* (Clarke et al. 1975). It is an amphiphilic protein whose mode of action on sensitive clostridia superficially resembles that of other membrane-active bacteriocins on their target organisms, including colicins E1, I and K on *Escherichia coli* (Clarke and Morris 1976a). In particular, rapid efflux of intracellular K^+ ions occurs immediately on exposure of *Cl. pasteurianum* to a minimal lethal concentration of butyricin 7423 (Clarke and Morris 1976a). Yet butyricin 7423 is unusual in that whilst it is bactericidal to vegetatively growing cells of *Cl. pasteurianum* it does not prevent the normal development of cells committed to sporulation (Clarke and Morris 1977). It is especially interesting that whereas butyricin 7423 acts in vitro as a potent inhibitor of the H^+-ATPase (BF_oF_1) associated with the cytoplasmic membrane of vegetative cells of *Cl. pasteurianum* (Clarke et al. 1979), the corresponding enzyme complex of the mother cell membrane in the sporulating organism has an altered protein subunit composition and is not inhibited by the bacteriocin (Clarke and Morris 1977, 1980).

It is the chief purpose of this communication to report that whilst the ability of butyricin 7423 to interact specifically with the BF_o component of the membrane H^+-ATPase of the sensitive target cell is possibly an essential prelude to its action, the lethality of the bacteriocin is not attributable merely to its in vitro ability to inhibit this enzyme complex.

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Non-standard abbreviations: H^+-ATPase = proton translocating adenosine 5′-triphosphatase (EC 3.6.1.3); DCCD = dicyclohexylcarbodi-imide

Materials and Methods

Organism, Maintenance and Growth. Clostridium pasteurianum 6013 was kindly supplied by Mrs W. Ego (University of Hawaii) as strain W-5, ATCC 6013. It was maintained as previously described (Mackey and Morris 1971) and was grown in batch culture in a minimal glucose (2% w/v) plus ammonium, salts and vitamins medium (Robson et al. 1974) incubated anaerobically at 37° C under $N_2:CO_2$ (19:1).

Isolation of Mutants. Petri plates containing agar (2%)-solidified glucose minimal medium supplemented with DCCD (80 μM) were overlaid with 3 ml of the same medium (but with 0.9% agar) seeded with approx. 3×10^7 cells of *Cl. pasteurianum* 6013 taken from a late exponential phase batch culture. A small quantity (approx. 0.5 mg) of N-methyl-N′-nitro-N-nitrosoguanidine (NTG) was placed on the plate at three widely separated sites. After anaerobic incubation at 37° C for 3 days, single colonies were selected and the isolates purified by sequential streaking on freshly prepared agar-solidified medium containing 80 μM DCCD. The final isolates were maintained as sporulated cultures on slopes of this medium. Mutants displaying diminished sensitivity to butyricin 7423 were obtained in a similar manner save that the bacteriocin was introduced into wells punched out of the seeded plate at suitable distances from the applied mutagen. Following isolation and purification on agar-solidified medium containing butyricin 7423, the selected mutant strains were stored as lyophilised cultures.

Preparation and Assay of Butyricin 7423. Butyricin 7423 was obtained from culture supernatant of *Clostridium butyricum* NCIB 7423 and was purified essentially by the procedure previously reported by Clarke et al. (1975). When large volumes of culture supernatant were employed (60–100 l) it was convenient to dispense with the use of Triton X-100 in the purification procedure which then involved hydrophobic

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affinity chromatography on Octyl Sepharose CL4B followed by concentation of the ethanolic eluate and ion exchange chromatography on CM-Sepharose CL6B. The final product was concentrated by lyophilisation and was desalted by passage through a small column of Sephadex G 25. Butyricin 7423 was assayed in Arbitrary Units (AU) by the diffusion zone method (Clarke et al. 1975) using *Cl. pasteurianum* 6013 as the indicator organism. The purified preparations displayed an activity of about 10^5 AU mg^{-1} of protein.

Assay and Purification of ATPases. ATP phosphohydrolase activity was assayed (a) by the semi-continuous measurement of release of inorganic phosphate from ATP (Clarke and Morris, 1976b) and (b) by continuously monitoring the release of protons that accompanies hydrolysis of ATP (Lloyd and Edwards 1976). In each case the unit of ATP phosphohydrolase activity was defined at 1 µmol of product formed per min at 37° C. The preparation of washed suspensions of cell membranes from vegetatively growing organisms, and the separate purifications of H^+-ATPase (BF_oF_1) and its soluble BF_1 component, have all been described previously (Clarke et al. 1979).

