APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Apr. 1989, p. 1040–1043
0099-2240/89/041040-04$02.00/0
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Vol. 55, No. 4

Effect of Carbonyl Cyanide *m*-Chlorophenylhydrazone on *Escherichia coli* Halotolerance

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Received 26 October 1988/Accepted 18 January 1989

The growth-inhibitory effect of carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) was less on members of the family *Enterobacteriaceae* (halotolerant organisms) than it was on species of *Vibrio* (moderately halophilic organisms). When sodium chloride concentration increased from 0.5 to 0.85 M, this effect was more pronounced for *Escherichia coli*; it remained relatively stable for *Vibrio* spp. The effect of carbonyl cyanide *m*-chlorophenylhydrazone was antagonized by the addition of glycine betaine or proline or by growth in a rich medium.

Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) is an H^+ ionophore which dissipates the H^+ gradient and thus uncouples electron transport from ATP synthesis. It can transport protons into the cell without the participation of ATP synthase (16). Therefore, the proton motive force (PMF) is cancelled out, and ATP can no longer be synthesized (1). Cellular respiration continues and may even be stimulated (9). CCCP is frequently used in the study of the active transport of substances. Studies have been carried out on halotolerant bacteria, such as *Escherichia coli* (7, 8) and *Klebsiella* sp. (3), halophilic bacteria, such as *Vibrio alginolyticus* (17, 18) and *Vibrio parahaemolyticus*, and moderately halophilic bacteria, such as *Vibrio costicola* (6) (for a review, see reference 3). Reactions to CCCP are varied; each type of bacterium according to its mode of energy transport (respiratory Na^+ pump, Na^+/H^+ antiport, etc.) shows a different response to concentrations of CCCP, this response being linked to pH (12, 18). Circulation of Na^+ ions across the membrane is essential for the different physiological functions of marine and halophilic bacteria (3). In halotolerant bacteria, the presence of Na^+ is not crucial, but growth can take place at a certain concentration of sodium chloride (NaCl) between 0.5 and 0.68 M in minimal medium. Compatible osmoprotectors, such as glycine betaine (GB) and proline (Pro) (10, 11), allow the bacteria to support even stronger concentrations of NaCl. Uptake of these substances occurs by active transport (4, 14).

We have studied the influence of CCCP on halotolerant (*Enterobacteriaceae*) and halophilic (*Vibrio* spp.) strains. Salt tolerance of the organisms and MIC of CCCP on the organisms were determined. A minimal medium supplemented with and without an osmoprotector and a complex medium were prepared at pH 7.2. Salt concentrations ranging from 0 to 1.2 M were used. All strains (*E. coli*, *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Salmonella* spp., *V. parahaemolyticus*, and *V. alginolyticus*) were of marine environmental origin.

The bacteria were incubated aerobically in 5 ml of Trypticase (BBL Microbiology Systems, Cockeysville, Md.) soy broth (TSB) (A.E.S. Laboratoire, Combourg, France) at 37°C for 18 h. They were then washed three times with 10 ml of saline water (0.9% [wt/vol] NaCl in distilled water). A volume of 5 μl of washed cells was used to inoculate U-bottom, 96-well polystyrene microdilution plates, for a total volume of 200 μl, to determine either the maximum NaCl tolerance or the MIC in a liquid medium. The cultures were then incubated at 37°C for 24 h. Bacterial growth was evaluated by visual assessment of turbidity. To determine the MIC on a solid medium, a drop of culture incubated aerobically in TSB for 18 h at 37°C was transferred via a multipronged replicator onto agar.

Minimal medium M63 (2) with 10 mM of glucose as the carbon and energy source was used. As indicated below, 1 mM of the osmoprotectors GB (Sigma Chemical Co., St. Louis, Mo.) or Pro (E. Merck AG, Darmstadt, Federal Republic of Germany) was added to the M63 after sterilization by passage through a membrane filter (0.22-μm pore size; Millipore Corp., Bedford, Mass.). TSB was used as the complex medium, and Trypticase-agar was the solid medium. The osmotic strengths of these media were increased by the addition of NaCl at the following concentrations: 0.085, 0.5, 0.68, 0.85, 1.02, and 1.20 M. The final pH of these media before sterilization was 7.2. Bacteria were incubated in the presence of CCCP (Sigma) dissolved in dimethyl sulfoxide at the following concentrations: 0, 0.312, 0.625, 1.25, 2.5, 5, 10, 20, 40, 50, 60, 80, and 100 μM. Blanks consisting of bacterial culture in the presence of dimethyl sulfoxide or each concentration of salt were included in each experiment. MICs were determined by duplicate tests. Comparison of a large number of strains was made possible by replication plating on solid media. Readings were taken after 18 h of incubation.

