Divalent Cations in the Envelope of a Psychrophilic *Achromobacter*

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SUMMARY

The function of Ca^{2+} in a psychrophilic *Achromobacter*, previously found to bind large amounts of these ions to its envelope, has been studied. Bacteria suspended in media of low ionic content showed decreases in wet weight, dry weight and growth capacity, and increases in light scattering and in the release of u.v.-absorbing substances into the medium. The permeability barrier to Ca^{2+} was also damaged, and there was a release of radioactivity from bacteria labelled with $^{45}\text{Ca}^{2+}$. These events occurred at the optimum growth temperature, and took place at increased rates at higher temperatures. Damage was prevented to about the same extent by 0.1 mM- CaCl_2 , BaCl_2 or MgCl_2 and by 10 mM- NaCl , KCl or LiCl . Ion competition experiments showed that Ca^{2+} was preferentially taken up and retained in comparison with Ba^{2+} , Mg^{2+} and Na^+ , in that order. Isolated envelopes gave similar results. The dry weight of envelopes was reduced by 35% when they were suspended in water at 40 °C.

It is clear that the function of certain envelope components in *Achromobacter* is highly dependent on divalent cations; and that both the integrity of the permeability barrier and the stability of the envelope are affected at low ion concentrations.

INTRODUCTION

Divalent cations have been assigned several important roles in the envelopes of Gram-negative bacteria. By joining adjacent negative groups they affect the structure and function of the various envelope layers. In the outer membrane of the envelope, divalent cations may provide association between some lipopolysaccharide molecules (Nikaido, 1973; Leive, 1974). Crosslinking by Mg^{2+} in the peptidoglycan molecule may contribute to the mechanical strength of this component (Rayman & MacLeod, 1975); and the integrity of lipid-protein membranes depends on divalent cations (Brown, 1964; Hendricks, 1964; Reynolds, 1972).

An earlier publication (Ledebø, 1974) described the uptake of Ca^{2+} by a psychrophilic *Achromobacter* strain at sites mainly outside the permeability barrier, i.e. in the envelope. The amount of Ca^{2+} taken up greatly exceeded that reported for other Gram-negative bacteria such as *Rhizobium trifolii* (Humphrey & Vincent, 1962) and *Pseudomonas aeruginosa* (Eagon, Simmons & Carson, 1965). Thus, studies of *Achromobacter* may throw additional light on the function of divalent cations in Gram-negative envelopes. The object of this investigation was to determine the effect of low cation concentrations on the integrity of the *Achromobacter*; and to study the cation-binding specificity of isolated bacterial envelopes.

METHODS

Preparation of bacterial suspensions. *Achromobacter* no. 102, a psychrophilic laboratory strain (Ledebø & Ljunger, 1973), was grown in 1% Bacto-peptone (Difco) at 20 °C in a shaking water bath until the bacteria reached the mid-exponential phase of growth (0.11 mg

dry wt/ml). Bacteria were harvested by centrifuging, washed once in deionized water, suspended in water and kept at 0 °C until the start of the experiment. All suspensions were prepared on the day of use.

Preparation of envelopes. Bacteria were harvested during the exponential phase of growth, one generation later than indicated above. They were washed once in 1 mM-CaCl₂ and disintegrated in a Ribi Cell Fractionator (Sorvall) at 25000 lbf in⁻². To separate intact cells, the treated suspension was centrifuged at 2700 g for 20 min at 2 °C. The supernatant was further centrifuged at 25000 g for 20 min to sediment the envelopes. These were washed three times in 1 mM-CaCl₂ and finally suspended in 1 mM-CaCl₂ to about 6 mg dry wt/ml. The suspension was divided into portions and frozen.

