Isolation and Characterization of a Glutathione-overproducing, Captan-resistant Mutant of *Azospirillum brasilense*

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The fungicide Captan inhibited the growth of *Azospirillum brasilense* at a concentration of 25 µg ml⁻¹. When cysteine and glutathione were added to the medium they removed the toxicity of the fungicide. A spontaneous mutant was isolated which was able to grow and fix nitrogen in the presence of 100 µg Captan ml⁻¹. Characterization of the mutant indicated very high levels of glutathione and glutathione transferase activity as compared to the parent strain. The role of these cellular components in the mechanism of resistance to Captan is discussed; the involvement of a selenium-independent glutathione peroxidase appears essential to Captan resistance in the mutant strain.

INTRODUCTION

The wide use of fungicides in modern agriculture has necessitated the study of the effects of these chemicals on soil micro-organisms, including nitrogen-fixing bacteria (Anderson, 1978; Simon-Sylvestre & Fournier, 1979). Captan or Captan-containing fungicides are toxic to *Rhizobium* sp. and to symbiotic N₂-fixation and related processes (Curley & Burton, 1975; Graham *et al.*, 1980; Rennie & Dubetz, 1984; Rennie *et al.*, 1985; Banerjee & Banerjee, 1987). Mutants resistant to various fungicides have been isolated from different species of *Rhizobium* (Odeyemi & Alexander, 1977; Ruiz-Sainz *et al.*, 1984; Rennie, 1986).

Conflicting results are available on the effects of pesticides on growth and nitrogenase activity of *Azospirillum*, a bacterium able to fix nitrogen in association with the roots of important cereals and whose agronomic use has been suggested (Döbereiner & Day, 1975; Okon, 1985*a, b*). Alvarez & Sleiman (1983) reported that three insecticides and ten herbicides had no effect on the growth and nitrogenase activity of different strains of *Azospirillum lipoferum* and *Azospirillum brasilense*, but Gadkari (1988) found that the herbicides Metribuzin and Ethiozin caused a marked decrease in nitrogenase activity.

Little information is available on the mechanism of action and the fate of Captan in bacterial cells (Agnihotri, 1971). Studies, mainly done on fungal and animal cells, suggest a possible involvement of cysteine, glutathione and other thiol compounds in antagonizing the action of Captan (Rup Lal & Saxena, 1980; Sisler, 1982; Somerville, 1986). In view of the possible utilization of *Azospirillum* sp. as a biofertilizer for cereal crops (Okon & Kapulnik, 1986) it is important to investigate the effect of fungicides on this bacterium. In this paper, the effects of three structurally related substituted phthalimide fungicides (Captan, Folpet and Captafol) on the growth and nitrogenase activity of *A. brasilense* (strain Cd) and the isolation and characterization of a mutant resistant to Captan are described.

Abbreviation: CHP, cumene hydroperoxide.

METHODS

Bacterial strains. *A. brasilense* Cd (ATTC 29710) and SPF 410, a spontaneous mutant resistant to Captan, derived from the Cd strain were used.

Media. MSP medium (Bani *et al.*, 1980) was used. Unless otherwise specified the medium was not supplied with any nitrogen source. Complete medium (CM) was MSP plus yeast extract (5 g l⁻¹). Growth conditions were as described by Bani *et al.* (1980).

Chemicals. Captan (*N*-trichloromethylthio-4-cyclohexene-1,2-dicarboximide), Folpet (*N*-trichloromethylthiophthalimide) and Captafol (tetrachloromethylmercaptocyclohexene-dicarboximide) were obtained from S. Ehrenstorfer, Augsburg, FRG. They were dissolved in dimethylsulphoxide (DMSO) before use.

Determination of fungicide minimal inhibitory concentration (MIC). Captan, Folpet and Captafol solutions in DMSO were mixed with MSP agar to obtain plates at increasing concentrations of fungicide (from 0 to 100 µg ml⁻¹). Suspensions of bacterial cultures suitably diluted (about 100 colonies per plate) were seeded onto the plates. After 48 h at 34 °C the plates were examined for growth of colonies. Agar plates containing 0.1% (v/v) DMSO were used as controls.

Removal of fungicide toxicity by natural metabolites. All natural amino acids, purine and pyrimidine bases and reduced glutathione (GSH) were assayed for the ability to remove the *A. brasilense* growth inhibition by the fungicide. Each metabolite was added, at different concentrations, to MSP solid medium plates containing 50 µg Captan ml⁻¹, 60 µg Folpet ml⁻¹ or 100 µg Captafol ml⁻¹. A suspension of 5 × 10⁷ cells ml⁻¹ of strain Cd was streaked onto the plates and growth estimated visually after 2 d incubation at 34 °C.

