Effects of the virulence plasmid ColV, I-K94 on the sensitivity of *Escherichia coli* to putative environmental inhibitory agents

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Summary. Derivatives of *Escherichia coli* carrying the virulence plasmid, ColV, I-K94 were more resistant than the ColV$^-$ parents to phage Mel but were more sensitive to the hydrophobic inhibitors deoxycholate, erythromycin and lysozyme. The basis for these changes in sensitivity has been examined in ColV$^+$ mutants with altered colicin or VmpA protein levels and in ColV$^+$ strains with repressed transfer properties.

Key words. Escherichia coli; ColV plasmid; inhibitory agents; environmental inhibitors, sensitivity to.

The outer membrane of the Gram negative bacterial cell forms a barrier to the penetration of such noxious agents as antiseptics, detergents, antibiotics, metal ions, lytic enzymes etc. In particular, in most Gram-negative bacteria isolated from the wild, the outer membrane forms an impenetrable barrier against naturally occurring hydrophobic agents.[2] The outer membrane also plays a role in the environmental response of Gram-negative bacteria to inhibitory bacteriocins and bacteriophages because the receptors for these agents are located in the outer membrane.[3] The presence of plasmids in Gram-negative bacteria frequently alters the composition and properties of the outer membrane[4,5,6] and where such changes alter the response of the bacterium to natural inhibitors, they may also affect survival in the environment. Such effects are of particular significance for plasmids of medical importance.

The plasmid considered here, ColV, I-K94, is a 95 megadalton element which specifies synthesis of colicins V and I and resembles the F plasmid in both transfer properties and incompatibility.[7] It is a virulence plasmid in the sense that, in common with other ColV elements, it increases the pathogenicity and serum resistance of certain *Escherichia coli* strains[8].

Strains of *E. coli* which harbor ColV, I-K94 show marked membrane protein alterations when compared to isogenic Col$^-$ strains. Firstly, like the sex factor F[5], ColV, I-K94 specifies membrane-associated transfer components e.g. membranes from ColV, I-K94$^+$ strains contain a 25,000 mol. wt protein, which, like the similarly sized Tra T gene product of F$^+$ cells[5], occurs in the outer membrane fraction and is absent if transfer properties are repressed[6]. Secondly, membranes from ColV$^+$ strains contain a major membrane component, the plasmid-encoded 33K VmpA gene product, which is absent from Col$^-$ strains[6,9].

Because ColV, I-K94 increases bacterial pathogenicity and alters the envelope of *E. coli* strains, a study has been undertaken in this laboratory to examine how the presence of ColV elements affects the response of enterobacteria to putative environmental inhibitors. This paper reports that the presence of ColV, I-K94 in *E. coli* increases sensitivity to deoxycholate, erythromycin and lysozyme but abolishes sensitivity to the phage Mel.

Methods. The strains of *Escherichia coli* K12 used in this study were P678-54 (*thr, leu, thi*) and 1829 (*trp*) together with their non-mucoid ColV, I-K94$^+$ (ColV$^+$) derivatives; for some experiments, *E. coli* KH262 (*thr, leu*) or *E. coli* C600 (*thr, leu, thi*) or *E. coli* PC0479 (*thr, leu, thi, pyr, thy, arg, ilv, his*) or their non-mucoid ColV$^+$ derivatives were used and in others, mutant derivatives isolated from strain P678-54 ColV as resistant to phage MS2. These mutants are numbered 2 and 32. Oxoid No. 2 broth was employed as growth medium and growth was with shaking at 37 °C unless otherwise stated. For solid medium, nutrient broth was solidified by adding 2% W/V Difco Bacto Agar. For plasmid elimination, organisms were grown for 48 h at 37 °C in nutrient broth +1% sodium dodecyl sulphate (SDS) with an inoculum of 10^5 organisms/ml. After this growth, the culture was diluted 1 in 100 in fresh broth and grown for 48 h at 44.5 °C. For membrane isolation, exponential brothgrown organisms were harvested, washed, sonically disrupted and whole membranes isolated essentially as described by Lugtenberg et al.[10]. For studies of the OmpC protein in ColV$^+$ strains, membrane proteins were further analyzed for location (by examining association with outer or cytoplasmic membrane[11]), trypsin sensitivity[12] or murein association (by examining behavior in 2% SDS at 60 °C[12]). Gels and electrophoresis conditions were essentially as described by Lugtenberg et al.[10]. Phage and colicin tests were made as described by Moores & Rowbury[6] and lysozyme sensitivity tested as described previously[13].