Radioactivity exposure and measurement. The radioisotope ⁴⁵Ca²⁺ (from The Radiochemical Centre, Amersham, Buckinghamshire) was added to an aqueous solution of peptone or salt; and the resulting radioactive solution was mixed with either a volume of the bacterial suspension, a heat-treated bacterial pellet, or a sample of envelopes. Samples corresponding to about 20 mg dry cells or 3 mg dry envelopes were normally used. Exposure to the isotope took place at room temperature (approximately 20 °C) for 1 h. The cells or envelopes were then separated by centrifuging at 2 °C, the supernatant was poured off rapidly, and any remaining fluid blotted away from the interior of the centrifuge tube. The entire pellet, which contained only the envelopes or cells and unavoidable extracellular fluid, was used for subsequent experiments or for radioactivity measurements.

The pellet was suspended in 0.5 ml 0.5 % peptone and the suspension mixed with scintillation liquid (10 ml) and transferred to a vial. The scintillation liquid contained 100 g naphthalene and 8 g butyl-PBD [2-(4'-t-butylphenyl)-5-(4''-biphenyl)-1,3,4-oxadiazole] dissolved in 1 l dioxan: all chemicals were scintillation grade. To eliminate chemiluminescence, vials were kept in the dark for 1 h before the radioactivity was counted in a liquid scintillation counter (β -Mate II, Beckman Instruments) for three 1 min periods. The counting efficiency was about 75 % as determined by the internal standard method. The radioactivity of the supernatant liquid was measured on 0.5 ml samples.

Measurement of light scattering. An EEL nephelometer head on an EEL Unigalvo Type 20 was used for recording the light scattering of bacterial suspensions. The values were related to a predetermined standard, the assigned unit being the same for all experiments.

Weight determination. After removing the residual medium, the wet weight of the cell pellet was determined. Dry weights were determined after heating overnight at 105 °C.

RESULTS

When washed *Achromobacter* organisms were suspended in water, the light-scattering capacity of the suspension remained constant at 0 °C but increased with increase in temperature (Fig. 1). Yet if calcium ions were added to the suspending medium there was no such increase in light scattering with temperature (Fig. 7*b*). Increased light scattering which could not be attributed to increases in bacterial numbers may be due to volume reductions of the cells (Koch, 1961). Thus, the relationship between the wet weight and the nephelometer value of cells suspended in water and treated at 25 and 35 °C was studied (Fig. 2). The increase in light scattering correlated closely with decrease in wet weight. The dry weight was practically unchanged at 25 °C and decreased by only about 15 % at 35 °C. Thus, the decrease in wet weight of the organisms was mainly due to loss of cell water.

The leakage of intracellular components was studied by measuring the u.v.-absorption of the surrounding medium (Fig. 3). Leakage was detected when the cells were suspended

Fig. 1

Fig. 2

Fig. 1. The light-scattering capacity of *Achromobacter* cells suspended in water at 0 °C (■), 10 °C (□), 25 °C (●) and 40 °C (○). Concentrated cell suspension in water (0.5 ml) was added to temperature-equilibrated test tubes containing 9.5 ml water and the nephelometer values recorded.

Fig. 2. Changes of wet weight (●) and light scattering (○) of *Achromobacter* suspended in water at 25 and 35 °C. A bacterial suspension in water (1 mg dry wt/ml) was treated at the temperatures stated. Samples (10 ml) were centrifuged at intervals and the pellets weighed. Other samples (1.0 ml) were diluted in 9 ml water and the light scattering measured. The values of weight and light scattering at 35 °C were adjusted so that they coincided at zero time and after 3 h and corresponding adjustments were made to all other values.

Fig. 3

Fig. 4

Fig. 3. Release of u.v.-absorbing substances from *Achromobacter* in water (●) and in 10 mM- CaCl_2 (○) at 25 °C (dashed lines) and 35 °C (full lines). A suspension (1 mg dry wt/ml) was treated as indicated and the u.v.-absorption of the supernatant was measured at 260 nm.