Analytical Methods. Intracellular ATP concentration was measured by the luciferin-luciferase procedure (Stanley and Williams 1969) after rapid transfer of the organisms into ice-cold perchloric acid (0.2 M) and subsequent holding therein for 30 min. Protein was measured by the method of Lowry et al. (1951) using bovine serum albumin, fraction V as a standard. Radioactivity was measured by scintillation counting using a Beckman LS200B spectrometer (Clarke et al. 1979). Electrophoresis of proteins in the presence of sodium dodecylsulphate was performed as previously described, with marker proteins of known molecular weights being simultaneously electrophoresed in adjacent gels (Clarke et al. 1975). Gels were stained for protein with Coomassie Blue by the method of Fairbanks et al. (1971) and were scanned at 609 nm using a Gilford 2400-S recording spectrophotometer. Radioactive gels were sliced into serial 1.5 mm sections each of which was solubilised in 1.5 ml of 9% (v/v) H_2O_2 by heating at 70° C for 18 h in capped tubes prior to the assay of radioactivity.

Chemicals. These were all obtained from previously disclosed sources (Clarke et al. 1979).

Results

In Vitro Inhibition by Butyricin 7423 of the Membrane H^+-ATPase of Clostridium pasteurianum 6013

Samples (1 ml) of a suspension of washed cell membranes (0.5 mg protein ml^{-1}) derived from *Cl. pasteurianum* 6013 harvested in mid-exponential phase of batch culture growth in glucose minimal medium, were anaerobically pre-incubated at 37° C for 30 min with various concentrations of butyricin 7423 in a buffer mixture consisting of 90 mM Tris-HCl, pH 7.6 plus 6 mM $MgCl_2$. The specific ATP phosphohydrolase activity of each preparation was then assayed by the phosphate release procedure (Methods) following the addition of 8 mM ATP. Table 1 shows that butyricin 7423 was an effective inhibitor of this H^+-ATPase (BF_oF_1) complex with 50% inhibition being achieved with a concentration of about 100 AU of butyricin mg^{-1} membrane protein. When the purified, soluble BF_1 component of this H^+-ATPase was similarly tested, its ATP phosphohydrolase activity was not inhibited by the bacteriocin (Table 1; also Clarke et al. 1979).

Table 1. Relative sensitivities to inhibition (a) by DCCD and (b) by butyricin 7423 of membrane-bound H^+-ATPase (BF_oF_1) preparations and of the soluble BF_1 component of the enzyme complex from *Cl. pasteurianum* 6013

Cl. pasteurianum-derived ATP phosphohydrolase preparation	Concentration of inhibitor causing 50% inhibition	
	DCCD (µg mg^{-1} protein)	butyricin 7423 (AU mg^{-1} protein)
Membrane BF_oF_1 of wild-type organism	9.3	100
Purified BF_1 of wild-type organism	251.0	not sensitive
Membrane BF_oF_1 of mutant DC3	322.3	not sensitive
Membrane BF_oF_1 of mutant DC5	305.2	138
Membrane BF_oF_1 of mutant DC6	316.8	189

Sensitivity to Inhibition by DCCD of H^+-ATPases of Mutant Strains Selected for Diminished Sensitivity to Butyricin 7423

Following mutagenesis of *Cl. pasteurianum* 6013 with NTG (Methods), several strains were isolated which demonstrated varying degrees of diminished sensitivity to the bactericidal action of butyricin 7423 (Table 2). When the membrane-associated H^+-ATPase activity of each was tested for sensitivity to inhibition (a) by butyricin 7423 and (b) by DCCD, the strains were found to fall into two distinct classes (Table 2). Class 1 consisted of mutant strains whose membrane H^+-ATPase was no less sensitive to inhibition either by butyricin 7423 or by DCCD than was the enzyme complex from the parent, wild-type organism. Cultures of Class 1 mutant strains demonstrated the least sensitivity to the bactericidal action of the bacteriocin. Class 2 mutant strains showed varying degrees of reduced sensitivity to butyricin 7423 and their membrane H^+-ATPases also displayed diminished sensitivities to inhibition by butyricin 7423. Yet the sensitivity to inhibition by butyricin 7423 that was displayed by the membrane H^+-ATPase of such a Class 2 mutant strain was not wholly predictable from the sensitivity of the organism to the bactericidal action of the bacteriocin. In the case of the membrane H^+-ATPase complexes of each of the Class 2 mutants however, diminished sensitivity to inhibition by butyricin 7423 was invariably accompanied by decreased sensitivity to inhibition by DCCD.