Results and discussion. For studies of the effects of ColV, I-K94 (referred to subsequently as ColV) on the membrane protein composition and inhibitor sensitivity of *Escherichia coli,* ColV$^+$ & ColV$^-$ derivatives of strains P678–54 and 1829 were used. For some experiments, ColV$^+$ and Col$^-$ derivatives of *E. coli* strain PC0479, C600 or KH262 were used also. The plasmid was stably maintained in these derivatives (overnight growth in broth at 37 °C with shaking gave cultures which had at least 99.5% ColV$^+$ organisms), but could be 'cured' as described in the Methods section.

The membranes of P678-54, 1829 and their ColV$^+$ derivatives were analysed on sodium dodecyl sulphate (SDS)-polyacrylamide gel. Figure la shows the analysis of membrane samples

Table 1. Effect of ColV on bacterial sensitivity to inhibitory agents

Strains	Chemical agents: % survival with stated additions to NA						Bacteriophages									Colicins		
	None	Copper* ions (50 µg/ml)	Ampicillin (1 µg/ml)	Erythromycin (20 µg/ml)	Novobiocin (20 µg/ml)	SDS (3%)	K3	λ	Mel	MS2	T4	T6	T7	Tula	TuII*	B	K	L
P678-54	100	98	100	64	88	40	S	R	S	R	S	S	S	S	S	S	S	S
P678-54 ColV	100	56	89	0.3	100	23	S	R	R	S	S	S	S	S	S	S	S	S
1829	100	96	30	100	100	58	S	R	S	R	S	S	S	S	R	S	S	S
1829 ColV	100	57	44	6	45	1	S	R	R	S	S	S	S	S	R	S	S	S

For test of the appropriate chemical agents, organisms were grown to exponential phase in broth at 37 °C and then organisms (200-300 per plate) were plated on NA with the stated supplements. Incubation was at 37 °C for 20–48 h. For colicin sensitivity tests, organisms of colicin producing strains were plated on NA and grown to produce colonies. These were surface killed with chloroform vapor and overlaid with soft agar containing the appropriate strain at 10^8 organisms/ml[6]. For bacteriophage sensitivity tests, organisms of the strain under test (10^8/ml) were plated, with appropriate dilutions of the phage under test, in soft agar on NA plates.[6] Incubation for colicin and bacteriophage tests was for 20 h at 37 °C. All tests in this Table were repeated at least 3 times with consistent results. R, resistant; S, sensitive. * Copper ions were added as cupric sulphate.

from P678-54 and derivatives after dissociation in 2% SDS at 100 °C (lanes 1, 3, 5, 7) or 30 °C (lanes 2, 4, 6, 8). In all the 100 °C treated samples, the major outer membrane proteins OmpF (ca 37,000 mol. wt), OmpC (circa 36,000) and OmpA (circa 33,000) can be readily observed. In the ColV$^+$ derivative (lane 3, the VmpA protein cannot be easily seen in the 100 °C sample; it rarely separates from the OmpA protein[6]. The VmpA protein can, however, be seen in the 30 °C treated sample (lane 4), because the OmpA protein runs faster under these conditions[14] (see lanes 2, 4, 6, 8) whereas the VmpA protein still runs at the 33 K position (lane 4). This figure also shows the absence of the 33 K VmpA protein from 2 'cured' derivatives (fig. 1a, lanes 6 and 8) obtained from P678-54 ColV by plasmid elimination (curing) as described in Methods. The levels of the OmpF and OmpC (37 K and 36 K) proteins do not appear (from this figure) to be appreciably affected by the presence of ColV. Membrane preparations from 1829 ColV behaved similarly to those from P678-54 ColV, showing the presence of the VmpA protein (data not shown) and levels of the OmpF and OmpC proteins apparently unaltered by the presence of ColV. Similar experiments to the above, showed that the VmpA protein is present in ColV$^+$ derivatives of strains C600, KH262 & PC0479 but not in the corresponding Col$^-$ strains.

