Attenuation by Divalent Cations of the Effect of the Phytoalexin Rishitin on *Erwinia carotovora* var. *atroseptica*

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Rishitin at 300 $\mu\text{g ml}^{-1}$ rapidly decreased oxygen uptake by *Erwinia carotovora* var. *atroseptica* suspended in 0.1% (w/v) peptone water. Variation in the composition of the suspending medium affected sensitivity to rishitin, with Mg^{2+} and, to a lesser extent, Ca^{2+} decreasing bacterial sensitivity. Addition of 100 μg rishitin ml^{-1} affected cell membrane permeability causing an increase in conductivity of the suspending medium. Inhibition of oxygen uptake by the cationic surfactant hyamine 2389 or by sodium lauryl sulphate was also alleviated by the addition of Mg^{2+} suggesting that rishitin may act directly on cell membranes of bacteria, possibly in a manner similar to a cationic surfactant or a membrane-active antibiotic. The results also suggest that the sensitivity of *E. atroseptica* to rishitin in potato tubers may be affected by variation in their Mg^{2+} or Ca^{2+} content. Phaseollin did not inhibit respiration of *E. atroseptica*.

INTRODUCTION

Potato tubers incubated in air and therefore resistant to rotting produce the phytoalexin rishitin when inoculated with the Gram-negative soft rot bacterium *Erwinia carotovora* var. *atroseptica* (*E. atroseptica*). Tubers incubated under low oxygen conditions, and consequently more susceptible to rotting, produce little or no rishitin (Lyon, 1972*a, b*). These observations, together with the demonstration that rishitin affects the viability of *E. atroseptica* *in vitro* (Lyon & Bayliss, 1975), led to the conclusion that rishitin may play a role in the resistance of tubers to *E. atroseptica*.

Lyon & Bayliss (1975) demonstrated a difference in the sensitivity to rishitin of *E. atroseptica* when bacteria were incubated in two different media, viz. 0.1% (w/v) peptone water and a defined growth medium (GA). The present work was initiated to determine which constituent(s) of the defined medium GA affected this difference and so to suggest a possible mode of action of rishitin. The effect of treatments on the sensitivity of *E. atroseptica* to rishitin was assessed by measuring decreases in oxygen uptake as an indicator of physiological damage.

METHODS

Bacteria. *Erwinia carotovora* var. *atroseptica* isolate 17, obtained by M. C. M. Pérombelon at this Institute from potato stem tissue showing blackleg symptoms, was grown at 23 °C in shaken cultures of nutrient broth [containing (g l^{-1}): beef extract, 1; yeast extract, 2; Bacto-peptone, 5; NaCl, 5; pH 7.4]. Cultures (24 h) were centrifuged and washed twice with 0.1% (w/v) peptone water pH 7.0 (Lyon & Bayliss, 1975) before being resuspended in peptone water for respiration experiments. For conductivity experiments, cultures were washed twice with, and then resuspended in, 0.3 M-sucrose.

Phytoalexins. Chromatographically pure rishitin was extracted from potato tubers inoculated with *E. atroseptica* using the method described by Lyon (1972*a*). Crystalline phaseollin was extracted from *Phaseolus vulgaris* pods treated with 3 mM- CuCl_2 using methods described by Bailey & Burden (1973).

Fig. 1. Oxygen uptake by a 1 ml suspension of *E. atroseptica* in 0.1 % peptone water after addition of: ○, 100 µg rishitin; ■, 200 µg rishitin; ▲, 300 µg rishitin; ▼, 300 µg phaseollin; ●, 30 µl ethanol. Rishitin and phaseollin were added as ethanolic solutions (10 mg ml⁻¹). Oxygen uptake is expressed as a percentage of the initial rate, before addition of phytoalexin.

Oxygen uptake. Measurements were made at 25 °C using an oxygen electrode (Rank Bros, Cambridge). The concentration of bacteria was adjusted so that the addition of 5 µl bacterial suspension to 1 ml medium gave a rate of oxygen uptake of approximately 6.5 nmol min⁻¹ (approximately 3×10^8 bacteria ml⁻¹ in peptone water). Calculations were based on the assumption that air-saturated water contained 260 nmol O₂ ml⁻¹ at 25 °C (Estabrook, 1967). After 5 min, when a steady rate of oxygen uptake was attained (initial rate), test compounds were injected into the cell and changes in oxygen uptake were continuously recorded, the rates being expressed as a percentage of the initial rate. The phytoalexins were added as ethanolic solutions (10 mg ml⁻¹) as required.