Sensitivity to Butyricin 7423 of DCCD-Insensitive Mutants

Following mutagenesis with NTG (Methods), sixteen mutant strains of *Cl. pasteurianum* 6013 were isolated, designated as

Table 2. Relative sensitivities to inhibition by DCCD and by butyricin 7423 of (a) growth, and (b) activity of membrane H^+-ATPase (BF_oF_1) of *Cl. pasteurianum* 6013 and of several of its mutant strains selected for diminished in vivo sensitivity to the bacteriocin. Sensitivity of each organism to the bactericidal action of butyricin 7423 was measured by the plate diffusion procedure (Methods), whilst the specific ATP phosphohydrolase activity of each cell membrane preparation (0.5 mg protein) was measured before and after anaerobic incubation for 15 min at 37° C with DCCD (100 μM) or butyricin 7423 (100 AU mg^{-1} membrane protein). Relative sensitivities to inhibition are recorded as % of that displayed by wild-type *Cl. pasteurianum* 6013 and its vegetative cell membrane H^+-ATPase (BF_oF_1)

Organism	Relative sensitivity in culture to butyricin 7423	Relative sensitivity of membrane H^+-ATPase to inhibition by	
		butyricin 7423	DCCD
Wild-type, strain 6013	100	100	100
Mutant A 2	< 1	100	100
Mutant B 5	< 1	100	100
Mutant T 2	< 1	100	100
Mutant B 23	5	< 1	< 1
Mutant T 9	9	20	12
Mutant A 8	33	25	< 1
Mutant B 25	43	< 1	10

strains DC1 to DC16, each of which was able to grow in the presence of a concentration of DCCD (80 μM) which was lethal to the wild-type organism. Fifteen of these mutant strains were indistinguishable from the parent wild-type organism with respect to their sensitivity to growth inhibition by butyricin 7423. Mutant DC3 differed, in that when growing in glucose minimal medium containing 50 mM potassium phosphate buffer pH 7 it was only one sixth as sensitive to the bacteriocin as was *Cl. pasteurianum* 6013 when growing on the same medium. However in semi-defined broth culture (OXOID, Reinforced Clostridium Medium) mutant strain DC3 was as sensitive to the bacteriocin as was the wild-type organism.

Sensitivity to Inhibition by Butyricin 7423 of H^+-ATPases of Mutant Strains Selected for Diminished Sensitivity to DCCD

The sensitivities to inhibition (a) by DCCD and (b) by butyricin 7423 of the membrane-bound H^+-ATPase (BF_oF_1) complexes of three DCCD-resistant mutant strains of *Cl. pasteurianum* were assayed after growth in glucose minimal medium; strains DC5 and DC6 were chosen as representative of the majority of these mutants to which butyricin 7423 was bactericidal at the usual concentrations, whilst mutant DC3 was examined because of its diminished sensitivity to the bacteriocin in glucose minimal medium. In each case the enzyme complex from the mutant was some 30 times less sensitive to inhibition by DCCD than was the H^+-ATPase activity associated with the cell membrane of the parent, wild-type organism. Yet, whilst the membrane H^+-ATPases of mutants DC5 and DC6 were only slightly less sensitive to inhibition by butyricin 7423 than was the H^+-ATPase of the wild-type organism, that of mutant DC3 was not inhibited by butyricin 7423 (Table 1).