The ColV$^+$ derivatives of P678-54 & 1829 were tested for sensitivity (table 1) to a range of colicins (colicins B, K, L) and bacteriophages (phages MS2, λ, T4, T6, T7, K3, Mel, Tula, TuII*). The plasmid only altered the response of the strains to 2 of the tested agents. Firstly, the ColV$^+$ derivatives of strains 1829 and P678-54 were sensitive to phage MS2 whereas the Col$^-$ parents were resistant. Further tests showed that the ColV$^+$ derivatives of strains C600, KH262 and PC0479 (but not the Col$^-$ parents) were also sensitive to phage MS2. This phage binds specifically to pili on strains which carry F-like plasmids with derepressed transfer properties. The plasmid used here is of this type. The sensitivity to such phages may appreciably reduce the environmental survival of ColV$^+$ enterobacteria compared to that of Col$^-$ ones because we find that ca 20% of all the bacteriophages in sewage which plate on ColV$^+$ enterobacteria are male-specific bacteriophages specific for cells harboring F-like plasmids.

The presence of ColV led to increased resistance to the T4-like phage Mel. This resistance was initially demonstrated for strains 1829 ColV$^+$ and P678-54 ColV$^+$ (table 1) but further studies showed that PC049 ColV$^+$ and KH262 ColV$^+$ are also resistant to phage Mel whereas the corresponding Col$^-$ strains are sensitive. Phage Mel uses the 36,000 (36K) OmpC protein as receptor[15] and, accordingly, resistance might have resulted from the absence of this protein. However, membranes of the P678-54 ColV$^+$ derivative have a major 36 K protein (fig. la, lane 3), which, like the OmpC protein, is a murein-associated outer membrane protein which is insensitive to trypsin, shows an apparent reduction in molecular weight on heat-treatment, and acts as receptor for phage T4. Accordingly the OmpC receptor protein for the attachment of phage Mel is present on ColV$^+$ bacteria. Two techniques have been used to establish that phage Mel particles can attach to ColV$^+$ bacteria. Firstly removal of free phage from broth by added ColV$^+$ bacteria has been estimated and secondly the ability of phage Mel particles to *kill* ColV$^+$ bacteria has been tested (the killing process is dependent on attachment). Firstly when organisms of strain 1829 ColV$^+$ were mixed with phage Mel in a circa 10^4:1 ratio in nutrient broth, then, if the culture was centrifuged after 15 min incubation at 37 °C, only 1% of the added phages remained in the supernatant fluid, i.e. the phages had attached to the ColV$^+$ bacteria. Secondly, when organisms of 1829 ColV$^+$ were incubated with phage Mel in a 1:10 ratio in broth, after incubation for 90 min at 37 °C, the viable count of the ColV$^+$ bacteria had fallen to ca. 0.05% of its original value. These experiments establish that phage Mel will attach to ColV$^+$ bacteria and will kill them. It is now intended to examine Mel phage DNA injection and multiplication in ColV$^+$ strains. Resistance to phage Mel in ColV$^+$ strains is not related to the production of transfer components by the ColV$^+$ strains because a strain in which the derepressed transfer properties of ColV have been repressed (by a *fi*$^+$ plasmid, ColB-K98) still shows resistance to phage Mel. Additionally it is probably not the presence of colicin or VmpA protein in ColV$^+$ strains which affects phage Mel because some mutants derived from P678-54 ColV$^+$ which produce normal levels of colicin and VmpA protein (compared to strain P678-54 ColV itself) show sensitivity to phage Mel (mutant 32, table 2).

The effects of ColV were also tested on the sensitivity of strains P678-54 and 1829 to copper ions, antibiotics, detergents