The MIC of CCCP for *E. coli* ZB400 on M63 containing 0.5 M NaCl was 10 μM, whereas *V. alginolyticus* and *V.*

TABLE 1. MIC of CCCP for *E. coli*, *V. alginolyticus*, and *V. parahaemolyticus* in TSB at different salt concentrations

[NaCl] (M) in TSB	MIC of CCCP (μM) for:			
	E. coli ZB 400	*E. coli* ZB 401	*V. alginolyticus*	*V. parahaemolyticus*
0.085	>100	>100	5	5
0.50	40	50	2.5	2.5
0.68	20	40	2.5	2.5
0.85	20	20	1.25	2.5
1.02	20	20	1.25	0.625
1.20	—[a]	—	0.312	0.312

[a] —, No growth.

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TABLE 2. Percentage of strains resistant to CCCP on Trypticase-agar at different salt concentrations

[NaCl] (M)	[CCCP] (μM)	Growth[a] of strains (*n* = no. of strains tested)											
		V. alginolyticus and *V. parahaemolyticus* (*n* = 24)			*E. coli* (*n* = 27)			*K. oxytoca* and *K. pneumoniae* (*n* = 18)			*Salmonella* spp. (*n* = 20)		
		NG	PI	No G	NG	PI	No G	NG	PI	No G	NG	PI	No G
0.5	2.5	100			100			100			100		
	5	66.7	8.3	25	100			100			100		
	10			100	100			100			100		
	15				100			100			100		
	20				100			100			100		
	30				100			100			100		
	40				100			100			100		
	50				96.2		3.7	94.4		5.5	100		
	60				70.3	22.2	7.4	72.2	11.1	16.6	45	30	25
	80				66.6	11.1	22.2	55.5	5.55	38.8	20	35	45
	100				48.2	27.6	24.1	38.8	11.1	50		20	80
0.68	2.5	100		100	100			100					
	5	66.7		33.3	100			100			100		
	10			100	100			100			100		
	15				100			100	100				
	20				100		88.8	11.1	95	5			
	30				96.2	3.7	55.5	33.3	11.1	50	30	20	
	40				62.9	22.2	14.8	44.4	5.5	50	30	20	50
	50				40.7	40.7	18.5	38.8		61.1		20	80
0.85	2.5	100	100	100						100			
	5	20.8	12.5	54.1	100	100				100			
	10			100	80	20	72.2	16.6	11.1	100			
	20				59.2	25.2	14.8	44.4	33.3	22.2	20	25	55
	30				24.1	41.3	34.4	22.2		77.7		20	80

[a] NG, Normal growth; PI, partially inhibited; No G, no growth.

parahaemolyticus were inhibited by lower concentrations of CCCP (ca. 2.5 and 0.650 μM).

In a complex liquid medium at 0.085 M NaCl, the MIC of CCCP was higher than 100 μM for *E. coli* ZB400 and *E. coli* ZB401 and 5 μM for the *Vibrio* species (Table 1). On Trypticase-agar, the results (Table 2) show that the members of *Enterobacteriaceae* were resistant to CCCP. *E. coli* and the *Klebsiella* species were more resistant than *Salmonella* spp. With 0.5 M NaCl and 100 μM CCCP, 48.2% normal growth was observed for *E. coli* and 38.8% for the *Klebsiella* species. Under identical conditions, no growth was observed for *Salmonella* spp. Furthermore, resistance to CCCP appears to be dependent on salt concentration. The *Vibrio* species were very sensitive to concentrations lower than 10 μM CCCP, whatever the salt content used.