Properties of Purified H^+-ATPases of the DCCD-Insensitive Mutants

The H^+-ATPase (BF_oF_1) complex of each of the mutant strains DC3, DC5 and DC6 was solubilised and partially purified (some 5-fold) as described in Methods. When the enzyme complex was derived from organisms harvested in the early exponential phase of batch culture in glucose minimal medium, its polypeptide subunit composition (determined by dodecyl sulphate polyacrylamide gel electrophoresis) was in each case indistinguishable from that of the H^+-ATPase (BF_oF_1) of vegetatively growing cells of wild-type *Cl. pasteurianum* 6013 (Clarke et al. 1979). In particular, each of the enzyme (BF_oF_1) preparations from the vegetatively growing mutant organism contained a protein subunit which was electrophoretically indistinguishable from that designated as f_a (molecular weight 15,000) which had been identified as the single, DCCD-binding protein subunit in the BF_o component of the H^+-ATPase in the vegetative wild-type organism (Clarke et al. 1979). To determine whether binding of DCCD by this f_a subunit was impaired in any of the H^+-ATPase preparations derived from the mutant strains, a sample of each preparation was incubated with ^{14}C labelled DCCD and was then subjected to dodecyl sulphate polyacrylamide gel electrophoresis. In each case substantial binding of the ^{14}C-labelled DCCD had occurred, predominantly to the f_a subunit (i. e. approximately 60% of total bound DCCD).

Changes in Intracellular ATP Concentration Provoked (a) by DCCD and (b) by Butyricin 7423

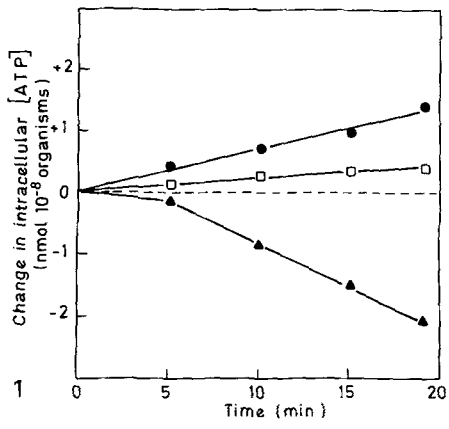
When exposed to a growth inhibitory concentration of DCCD, *Cl. pasteurianum* 6013 evidenced a substantial increase in intracellular ATP concentration, an effect not, of course, displayed by any of its DCCD-insensitive mutant strains when these were challenged with the same concentration of DCCD.

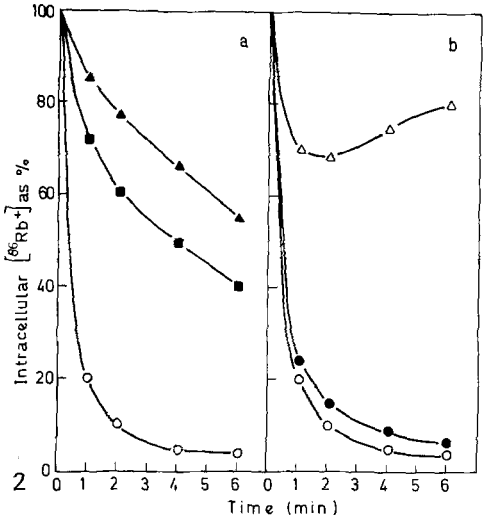
Exposure of *Cl. pasteurianum* 6013 to concentrations of butyricin 7423 greater than the minimum lethal dose also caused an increase in ATP concentration (Clarke and Morris 1975). Whilst treatment of mutants DC5 and DC6 with excess butyricin 7423 similarly caused their intracellular concentrations of ATP to rise, exposure of mutant DC3 to the same concentrations of the bacteriocin provoked a substantial decrease in the intracellular concentration of ATP (Fig. 1).

All of these experiments were carried out with washed suspensions of organisms which had been grown in a medium supplemented with 3 mM of 2-^3H adenine (0.3 μCi μmol^{-1}) and it was demonstrable that no ATP or other adenine-containing metabolites had been lost from the cells into the medium.

Efflux of Intracellular $^{86}Rb^+$ ions Provoked by Butyricin 7423

Cells of *Cl. pasteurianum* 6013 preloaded with $^{86}Rb^+$ ions by growth in a medium deficient in K^+ (< 2 mM) and supplemented with 0.13 μCi ml^{-1} of $^{86}RbCl$ (0.35 mg mCi^{-1}), when treated with the minimum lethal dose of butyricin 7423 quickly lost into the suspension medium the majority of this accumulated $^{86}Rb^+$ (Fig. 2; also, Clarke and Morris 1976a). This loss of intracellular $^{86}Rb^+$ was remarkably rapid when the organisms were suspended in an anaerobic isotonic buffer mixture containing 15% (w/v) sucrose and 50 mM sodium