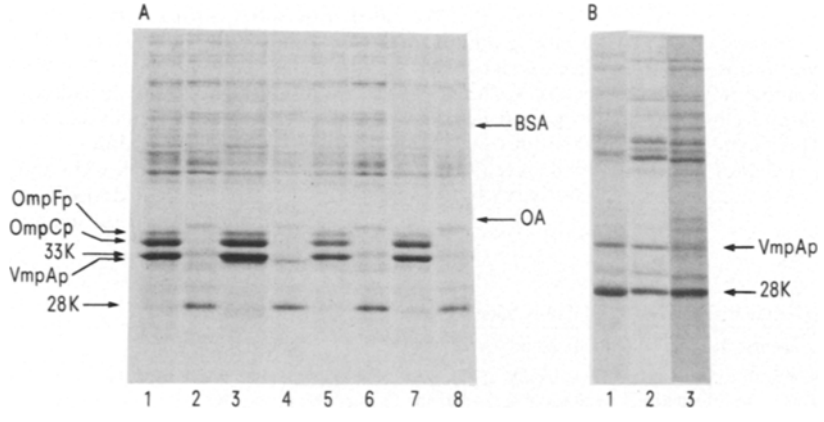

Figure 1. *a* Envelope proteins changes in a ColV$^+$ strain of *E. coli*. Organisms of *E. coli* P678-54, its ColV$^+$ derivative and 2 Col$^-$ isolates obtained from the latter by curing (see methods) were grown and membranes prepared and analyzed on SDS polyacrylamide gels as decribed in 'Methods'. Duplicate membrane samples were dissociated at 100 °C and 30 °C. Lane 1, P678-54 membranes dissociated at 100 °C; lane 2, P678-54 membranes dissociated at 30 °C; lane 3, P678-54 ColV$^+$, 100 °C; lane 4, P678-54 ColV$^+$, 30 °C; lane 5, cured strain No. 7 isolated from P678-54 ColV, 100 °C; lane 6, cured strain No. 7, 30 °C; lane 7, cured strain No. 12, 100 °C; lane 8, cured strain No. 12, 30 °C. The positions of standards (bovine serum albumen (BSA), 68 000; ovalbumen (OA), 43 000) and of OmpC, OmpF, OmpA (both 28 K and 33 K positions) and VmpA proteins are indicated. Even in the 100 °C sample from P678-54 ColV (lane 3), the VmpA protein can be just detected by the characteristic 'broadening' effect it confers on the OmpA protein band. *b* VmpA protein in mutant ColV$^+$ strains. Organisms of P678-54 ColV and 2 mutant derivatives of it were grown and membranes prepared and analyzed as for *a*. Dissociation of membrane samples was at 30 °C. Lane 1, P678-54 ColV; lane 2, mutant 32; lane 3, mutant 2.

and the bacteriolytic enzyme lysozyme. The presence of ColV increased the sensitivity of test strains to copper ions (table 1). The effect of ColV was not a large one but was consistently observed and also occurred for the other tested *E. coli* strains e.g. with 50 μg/ml copper ions, the percentage of organisms forming colonies (compared to control plates without copper) was for strain C600 48%; C600 ColV$^+$ 25%; PC0479 50%; PC0479 ColV$^+$ 31%. The presence of ColV did not appreciably alter bacterial sensitivity to the hydrophilic antibiotic ampicillin (table 1). Ampicillin uses the OmpF and OmpC porins for entry and so this unchanged sensitivity supports the view (see above) that these porins are essentially unaffected by the presence of ColV in these strains.

ColV$^+$ strains were markedly more sensitive than were Col$^-$ ones to the hydrophobic antibiotic erythromycin (table 1). This sensitivity was originally found for ColV$^+$ derivatives of 1829 and P678-54 but was also observed for KH262 ColV$^+$ and PC0479 ColV$^+$. The effect of erythromycin was studied further in liquid medium. The ColV$^+$ derivative of P678-54 was markedly more sensitive than was the Col$^-$ parent (fig. 2a) but resistance was restored in a derivative cured of the plasmid (fig. 2a). The ColV plasmid had essentially no effect on the sensitivity of strain P678-54 to novobiocin and only a small effect on the sensitivity of strain 1829 (table 1). Strain C600 resembled P678-54; on NA plus novobiocin at 20 μg/ml, the percentages of organisms forming colonies (compared to growth on NA) were as follows: C600, 93%; C600 ColV, 98%. Table 1 shows that ColV markedly sensitized 1829 to SDS but had only a slight effect on the sensitivity of P678-54 to this agent. In contrast, both ColV$^+$ derivatives were more sensitive than their parents to sodium deoxycholate (DOC). This sensitivity was noted on NA but the results on solid media were not reproducible probably because DOC at high concentrations tends to precipitate from NA. Accordingly the effects of DOC were examined in nutrient broth. At 3%, DOC doubled the mean generation time of P678-54 ColV in broth at 37 °C without affecting that of P678-54. A strain cured of ColV behaved like P678-54. Strain 1829 ColV was also more sensitive in broth to growth inhibition by DOC than was 1829 itself. ColV also sensitized strains KH262 and PC0479 to growth inhibition by DOC. The plasmid also affected the response of *E. coli* P678-54 and 1829 to lysozyme. Most strains of *E. coli* including P678-54 and 1829 are insensitive to lysozyme unless the chelating agent EDTA is also present. In contrast, P678-54 ColV and 1829 ColV were lyzed by lysozyme in the absence of EDTA. The data for strain P678-54 is shown in figure 2b.

