Permeability of Phenyl-Hg⁺-resistant and Phenyl-Hg⁺-susceptible Isolates of *Pyrenophora avenae* to the Phenyl-Hg⁺ Ion

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SUMMARY

The permeability of two phenyl-Hg⁺-resistant and two phenyl-Hg⁺-susceptible isolates of *Pyrenophora avenae* Ito & Kuribay. to the phenyl-Hg⁺ ion was investigated. Similar amounts of phenyl-Hg⁺ entered the living mycelium of both resistant and susceptible isolates. No difference was detected in the numbers of thiol groups present on the wall, although the apparent free space of susceptible isolates toward the phenyl-Hg⁺ ion was greater than that of resistant isolates. The mechanism of resistance to the phenyl-Hg⁺ ion is probably based on a detoxification mechanism involving the binding of the toxic ion to metabolically inessential molecules within the mycelium.

INTRODUCTION

Isolates of *Pyrenophora avenae* Ito & Kuribay. (a fungus causing leaf stripe disease of oats) resistant to organo-mercurial seed dressings have been reported from Holland (Dekker, 1969), New Zealand (Sheridan, Tickle & Chin, 1968), Norway (Magnus, 1970), the United Kingdom (Noble, MacGarvie, Hams & Leaf, 1966; Malone, 1968; Old, 1968) and the United States of America (Crosier, Waters & Crosier, 1970). Isolates of the fungus resistant to phenyl-Hg⁺ are clearly distinct from those susceptible to this ion (Greenaway & Cowan, 1970).

Ions of mercury and its compounds form covalent bonds primarily with thiol groups (Hughes, 1957; Dorne & Hirth, 1969) and can thus act as inhibitors of many enzymes (Webb, 1966). Enzyme inhibition is probably the main cause of the toxicity of Hg²⁺ and –Hg⁺ ions, although in yeast the toxicity of Hg²⁺ has been considered to be caused by its reaction with thiols present in the cell membrane (Rothstein, 1959).

Micro-organisms resistant to mercury must either prevent Hg²⁺ or –Hg⁺ ions from entering the cytoplasm or detoxify them once they have entered. Exclusion of Hg²⁺ or –Hg⁺ ions might be accompanied by modification of the physical structure or of the chemical composition of the cell wall. Physical structure of the wall is considered important in the toxicity of copper(II) complexes of substituted eight quinolinols (Gershon, McNeil & Hinds, 1969; Gershon, Kilroy & Schulman, 1971) and an increase in the number of thiol groups that are occasionally present in the walls of fungi (Robson & Stockley, 1962; Pitt, 1969) could also bind Hg²⁺ or –Hg⁺ ions.

In the experiments described here the permeability of phenyl-Hg⁺-resistant and phenyl-Hg⁺-susceptible isolates of *Pyrenophora avenae* was investigated by assessing the quantities of phenyl-Hg⁺ removed by these isolates from aqueous solutions of phenyl-mercuric acetate (PMA). The proportions of phenyl-Hg⁺ loosely associated with or tightly bound to the wall were assessed separately and the amounts of phenyl-Hg⁺ entering the mycelium deduced.

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METHODS

Fungal isolates. Two phenyl-Hg⁺-susceptible isolates (5, 19) and two phenyl-Hg⁺-resistant isolates (29, 36) of *Pyrenophora avenae* were used. Susceptible cultures were maintained on complete medium and resistant cultures on complete medium supplemented with PMA (5 p.p.m. mercury) to prevent the development of susceptible sectors.

Growth media and chemicals. Complete medium and thiol-deficient medium were used. Complete medium contained: glucose, 20 g; asparagine, 2 g; yeast extract, 0.75 g; casein hydrolysate, 0.75 g; malt extract, 1.2 g; ammonium tartrate, 0.5 g; thiamine hydrochloride, 100 µg; KH₂PO₄, 1.0 g; MgSO₄·7H₂O, 0.1 g; Na₂SO₄, 0.14 g; Na₂HPO₄, 2.25 g; Oxoid no. 3 agar, 15 g; distilled water to 1 l.

Thiol-deficient liquid medium was prepared as above, except that yeast extract, casein and Oxoid no. 3 agar were omitted.

Media were autoclaved at 121 °C for 15 min and where required they were supplemented with an aqueous solution of phenyl-mercuric acetate (PMA), (British Drug Houses, Godalming, Surrey) micro-analytical reagent, after autoclaving and when the medium had cooled to 50 °C.