The effect of CCCP on a population of *E. coli* (Fig. 1) shows that a concentration of 20 μM CCCP reduced the number of strains which grew in M63, M63 + GB, and M63

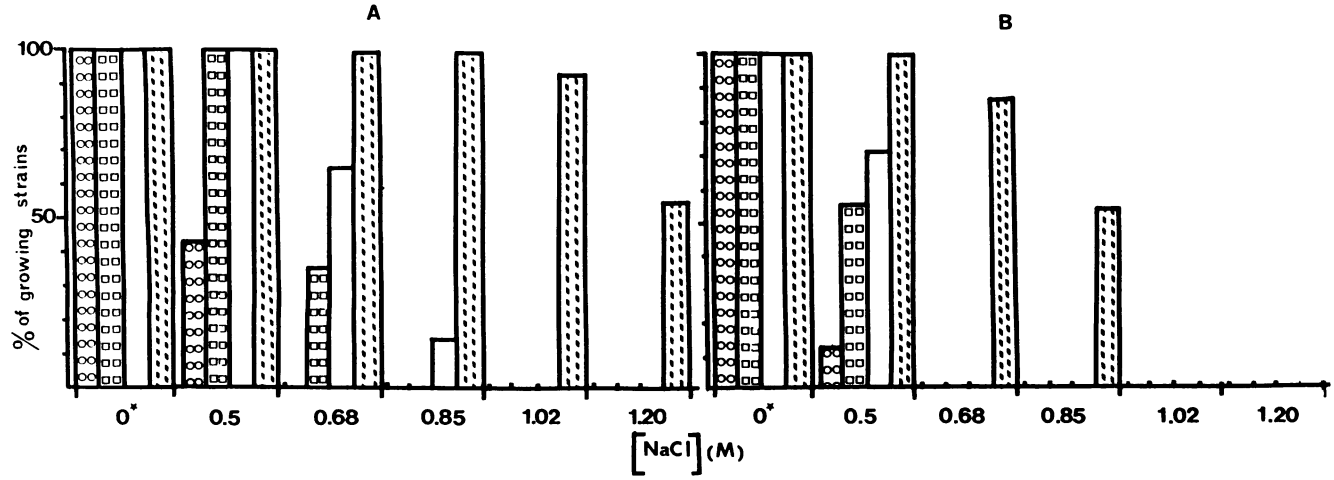

FIG. 1. Growth percentage of *E. coli* of marine origin (16 strains) at different NaCl concentrations in the absence (A) or presence (B) of 20 μM CCCP. Media: ▩, M63; □, M63 + GB (1 mM); ▩, M63 + Pro (1 mM); ▩ TSB; ★, TSB containing 5 g/l NaCl.

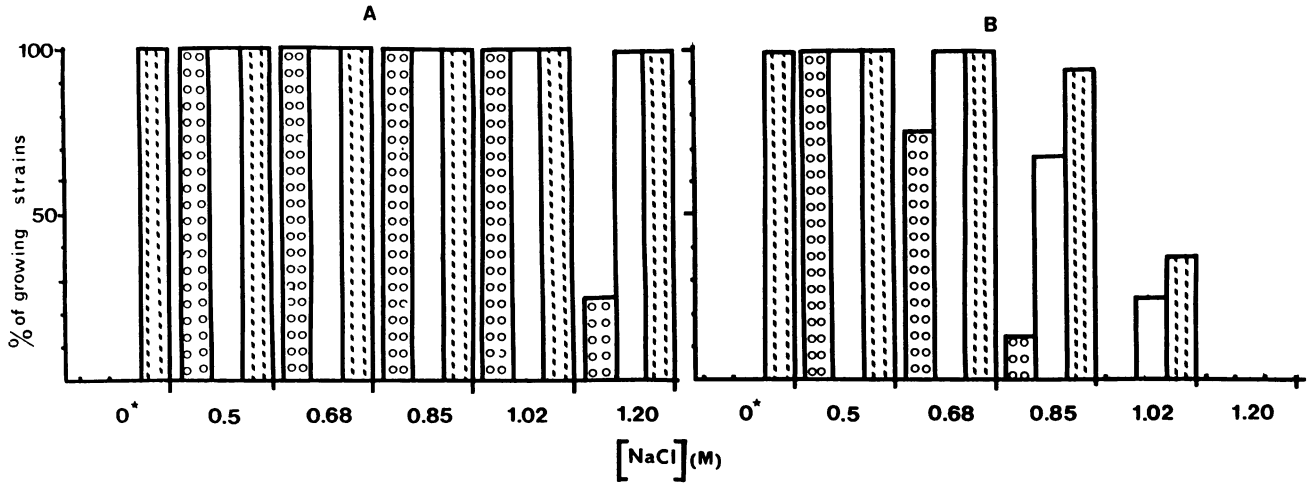

FIG. 2. Growth percentage of 16 *Vibrio* strains of marine origin (*V. alginolyticus*, eight strains; *V. parahaemolyticus*, eight strains) at different salt concentrations in the absence (A) or presence (B) of 1.25 μM CCCP. Media: ▨, M63; □, M63 + GB (1 mM), ▨, TSB; ★, TSB containing 5 g of NaCl per liter.