The chemical agents to which the ColV$^+$ strains became sensitive, namely sodium deoxycholate, erythromycin and lysozyme (and SDS in strain 1829 ColV$^+$) normally affect enterobacteria relatively little because they fail to penetrate the lipopolysaccharide (LPS)-containing outer leaflet of the outer membrane. Sensitivity of enterobacteria to hydrophobic agents results from treatments or mutations which allow the establishment of outer membrane phospholipid bilayer regions (permeable to hydrophobic agents). These can result from changes in LPS

Table 2. Inhibitor sensitivity in mutant ColV$^+$ strains

Strain	Level of ColV-specified components		Inhibitor sensitivity		
	Colicin (zone size, mm)	VmpA protein level	Phage Mel	Deoxycholate	Erythromycin
P678-54	0	Absent	S	R	R
P678-54 ColV	5.0	Normal	R	S	S
mutant 2	1.0	Low	R	S	S
mutant 32	5.5	Normal	S	S	S

Mutant 2 and 32 were isolated from P678-54 ColV as resistant to phage MS2. These mutants together with P678-54 and P678-54 ColV were tested for colicin production by overlaying chloroform-killed colonies with *E. coli* C600 and measuring the diameter of the inhibitory zones after incubation overnight at 37 °C. The stated levels of the VmpA protein derive from observations of PAGE gels dissociated at 30 °C (fig. 1a, b). Figure 1a (lane 2) shows the absence of VmpA protein from P678-54. Figure 1b shows that the level in mutant 32 (lane 2) is similar to that in P678-54 ColV (lane 1); this level is designated normal as it is that present in the normal ColV$^+$ strain. The level of VmpA protein in mutant 2 is designated low as it is markedly reduced compared to P678-54 ColV (fig. 1b, lanes 1 and 3). Mutant strains were designated sensitive (S) to deoxycholate and/or erythromycin if they resembled P678-54 ColV in response to these agents on solid and liquid medium; the designation resistant (R) was used if they resembled P678-54. Experiments were repeated 3 times with consistent results.

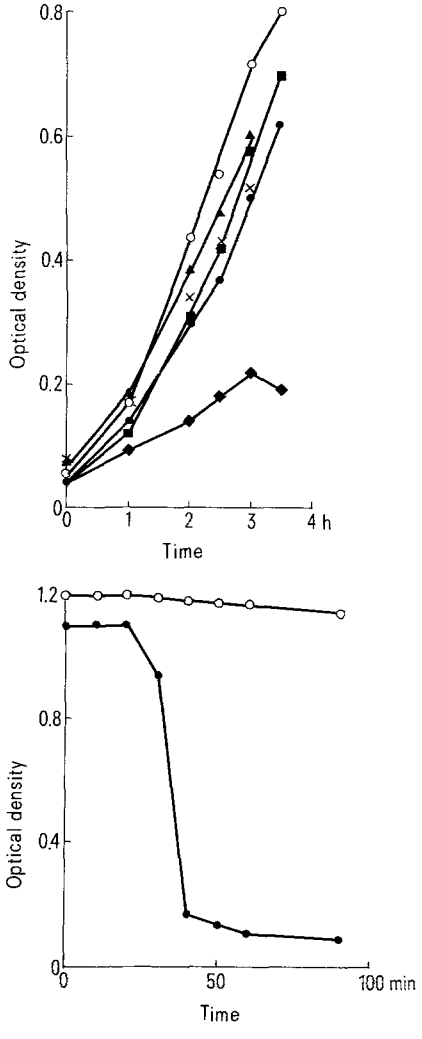

Figure 2. *a* Growth inhibitory effect of erythromycin on ColV$^+$ and ColV$^-$ strains. Organisms of P678-54, P678-54 ColV, and a cured derivative of the latter lacking ColV were grown to exponential phase in nutrient broth at 37 °C and then diluted in duplicate into fresh broth to O.D. circa 0.05 (time zero). Incubation was continued at 37 °C with shaking, optical density readings being taken at the stated intervals. Erythromycin (20 μg/ml) was added to the stated incubations at time zero. O, P678-54; ●, P678-54 + erythromycin. ■, P678-54 ColV; ♦, P678-54 ColV + erythromycin. ▲, cured strain; X, cured strain + erythromycin. *b* Effect of lysozyme on a ColV$^+$ strain of *E. coli*. Organims of P678-54 and P678-54 ColV were grown to exponential phase in broth at 37 °C with shaking, harvested, washed in 0.1 M Tris-buffer pH 8.0 resuspended in the same buffer to O.D. 1.1–1.2 and incubated at 37 °C static with lysozyme (150 μg/ml) O, P678-54; ●, P678-54 ColV.