Conductivity measurements. The conductivity of a 9 ml suspension of approximately 10^9 cells ml⁻¹ in 0.3 M-sucrose solution was measured using an Electronic Switchgear (London) type MC1 conductivity measuring bridge with cell type CB/10.

RESULTS

Effect of rishitin and phaseollin on respiration

Rishitin at 300 µg ml⁻¹ rapidly inhibited oxygen uptake by *E. atroseptica* in 0.1 % peptone water and slightly reduced the rate at 100 µg ml⁻¹ (Fig. 1). The effect of rishitin over incubation periods longer than 30 min was not studied. Phaseollin did not inhibit oxygen uptake but caused an immediate temporary increase followed by a return to a rate similar to that of the control [3 % (v/v) ethanol].

Variation in the composition of the medium in which the bacteria were resuspended affected their sensitivity to rishitin (Fig. 2). The rate of oxygen uptake by bacteria suspended in 0.1 % peptone water was affected more than that of suspensions in a mixture of KH₂PO₄, NH₄Cl and MgSO₄ [the salts used by Lyon & Bayliss (1975) in their defined medium GA]. The addition to 0.1 % peptone water of 0.81 mM-MgSO₄ slightly increased the ability of the bacteria to tolerate the rishitin (Fig. 2) and the protective action of a higher concentration (2.0 mM) against 200 µg rishitin ml⁻¹ is shown in Fig. 3. Equimolar concentrations of Na₂SO₄ and CaSO₄ were also tested for their protective ability. The results show that the cation was the active principle and that Mg²⁺ was more effective than Ca²⁺ in conferring tolerance. Measurements of the effects on respiration of rishitin at a range of concentrations showed that, after 20 min, 50 % inhibition occurred with 175 µg rishitin ml⁻¹ in the absence of Mg²⁺ whereas 271 µg rishitin ml⁻¹ was required to cause 50 % inhibition in the presence of 2.0 mM-Mg²⁺.

Effect of surfactants on respiration

An anionic surfactant, Triton GR-5 [60 % dioctyl sodium sulphosuccinate in propan-2-ol/water (1:1, v/v)], and a non-ionic surfactant, Triton X-100 (iso-octylphenoxypolyethoxyethanol), did not affect oxygen uptake at final concentrations of 0.1 % (v/v). However,

Fig. 2. Effect of 300 μg rishitin ml^{-1} on oxygen uptake by *E. atroseptica* suspended in: ●, salts solution as in defined medium GA [Lyon & Bayliss, 1975; containing (g l^{-1}): KH_2PO_4 , 2; NH_4Cl , 1; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2; pH 7.0]; ■, 0.1 % peptone water pH 7.15; ▲, 0.1 % peptone water plus 0.81 mM- MgSO_4 . Oxygen uptake is expressed as a percentage of the initial rate, before addition of rishitin (as a 10 mg ml^{-1} ethanolic solution).

Fig. 3. Effect of divalent cations on the sensitivity of *E. atroseptica* to rishitin. Oxygen uptake (percentage of the initial rate) was determined after addition of 200 μg rishitin ml^{-1} to *E. atroseptica* suspended in: ○, 0.1 % peptone water; ■, 0.1 % peptone water plus 2 mM- MgSO_4 ; ▲, 0.1 % peptone water plus 2 mM- Na_2SO_4 ; ▼, 0.1 % peptone water plus 2 mM- CaSO_4 ; or ●, after addition of 20 μl ethanol to bacterial suspension in 0.1 % peptone water.

the cationic surfactant Hyamine 2389 (50% methyl dodecyl benzyl trimethyl ammonium chloride and methyl dodecyl xylene bis trimethyl ammonium chloride in water) did inhibit oxygen uptake by *E. atroseptica* resuspended in peptone water, and, as with rishitin, this effect was alleviated by the addition of Mg^{2+} . In peptone water, 17.5 (S.E.M. 1.8) μg Hyamine 2389 ml^{-1} caused a 50% inhibition of respiration after 15 min whereas 51.0 (S.E.M. 4.4) μg ml^{-1} was required to cause 50% inhibition following the addition of 2.0 mM- Mg^{2+} .

Addition of sodium lauryl sulphate to *E. atroseptica* in peptone water also inhibited respiration, again to a lesser extent in the presence of Mg^{2+} (Fig. 4).

Effect of cyanide on respiration

Oxygen uptake was completely inhibited 20 min after adding 10 μl 10 mM- NaCN to a 1 ml suspension of *E. atroseptica*, although the bacteria remained viable and motile.