Fig. 4. Uptake of Ca^{2+} by *Achromobacter* after treatment in water (●) and in 10 mM- CaCl_2 (○) at 35 °C for various times, followed by exposure to 10 mM- $^{45}\text{CaCl}_2$.

in water at 25 and 35 °C, but was insignificant in 10 mM- CaCl_2 at these temperatures. No lysis was seen microscopically.

When the permeability barrier of *Achromobacter* is destroyed with, for example, toluene, the uptake of calcium ions increases greatly (Ledebø, 1974). This was interpreted as indi-

Fig. 5. Growth of *Achromobacter* in peptone at 25 °C (a). After 2 h exponential growth, the bacteria were separated from the medium, washed, suspended in water (b) or 1 mM- CaCl_2 (c), and incubation was continued. After another 2 h the bacteria were resuspended in their original growth medium and reincubated.

cating that in untreated organisms, calcium ions are bound mainly to the envelope, whereas in leaky cells the ions also gain access to the cell interior. On this basis, the protective effect of calcium ions on the permeability barrier of *Achromobacter* was examined. The calcium uptake of bacteria which had been treated in water at 35 °C increased with increasing duration of the heat treatment (Fig. 4). However, when the same heat treatment was done in 10 mM- Ca^{2+} , the uptake of Ca^{2+} remained constant, indicating an unaffected permeability barrier.

These observations suggested that water treatment at 35 °C greatly damaged the *Achromobacter*. In 1% peptone, 35 °C is above the maximum growth temperature and reduces survival to about 30% after 2 h. The optimum growth temperature is approximately 25 °C and growth capacity after treatment in water and 1 mM- CaCl_2 at this temperature is shown in Fig. 5. After suspension in 1 mM- CaCl_2 for 2 h at 25 °C, the bacteria resumed exponential growth immediately, whereas suspension in water for the same time markedly reduced the growth rate during the following 3 h.

It was shown in several experiments that Ca^{2+} in the suspending medium prevented certain types of damage to *Achromobacter*. The large capacity of envelopes for binding Ca^{2+} is probably responsible for the protective effect of the ionic solutions. However, even before suspension in Ca^{2+} solution, the cells contained Ca^{2+} taken up during growth, since the growth medium contained 0.05 to 0.1 mM- Ca^{2+} . The following experiment demonstrated what happened to the Ca^{2+} bound to the envelope during growth, when the cells were suspended in water.

The cells were labelled with radioactive calcium in 0.5% peptone so that the Ca^{2+} concentration did not exceed that of the normal growth medium. During treatment in water at 25 or 40 °C, the cells gradually released radioactivity into the medium (Fig. 6). At 40 °C a maximum of about 35% was reached within 2 h and was still maintained after 24 h. At 25 °C, the release was slower, being about 25% after 24 h. At 0 °C, the release of radio-

Fig. 6. Release of radioactivity from *Achromobacter* cells (●) and envelopes (○). Cells and envelopes were labelled with $^{45}\text{CaCl}_2$ in peptone, washed, and suspended in water at 25 and 40 °C at densities of 1 mg dry cells/ml or 0.6 mg dry envelopes/ml. Samples were taken at intervals and the radioactivity of the pellet and of the supernatant measured.

Fig. 7. Light scattering by *Achromobacter* suspended in solutions of (a) NaCl and (b) CaCl_2 at room temperature. Concentrations of solutions were: 0.1 mM (□); 1 mM (●); 10 mM (○); water only (■).

activity was negligible. The released radioactivity could be filtered through Millipore filters with a pore diameter of $0.22\ \mu\text{m}$ and dialysed to the same extent as Ca^{2+} in aqueous solution. This indicated that the released radioactive material consisted of very small moieties, perhaps the calcium ions alone. However, dry weight studies on the treated cells showed reductions of up to 15% after 2 h at 40 °C. The loss of internal solutes observed earlier could not account for such large values, so it was assumed that envelope components were released at the same time as the Ca^{2+} . This was confirmed by repeating the experiment with isolated envelopes, which also released radioactivity when suspended in water at 40 °C (Fig. 6). The maximum release of radioactivity, about 17%, was reached within 2 h and was only slightly higher after 24 h at 40 °C. The dry weight of the envelopes was reduced by about 15% after 2 h at 40 °C but continued to decrease and, after 24 h, about 35% of the envelope dry matter had been released into the medium.