structure[2], reduction in porin level or from the presence of plasmid-specified major envelope components. The possibility that LPS structure is altered in ColV$^+$ strains has not been studied so far, but the other 2 possibilities have been considered with respect to sensitivity to deoxycholate and erythromycin. Firstly it seems unlikely that the increased sensitivity of ColV$^+$ strains to these 2 hydrophobic agents results from a reduction in porin levels because membrane analysis (fig. 1a), studies of ampicillin sensitivity (table 1) and tests of sensitivity to phages T4 and Tula (table 1) suggest that porin levels are normal in the tested ColV$^+$ strains. It seems likely that sensitivity to deoxycholate and erythromycin is not dependent on the presence of the ColV-specified colicin or VmpA protein because some mutants derived from P678-54 ColV form reduced levels of colicin and VmpA protein but are still sensitive to inhibition by the 2 agents (mutant 2, table 2). The presence of ColV-encoded transfer components may be responsible for sensitivity to deoxycholate because repression of such transfer properties (by the *fi*$^+$ plasmid ColB-K98) led to complete DOC resistance.

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The role of zinc in regulating tabtoxin production[1]

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Summary. The phytotoxin, tabtoxinine-β-lactam, is produced by several *Pseudomonas syringae* pathovars if adequate Zn is available, otherwise its biologically inactive form, tabtoxin, is produced. The Zn is required for the action of a peptidase which cleaves tabtoxin, releasing the toxic β-lactam.
Key words. Pseudomonas syringae; phytotoxin; zinc, regulating tabtoxin; tabtoxin; tabtoxin-β-lactam.

The phytopathogenic bacterium *Pseudomonas syringae* pv. *tabaci* and several closely related pathovars are known to produce tabtoxin, a dipeptide which is responsible for the chlorotic leaf symptom associated with infection by these pathogens[2]. Tabtoxin per se is not toxic but when hydrolyzed by either plant or bacterial enzymes produces serine, threonine and the toxic entity, tabtoxinine-β-lactam[3]. This compound irreversibly inactivates glutamine synthetase[4].

It has been known for many years that log-phase cultures of pv. *tabaci* synthesize only tabtoxin. Recently, we found that the addition of 20% intercellular fluid from leaves would induce tabtoxinine-β-lactam, rather than tabtoxin, production[5]. At lower levels of fluid ($< 5\%$) a mixture of tabtoxin and tabtoxinine-β-lactam was produced. This communication reports on an examination of the fluid to determine what factor, or factors, is responsible for regulating which compound is produced.

Materials and methods. Initially, all experiments were done using *P. syringae* pv. *tabaci,* isolate 113. Once conclusive results had been obtained, additional isolates were tested to ascertain if they behaved similarly. The bacteria were grown with agitation in Woolley's medium at 23 °C for three days. The medium was supplemented with 20% filter-sterilized intercellular fluid (v/v), or various treatments of the fluid equivalent to the same concentration. The fluid was obtained by Klement's method[6] from mature leaves of tobacco, *Nicotiana tabacum* 'Havana 38'.

The culture filtrates were quantitatively assayed for tabtoxin and/or tabtoxinine-β-lactam content by running 0.2 ml aliquots of the crude filtrates on an amino acid analyzer (Beckman 118 BL containing W-2 resin).

Results and discussion. Results from the first test showed that the regulating factor in the fluid was heat stable (121 °C for 30 min), thus eliminating the presence of hydrolytic enzymes in the fluid as a possible explanation. This was further substantiated when ultra filtration (YCO5, Amicon Corp.) showed that all the activity was associated with a fraction of mol.wt $\lesssim 500$. Absorption of this fraction onto Chelex-100 (BioRad) showed that the active component(s) was cationic. When ashed (870 °C for 4 h), the low-molecular-weight fraction still retained complete activity.

Next, the medium was supplemented singly with various metal salts at 25 µmolar (i.e., Ca, Mg, Zn. Cu, Co, Ni, Sr, Fe, Mn and Cd). Of these compounds only the one containing Zn was active. Atomic absorption spectrometry of the intercellular fluid showed that its Zn concentration was 2.5 µmolar (analysis was done on a Perkin Elmer 603 with a D_2 arc background corrector, using an air-acetylene flame at 213.9 nm and zinc detection limits of 18 ppb[7]). Supplementation of the medium to this Zn concentration induced tabtoxinine-β-lactam produc-