Conductivity

Addition of 100 μg rishitin ml^{-1} to *E. atroseptica* in 0.3 M-sucrose caused an increase in conductivity (Fig. 5). Hyamine 2389 at 50 μg ml^{-1} caused a more rapid loss of electro-

Fig. 4. Effect of addition of 20 μ l 5 % sodium lauryl sulphate on oxygen uptake by *E. atroseptica* suspended in 1 ml 0.1 % peptone water (○) or 0.1 % peptone water plus 2 mM-MgSO₄ (■).

Fig. 5. Conductivity of a 9 ml suspension of approx. 10^9 *E. atroseptica* ml⁻¹ in 0.3 M-sucrose before and after the addition at 15 min (arrowed) of: ■, 100 μ g rishitin ml⁻¹ (added as a 10 mg ml⁻¹ ethanolic solution); ○, 50 μ g Hyamine 2389 ml⁻¹ (added as a 10 %, v/v, aqueous solution); ●, 10 μ l ethanol ml⁻¹.

lytes; this reached a maximum after 15 min when all cells had lysed. In the absence of bacteria the same amount of Hyamine increased the conductivity of the medium by 4.15 μ S.

DISCUSSION

The biological activity of rishitin assessed as inhibition of O₂ uptake agreed with data published by Lyon & Bayliss (1975) which showed that at 360 μ g ml⁻¹ rishitin caused complete loss of viability and at 106 μ g ml⁻¹, a 50 % reduction after 4 h. The inability of phaseollin to inhibit respiration of *E. atroseptica* confirmed the report by Stholasuta *et al.* (1971) that, unlike rishitin, it does not affect bacterial viability.

Inhibition of respiration of *Escherichia coli* by polymyxin B is thought to reflect either membrane damage resulting in the efflux of metabolites used for reducing potential in electron transport, or disorganization of the electron-transport chain (Storm *et al.*, 1977). Divalent cations such as Mg²⁺ and Ca²⁺ affect the activity of polymyxin B (Newton, 1953, 1955) possibly by competing for negatively charged phosphate groups on membrane lipids. The detergents sodium lauryl sulphate (Reynolds, 1973) and compounds related to Hyamine 2389 (Gilby & Few, 1960) act directly on cell membranes and the protective action of Mg²⁺ against another surfactant, Triton X-100, has also been reported (Unemoto & Macleod, 1975). Rishitin also, directly or indirectly, affected membrane permeability of *E. atroseptica* causing an increase in the conductivity of the suspending medium. That the action of

rishitin on *E. atroseptica* was also alleviated by the presence of Mg^{2+} , and to a lesser extent by Ca^{2+} , suggests that its mode of action may well be similar to the detergents used in these experiments or to a membrane-active antibiotic such as polymyxin. The site of action of rishitin is still uncertain but the results could be explained in terms of a disorganization of the cell membrane which controls the osmotic equilibrium of the cell. As bacterial respiration requires an intact sealed cytoplasmic membrane (Simoni & Postma, 1975), any damage to membrane function would also affect respiration. The divalent cations may be acting by 'stabilizing' the membrane and decreasing its affinity for rishitin, but it is also possible that they affect the permeability of the membrane to rishitin and hence decrease uptake, the site of action being internal to the membrane.

That the sensitivity of *E. atroseptica* to rishitin is reduced by the addition of 0.1% glucose to peptone water (Lyon & Bayliss, 1975) suggests that there is an active energy-requiring mechanism, possibly involving either membrane synthesis, which is able to repair damage done by rishitin, or an active exclusion of the phytoalexin.

Unlike the other treatments, where cell death and inhibition of O_2 uptake were coincident, the inhibition of O_2 uptake by cyanide did not affect cell viability, thus excluding the unlikely possibility that rishitin acts at the same site as cyanide.

Not only do these results provide evidence for a possible site of action for rishitin but they also demonstrate that sensitivity of *E. atroseptica* to rishitin can be affected by alterations in the composition of the suspending medium. Lyon (1977) suggested that because the sensitivity of *E. atroseptica* to rishitin was affected by glucose concentration and medium pH *in vitro* then it was possible that variation in potato tuber composition could affect the resistance of *E. atroseptica* to rishitin *in vivo* and hence resistance to rotting. The demonstration that the sensitivity of *E. atroseptica* to rishitin is also affected by Mg^{2+} and Ca^{2+} concentration *in vitro* suggests that these factors may also play a role in resistance to rotting. Certainly the contents of Ca^{2+} and Mg^{2+} in potato tubers are in a range similar to those used in the present experiments. They also vary considerably [10 to 130 and 46 to 217 mg (100 g dry matter)⁻¹ respectively (Burton, 1966)] although no relationship has yet been shown between such variations and tuber susceptibility to rotting.

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