Cysteine hydrochloride (BDH biochemical reagent) and disodium ethylenediaminetetraacetic acid (EDTA) (BDH laboratory reagent) were used.

Growth of isolates. Colonies for experiments on uptake of phenyl-Hg⁺ were grown singly in a thin layer of thiol-deficient liquid medium in plastic Petri dishes and incubated in Astell cooling incubators at 20 °C for 10 days.

Assessment of phenyl-Hg⁺ absorption by mycelia. A preliminary experiment was carried out to establish the toxicity of PMA (25 µg mercury/ml) to resistant and susceptible isolates of the fungus. Three replicates of colonies 5, 19 (phenyl-Hg⁺-susceptible) 29 and 36 (phenyl-Hg⁺-resistant) were each rinsed in 100 ml sterile distilled H₂O and then soaked in solutions of PMA (25 µg mercury/ml) for periods of between 1 s and 10 min. After three further rinses in 100 ml distilled H₂O their growth was measured on complete medium. Susceptible colonies (5, 19) did not grow after 5 s of soaking in the PMA solution, whereas resistant colonies (29, 36) were unaffected by 10 s of soaking, their growth being halved by 1 min soaking, and ceasing only after soaking for 10 min.

During phenyl-Hg⁺ uptake experiments susceptible colonies were soaked in PMA solutions (25 µg mercury/ml) for 1 s, 2.5 s, 5 s, 10 s and 20 s. Resistant colonies were soaked for the above times and in addition for 40 s, 1 min, 2.5 min and 5 min. For times of up to 10 s duplicate colonies were soaked owing to the difficulty of precisely terminating short periods of soaking.

For the main phenyl-Hg⁺ absorption experiments entire colonies were washed with water to remove traces of the growth medium and soaked in 20 ml of PMA solution (25 µg mercury/ml) for the required period of time. A 5 min wash in 100 ml distilled water after the PMA soak removed phenyl-Hg⁺ ions lightly associated with the mycelium including those that had entered the free-space of the mycelium available to the phenyl-Hg⁺ ion. The colony was then rinsed for 5 min in a cysteine solution (2 mM) which removed the phenyl-Hg⁺ which was strongly bound to the wall or membrane of the mycelium. EDTA (1 mM) was added to the cysteine wash to discourage the Fe²⁺- or Cu²⁺-catalysed oxidation of the thiol groups of cysteine resulting in cystine formation (Boyer, 1961; Tarbell, 1961). Assays using 2-chloromercuri-4-nitrophenol (McMurray & Trentham, 1969) confirmed the effectiveness of EDTA in preventing the metal-catalysed oxidation of cysteine. As an additional precaution no cysteine solution was kept for more than 15 min before use. The weight of the colony

Fig. 1. Phenyl-Hg⁺ (µg mercury) removed from aqueous solutions of phenyl-mercuric acetate by phenyl-Hg⁺-susceptible (5, 19) and phenyl-Hg⁺-resistant (29, 36) isolates of *Pyrenophora avenae*. ○, Isolate no. 5; ●, isolate no. 19; □, isolate no. 29; ■, isolate no. 36.

after drying at 90 °C for 24 h was then ascertained. For each time experiment the three solutions which were retained – namely (A) the PMA-soak solution, (B) the H₂O rinse after soaking in A, and (C) the cysteine-rinse solution – were assayed for phenyl-Hg⁺ ions by using a dithizone colorimetric assay based on the method of Johnson (1964). One ml of solution A (diluted to 5 ml with distilled H₂O), 50 ml of solution B, and 10 ml of solution C, acidified by the addition of 5 ml of 0.1 N, 1.0 N and 0.15 N-HCl (BDH Analar) respectively, were assayed for their mercury content. Amounts of mercury in the assay solution were estimated by reference to a standard graph. It was possible to assay solutions B and C only once whereas duplicates of solution A were routinely assayed.

The presence of 2 mM-cysteine and 1 mM-EDTA in the cysteine-wash solutions did not affect the dithizone assay.