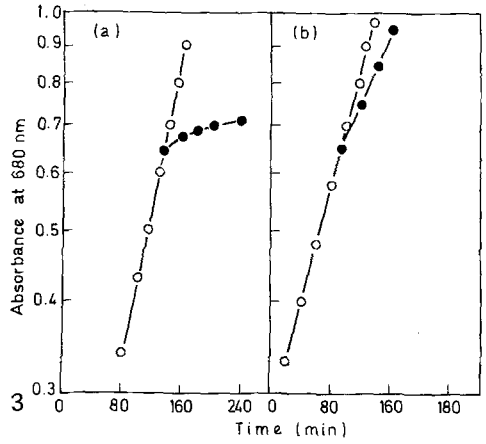


Fig. 1. Changes in intracellular concentration of ATP provoked by exposure to butyricin 7423 (3.5 AU 10^{-7} organisms) of cell suspensions of the DCCD-resistant mutant strains DC 5 (□---□), DC 6 (●---●), and DC 3 (▲---▲). The intracellular concentration of ATP at 0 min, when the bacteriocin was added, was between 2.1 and 2.5 nmol 10^{-8} organisms

Fig. 2a and b. Rates of efflux of $^{86}Rb^+$ ions from *Clostridium pasteurianum*, and from several of its DCCD-resistant mutants, provoked by inhibitors of H^+-ATPase including butyricin 7423. (**a**) *Clostridium pasteurianum* 6013 exposed to butyricin 7423, 0.6 AU 10^{-7} organisms (○---○), to DCCD, 20 μM (▲---▲), and to efrapeptin, 4 μg ml^{-1} (■---■). (**b**) Exposure to butyricin 7423 (0.6 AU 10^{-7} organisms) of mutant DC 5 (●---●), mutant DC 6 (○---○), mutant DC 3 (△---△). All inhibitors were added at 0 min to an anaerobic suspension of cells in 50 mM sodium phosphate pH 7 containing 15% (w/v) sucrose

Fig. 3a and b. Protection against the lethal action of butyricin 7423 afforded to *Clostridium pasteurianum* by a high extracellular concentration of K^+ ions. (**a**) Culture of *Clostridium pasteurianum* growing anaerobically at 37° C in glucose minimal medium containing 50 mM K^+ (○---○) with butyricin 7423 (5 AU 10^{-7} organisms) being added to a duplicate culture at the time indicated (●---●). (**b**) As in (**a**) but with the culture medium containing 200 mM K^+

phosphate pH 7 (Fig. 2a). Exposure of such organisms to DCCD (20 μM) or to the BF_1-directed ATPase inhibitor efrapeptin (4 μg mg^{-1}) provoked a much slower rate of efflux of $^{86}Rb^+$ ions (Fig. 2a).

With all of the DCCD-insensitive mutant strains other than mutant DC3, exposure to butyricin 7423 provoked $^{86}Rb^+$ efflux from $^{86}Rb^+$-preloaded cells at a rate and to an extent comparable with that observed with the parent, wild-type organism (Fig. 2b). In the case of mutant DC 3 however, although the immediate effect of exposure of $^{86}Rb^+$-preloaded cells to butyricin 7423 was again the promotion of a net efflux of $^{86}Rb^+$ ions, only a fraction of the total intracellular $^{86}Rb^+$ ion content had been lost from the cells when net efflux ceased, apparently due to reimportation of $^{86}Rb^+$ ions that had leaked from the organisms (Fig. 2b).

Protective Effect of Elevated K^+ ion Concentration

In the usual glucose minimal medium (containing about 50 mM K^+ ions) growth of *Cl. pasteurianum* 6013 was abruptly halted by a relatively small concentration of butyricin 7423 (Fig. 3). Yet when this defined medium was fortified by the addition of a high concentration of K^+ ions (so that the total K^+ ion concentration was greater than about 200 mM) continued growth and multiplication of the organism occurred, though at a somewhat diminished rate, in

the presence of what had previously proved to be a lethal concentration of the bacteriocin (Fig. 3). Only when concentrations of butyricin 7423 were employed that were very much in excess of the normally minimum lethal dose was the bacteriocin still growth inhibitory in K^+-enriched medium, lethality now being ascribable to relatively nonspecific leakage of a variety of small molecular weight, intracellular metabolites (cf. Clarke and Morris 1976a). Conversely, enhanced sensitivity to butyricin 7423 was displayed by *Cl. pasteurianum* 6013 growing in media low in K^+ ion concentration (below 10 mM K^+). Increased concentrations of ions other than K^+ (e. g. Na^+ and Mg^{2+}) did not similarly protect the organism from the lethal action of butyricin 7423, though in media low in K^+ (e. g. 5 mM K^+), increasing the NH_4^+ ion content of the medium (> 100 mM NH_4^+) slightly diminished the sensitivity of the culture to the bacteriocin.