In the above experiments, only Ca^{2+} had been used. However, other inorganic cations

Table 1. *Exchange between Ca^{2+} bound to intact Achromobacter and to envelopes and other ions in the medium*

Samples were first exposed to 1 mM- $^{45}\text{CaCl}_2$, then centrifuged and the pellet suspended in the various ionic solutions (1 mM). Exchange of ions was calculated from the released radioactivity.

Suspending medium	Exchange (%)* with Ca^{2+}	
	Bacteria	Envelopes
CaCl_2	100.0	100.0
BaCl_2	78.0	94.9
MgCl_2	73.6	62.9
NaCl	31.7	26.1

* Standard error, ± 0.5 .

Table 2. *Competitive binding of Ca^{2+} and other ions to intact cells and envelopes of Achromobacter*

The cells and envelopes were exposed to the ionic solutions for 1 h at room temperature and the uptake of $^{45}\text{Ca}^{2+}$ was measured. Solutions were 0.5 mM except where indicated.

Suspending medium	Radioactivity (%)*	
	Bacteria	Envelopes
$^{45}\text{CaCl}_2$ (1.0 mM)	100.0	100.0
$^{45}\text{CaCl}_2 + ^{40}\text{CaCl}_2$	50.5	48.3
$^{45}\text{CaCl}_2 + \text{BaCl}_2$	53.8	49.7
$^{45}\text{CaCl}_2 + \text{MgCl}_2$	57.7	55.6
$^{45}\text{CaCl}_2 + \text{NaCl}$	81.0	76.7

* Standard error, ± 0.4 .

might be expected to serve a similar function and so various ionic solutions were examined for the capacity to prevent increases in light scattering of a bacterial suspension. Figure 7 shows the light scattering of organisms suspended in NaCl and CaCl_2 . Concentrations of 10 mM were needed before the monovalent ions Na^+ , K^+ and Li^+ fully suppressed the increase of light scattering at room temperature; whereas the divalent ions Ca^{2+} , Mg^{2+} and Ba^{2+} prevented any such increase even at the lowest concentration studied (0.1 mM).

To examine the specificity of ion binding, the exchange and competition between calcium ions and ions of barium, magnesium and sodium was studied. Both whole cells and isolated envelopes were used and were found to take up (from 1 mM- $^{45}\text{Ca}^{2+}$) 0.13 and 0.52 $\mu\text{mol Ca}^{2+}$ /mg dry wt, respectively. The Ca^{2+} taken up by the cells could be completely exchanged with Ca^{2+} in the medium during a subsequent suspension (Table 1). Ba^{2+} and Mg^{2+} exchanged with Ca^{2+} to a lesser extent, and Na^+ the least. Ion exchange with envelopes was similar. When the bacteria and the envelopes were suspended in a mixture of two cations at equimolar concentration, Ca^{2+} was taken up preferentially before Ba^{2+} and Mg^{2+} , in that order, and to a large extent before Na^+ (Table 2).

DISCUSSION

When suspended in water or media of low ionic content, *Achromobacter* underwent several changes which indicated damage to the envelope. Clearly, the permeability barrier was affected, since intracellular components were released, and previously excluded Ca^{2+} was admitted into the interior of the cells. Although the main permeability barrier of bacteria

is the cytoplasmic membrane, the outer membrane of Gram-negative organisms has some barrier function (Leive, 1974). Other manifestations of damage to *Achromobacter* when suspended in water included a much reduced wet weight which, assuming that no cell lysis occurred, is interpreted as arising from a reduced cell volume. This change could also be observed as an increased light-scattering capacity of the suspension. Large volume reductions are known to occur when bacteria become plasmolysed or when the wall of Gram-positive bacteria is exposed to salt solutions (Marquis, 1968). Neither cause could be responsible for the present volume reduction of the *Achromobacter* cells since they were suspended in pure water. However, a component involved in the mechanical stability of the envelope is probably affected by the treatment, since the large volume reduction observed is not compatible with a rigid envelope structure.