RESULTS

Removal of phenyl-Hg⁺ from soak solution. The amount of phenyl-Hg⁺ removed by colonies from the PMA-soak solution is shown in Fig. 1. Values shown in Fig. 1 were obtained by deducting the phenyl-Hg⁺ measured in 1 ml of solution A from the initial phenyl-Hg⁺ content of 1 ml of the soak solution (25 µg mercury). The resulting figure was then adjusted to correspond to the amount of phenyl-Hg⁺ which would have been removed by a colony of 50 mg dry wt, which was the average dry wt of a colony 35 mm in diameter, and the total amount of phenyl-Hg⁺ removed calculated. The average dry weights (mg) of colonies used in the experiments were: isolate no. 5, 63.0; no. 19, 54.5; no. 29, 55.5; and no. 36, 47.2. No explanation can be offered for the low amount of phenyl-Hg⁺ removed by the resistant colonies soaked for 20 s.

Removal of phenyl-Hg⁺ from the solution in the first 5 s of soaking was more rapid in isolate 19 (susceptible) than in the other three isolates, which all removed similar but smaller amounts of phenyl-Hg⁺ from the PMA solution. Total phenyl-Hg⁺ removed by resistant colonies, which survived 5 min soaking in the PMA solution, was greater than that removed by susceptible colonies, to which 5 s soaking was lethal.

Phenyl-Hg⁺ removed from colonies by water and cysteine rinses. Experimental results were expressed on the basis of a standard colony dry wt of 50 mg, and the total amount of phenyl-Hg⁺ in the rinse solutions was calculated (Table 1).

Water rinses removed more phenyl-Hg⁺ from susceptible colonies than from resistant

Table 1. *Phenyl-Hg⁺ removed by water and cysteine rinses from colonies of isolates 5, 19, 29 and 36 grown on thiol-deficient medium and soaked in 20 ml of a solution of PMA (25 µg mercury/ml)*

Duration of PMA soak	Phenyl-Hg ⁺ (µg mercury) removed by water rinse				Phenyl-Hg ⁺ (µg mercury) removed by cysteine rinse			
	5*	19*	29†	36†	5	19	29	36
1 s	5	11	0	1	1	2	0	1
2.5 s	6	15	2	0	1	2	1	0
5 s	9	21	1	4	2	2	2	1
10 s	13	21	7	7	2	4	2	2
20 s	9	24	13	13	2	3	3	1
40 s	—	—	13	17	—	—	4	5
1 min	—	—	10	10	—	—	5	4
2.5 min	—	—	29	29	—	—	8	10
5 min	—	—	32	54	—	—	19	30

* Phenyl-Hg⁺-susceptible isolates.

† Phenyl-Hg⁺-resistant isolates.

colonies, although the cysteine rinse removed similar amounts of phenyl-Hg⁺ from all colonies. More phenyl-Hg⁺ was removed from colonies of isolate 19 by the water wash than from colonies of other isolates. Because of this, the amount of phenyl-Hg⁺ remaining within the mycelium of all isolates after the first 20 s of soaking in PMA and subsequent washing, showed no marked difference.

DISCUSSION

Because experiment shows that similar amounts of phenyl-Hg⁺ ions appear to enter both resistant and susceptible isolates of *Pyrenophora avenae* during the period of soaking in PMA which is lethal to the susceptible isolates, permeability is not thought to be a factor in the resistance of this fungus to PMA. The greater quantity of phenyl-Hg⁺ removed from susceptible colonies by a water wash (Table 1) presumably indicates these to have a larger free-space in the mycelium available to phenyl-Hg⁺ ions than have resistant colonies. Further investigation of the observed differences in the free-space of the mycelia for the phenyl-Hg⁺ ion are being made.

As cysteine washes remove similar quantities of phenyl-Hg⁺ ions from both resistant and susceptible strains (Table 1), the number of thiol groups in the walls of these strains are thought to be similar. It is therefore concluded that resistance to phenyl-Hg⁺ is based on an intracellular process in *Pyrenophora avenae*.

In experiments described here large numbers of phenyl-Hg⁺ ions entered the mycelium of resistant colonies in a short space of time and yet the mycelium survived. It is probable that survival under conditions where there is a rapid influx will require a pre-existing pool of metabolically inessential compounds capable of binding the toxic ion and inactivating it. These considerations lead to the tentative conclusion that in *Pyrenophora avenae* the mechanism of detoxification is initially based on ligand saturation of the mercuric ion. It has already been suggested that anthraquinone molecules may be involved in such a binding process (Greenaway, 1971). Further investigations of this proposed mechanism are being made.

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