Discussion

Butyricin 7423 is an in vitro BF_o-directed inhibitor of the membrane H^+-ATPase complex of vegetatively growing cells of *Cl. pasteurianum*. Evidence that butyricin 7423 interacts with the DCCD-binding protein in this ATPase is supplied (1) by the insensitivity to butyricin 7423 of the purified BF_1 ATP phosphohydrolase and the sensitivity of the H^+-ATPase (BF_oF_1) which in the BF_o component contains the DCCD-binding protein f_a; (2) by the isolation of mutant DC3 which although selected for its diminished sensitivity to DCCD possessed a H^+-ATPase which was also relatively insensitive to inhibition by butyricin 7423; (3) by the isolation of mutant strains (Class 2) selected for diminished sensitivity to butyricin 7423 yet whose H^+-ATPases were less sensitive to inhibition by DCCD as well as by the bacteriocin. It would seem however that other membrane protein(s) may also bind butyricin 7423 for some 25% of the ^{125}I-labelled butyricin 7423 which bound tightly to cell membrane preparations from *Cl. pasteurianum* was found to be associated with proteins other than the H^+-ATPase (C. D. Morley, unpublished findings). The existence of some additional receptor could also be inferred from the existence of the butyricin-resistant mutants (Class 1) whose membrane H^+-ATPases displayed undiminished sensitivity to inhibition by the bacteriocin.

Our present inability to analyse the genetic lesions in these mutant strains means that we must be cautious in attempting to explain their properties. Even so, the response of mutant DC 3 when challenged with butyricin 7423 was most interesting, for unlike its parent wild-type organism, its intracellular ATP content decreased and whilst there was an initial efflux of $^{86}Rb^+$ (here employed as a homologue of K^+) a substantial proportion of the initial cellular content of this ion was retained, presumably as a result of the continued operation of the H^+-ATPase. When, during the course of sporulation of mutant DC3 the specific activity of this H^+-ATPase progressively declined, then treatment with butyricin 7423 evoked more rapid and more complete efflux of $^{86}Rb^+$ (Clarke 1978).

The same high extracellular concentrations of K^+ ions which protected *Cl. pasteurianum* 6013 from the lethal action of butyricin 7423, also afforded protection to this organism against otherwise lethal concentrations of gramicidin D (Morley, unpublished findings). As with *Streptococcus faecalis* which survived exposure to gramicidin in a nutrient medium at slightly alkaline pH when this was supplemented with K^+ ions (Harold and van Brunt 1977) we can conclude that transmembrane proton circulation is not essential for the maintenance of growth and division of *Cl. pasteurianum* at high external K^+ concentrations in a glucose minimal medium. Kopecky et al. (1975) similarly found that a normally lethal dose of colicin K failed to kill *Escherichia coli* when its growth medium was fortified with K^+ and Mg^{2+} ions.

It might at first sight thus appear that the bacterial action of butyricin 7423 may be explained by its inhibition of the H^+-ATPase which is the sole means of generating a transmembrane protonmotive force in *Cl. pasteurianum*. If this were the case, why should butyricin 7423 have elicited any K^+ efflux from cells of mutant DC3, and why should this organism not be wholly indifferent to the bacteriocin in glucose minimal medium? Furthermore, if butyricin 7423 acts in an analogous manner to other membrane-active bacteriocins which demonstrate single hit kinetics of killing (Konisky 1978) any explanation of its mode of action which is based on stoichiometric inhibition of the membrane H^+-ATPase can be ruled out. On the other hand, interaction with the DCCD-binding BF_o protein could yet be a necessary prelude to its lethal action since when, as in sporulating cells *Cl. pasteurianum* this interaction is not demonstrable (Clarke and Morris 1977, 1980) the organism displays a butyricin-resistant phenotype. If this is so, then this would appear to be the first instance of a cytoplasmic membrane protein of well-characterised function serving as a necessary receptor/target for a membrane-active bacteriocin.

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