+ Pro containing 0.5 M NaCl. Total inhibition occurred at a salt concentration of 0.68 M. However, *E. coli* strains showed higher resistance in a complex medium. On the other hand, no growth was observed with a *Vibrio* population exposed to 10 μM CCCP. Therefore, 1.25 μM CCCP was used. Partial inhibition was already noted in M63 at 0.68 M NaCl and 0.85 M for rich medium. Inhibition in M63 + GB was intermediate (Fig. 2).

In summary, resistance of *E. coli* to CCCP was dependent on the nature of the medium. MICs in the region of 50 μM in M63 and in excess of 100 μM in TSB for a salt concentration of 0.085 M were obtained. These results are in agreement with those of Kinoshita et al. (8).

In a rich medium, the MIC fell sharply as salt concentration rose, reaching only 20 μM between 0.68 and 1.02 M NaCl (Table 1). In *E. coli*, it is the Na^+/H^+ antiport which functions, and cells can thus grow in the absence of PMF when glucose is used as an energy source (8). This accounts for the high MICs observed in particular at low salt concentrations. Our results can be interpreted as a combination of the effects of CCCP and osmotic stress to prevent growth; when the salt concentration is high, glycolytic energy no longer suffices to allow cells to resist.

On the contrary, for *Vibrio* strains, the type of growth medium, rich or minimal, seems to have no influence on the inhibitory action of CCCP; in both cases, the strains were highly sensitive. At pH 7.2, the MIC was on the order of 2.5 μM CCCP. These results agree with those of Tokuda and Unemoto (18). At this pH, sensitivity to CCCP was fairly high, since it is the H^+ pump which functions and not the Na^+ pump, whose maximum activity occurs at pH 8.5, thus rendering the *Vibrio* species resistant to CCCP (18). Furthermore, it is interesting that this inhibition was independent of salt concentration.

To observe the phenomenon of inhibition by CCCP on a population of *E. coli* (Fig. 1B) and *Vibrio* spp. (Fig. 2B), cells were grown at a subinhibitory concentration (20 and 1.25 μM, respectively). Halotolerant strains of *E. coli* were sensitive to 20 μM CCCP during growth in M63. They showed much stronger resistance when the M63 contained an osmoprotector, GB or Pro, which does not undergo any metabolic pathway under these conditions (4, 5, 10, 14). This resistance was particularly notable in a rich medium. Indeed, in this case, more than 50% of strains grew in the presence of 20 μM CCCP at a salt concentration of 0.85 M. Thus, it can be observed that the presence of osmoprotectors and nutrients in the TSB increased the resistance of *E. coli* to CCCP. Roth et al. (15) reported that in NaCl-upshocked cells, GB stimulates ATP production via an increase in glucose transport. However, this resistance is dependent upon the NaCl concentration (Fig. 1B). Inhibition in M63 is due to partial dissipation of the PMF by 20 μM CCCP (8). The cell can no longer provide enough energy to combat the osmotic stress induced by the salt; glycolytic energy alone is thus insufficient. In M63 with added GB or Pro, or in TSB, the inhibition observed is due to slowing down of the active transport of compatible substances, which reduces the osmoprotective effect (4, 13, 14). At high salt concentrations, osmotic stress is so great that even the slightest partial inhibition of PMF, induced by a reduction in energy supply, prevents growth of the cell.

For *Vibrio* spp. in a rich medium and in M63 + GB, the percentages of growing strains fell at high salt concentrations, as can be expected if the active transport of compatible factors of TSB and GB is partially inhibited by CCCP. In M63 the fall in these percentages is noticeable. This can be attributed to the absence of osmoprotectors. Given the slowing down of cellular energetic processes, the cells can no longer resist the osmotic stress induced by the NaCl. At a concentration of 10 μM, CCCP had a toxic effect on cells whatever the medium used.

In all cases, osmotic stress caused by salt combined with inhibition, even partial, of the PMF to slow the growth of the bacteria.

We are indebted to R. R. Colwell for helpful discussion and for reviewing the manuscript, and we thank D. McKeown for revising the English in this article.

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