Thus, both the barrier function and the stability function of the envelope were impaired when the cells were suspended in water at 25 to 40 °C. The nature of the damage to the envelope is suggested by the way it can be prevented. Ca^{2+} in the medium was effective in keeping the permeability barrier intact and also maintained the original light-scattering capacity. Since the presence of Ca^{2+} preserves the integrity of the envelope, it can be assumed that the absence of this ion causes the damage. Furthermore Ca^{2+} was released from the envelope into the medium under the damaging conditions. This release was as dependent on temperature as the other damage observed. Bacteria suspended in water at 40 °C lost about 35% of their bound Ca^{2+} as well as dry matter, part of which originated from the envelope. The release of envelope components continued after the maximum release of ions had been reached. It appeared that the release of Ca^{2+} and envelope components that took place during the first two or three hours of water treatment at 40 °C was the primary damaging event.

Although Ca^{2+} is assumed to be bound primarily to the envelope, the release of $^{45}\text{Ca}^{2+}$ from whole cells and isolated envelopes is not fully comparable. It seems that a smaller part of the radioactivity is released from the isolated envelopes. This may be because the envelopes have lost components with high calcium-binding capacity during the isolation procedure. Such a loss is also indicated by the relationship between calcium uptake in whole cells and in isolated envelopes. The dry weight of the Gram-negative envelope is usually considered to be about 15% of the dry weight of the cell (Salton, 1964). If this is applied to *Achromobacter*, the calcium-binding capacity of the envelopes is only 60% of that of the whole cells according to the results for uptake in 1 mM- CaCl_2 .

The ions and the envelope components in *Achromobacter* are readily released by suspension in water without addition of a chelating agent. In other terrestrial species, ethylenediaminetetraacetate has to be added to achieve a similar release. In *Pseudomonas aeruginosa* (Cox & Eagon, 1968), *Escherichia coli* (Leive, 1968) and *Salmonella enteritidis* (Chipley & Edwards, 1972), removal of divalent cations from the envelope has been shown to release lipopolysaccharide and to alter the permeability. Lipopolysaccharide is located in the outer membrane of the Gram-negative envelope, where it seems to be involved in maintaining the envelope's passive barrier function (Nikaido, 1973; Costerton, Ingram & Cheng, 1974; Leive, 1974). The stability of the envelope may also be dependent on the outer membrane. Leive (1968) and Burman, Nordström & Bloom (1972) treated *E. coli* with penicillin under various conditions and concluded independently that the peptidoglycan is not solely responsible for the mechanical stability of the envelope: the state of the outer membrane is also important. The outer membrane is associated with the peptidoglycan through covalent links (Braun & Sieglin, 1970) and with the cytoplasmic membrane through numerous points of adhesion (Bayer, 1968). The latter may exist through ionic interactions (Costerton *et al.*,

1974). Thus, it seems possible that any damage in the outer membrane may affect the inner envelope layers as well.

In conclusion, divalent cations in *Achromobacter* envelopes seem to have the function of holding in position certain envelope components which are directly or indirectly involved in maintaining the integrity of the permeability barrier and the mechanical stability of the envelope. These observations agree well with the models of the outer membrane presented by Nikaido (1973) and Leive (1974) in which divalent cations hold part of the lipopolysaccharide molecules in place. Both Ca^{2+} and Mg^{2+} may be important for this function, the former being more active in the *Achromobacter* envelope.

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