Isolation of Captan-resistant mutants. Spontaneous Captan-resistant mutants were obtained by plating cells from a late exponential phase culture of *A. brasilense* strain Cd on MSP medium containing 50 µg Captan ml⁻¹. After 3 d at 34 °C, resistant colonies were picked up and subcultured on the same selective medium to check the level of resistance to Captan.

Cell-free extract preparation. Bacterial cells (strain Cd or SPF 410) were grown overnight in 20 ml CM and then diluted in 1200 ml of the same medium. Growth was followed up to the end of the exponential phase. The cells were harvested by centrifugation and washed twice with saline. The cell pellet was broken by grinding in a mortar with alumina (type 305, Sigma) and resuspended 1:5 (w/v) in 0.1 M-phosphate buffer (pH 6.5) containing 2 mM-EDTA. Alumina and cell debris were removed by centrifuging (6000 g, 10 min), the supernatant was collected and centrifuged at 13000 g for 45 min. The cell-free extract obtained was used to assay GSH, enzymic activities and protein content.

GSH determination. The determination of total glutathione, reported as the sum of reduced (GSH) and oxidized (GSSG) forms of glutathione (GSH + 2 GSSG), was done as described by Akerboom & Sies (1981). To 800 µl supernatant were added 3.2 ml 1 M-HClO₄ containing 2 mM-EDTA and the resulting mixture was centrifuged at 10000 g for 15 min. The supernatant was adjusted to about pH 7 with 0.3 M-MOPS containing 2 M-NaOH. The volume changes were taken into account in the calculation of the GSH concentration.

Enzyme assays. Nitrogenase activity of whole cells was assayed by the acetylene reduction method (Postgate, 1972); the cultures for nitrogenase assay were prepared as previously described (Gallori & Bazzicalupo, 1985). The number of viable cells in the presence of different concentrations of Captan was determined by plating cultures on CM medium for 48 h.

GSH transferase (EC 2.5.1.18) was assayed using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate (Habig *et al.*, 1974). The formation of conjugated derivatives was followed spectrophotometrically at 340 nm as a function of time. The standard assay mixture contained 0.1 M-potassium phosphate buffer (pH 6.5), 1 mM-EDTA, 2 mM-GSH, 1 mM-CDNB and a volume of cell-free extract corresponding to 0.5–1.0 mg protein.

GSH peroxidase activity was determined by the method described by Lawrence & Burk (1976) using 1.2 mM-cumene hydroperoxide (CHP). In this assay, the GSSG produced by the hydroperoxide-dependent oxidation of GSH was reduced by GSH reductase with concomitant consumption of NADPH, which was followed spectrophotometrically at 340 nm. Selenium-dependent GSH peroxidase (EC 1.11.1.9) activity was determined as above, but CHP was replaced by 0.25 mM-H₂O₂. Selenium-independent GSH peroxidase activity was computed by the difference between CHP and H₂O₂ activities (Wendel, 1980; Tappel *et al.*, 1982).

GSH reductase (EC 1.6.4.2) activity was assayed as described by Carlberg & Mannervik (1975), by following the oxidation of NADPH.

All three enzymic assays were done at room temperature in a Beckman model 35 spectrophotometer. Cell-free extracts, separately prepared from different cultures of Cd or SPF 410, were assayed. Each cell-free extract was assayed in duplicate both for protein (by the Lowry method) and for enzyme activities. Each assay was linear as a function of time and protein concentration.

RESULTS

Effect of substituted phthalimide fungicides on *A. brasilense* growth. On MSP solid medium Captan (25 µg ml⁻¹) blocked the growth of strain Cd, whereas the MICs of Folpet and Captafol

Table 1. Removal of Captan, Folpet and Captafol toxicity by cysteine or glutathione in *A. brasilense* strain Cd

Fungicide	Growth					
	Cysteine concn [g (g fungicide) ⁻¹]			GSH concn [g (g fungicide) ⁻¹]		
	0.5	1	1.5	0.5	1	1.5
Captan (50 µg ml ⁻¹)	—	—	±	—	±	+
Folpet (60 µg ml ⁻¹)	—	±	+	—	±	+
Captafol (100 µg ml ⁻¹)	—	±	+	—	+	+

—, No growth; ±, very reduced growth; ±, reduced growth; +, growth.

Table 2. Acetylene-reduction activity and survival of *A. brasilense* parent strain and Captan-resistant mutant SPF 410 in the presence of various concentrations of Captan

Values are the mean ± SD of four experiments with two replicates per experiment.

Strain	Captan concn (µg ml ⁻¹)	Acetylene reduction*	10 ⁻⁷ × No. of cells
Cd	0	1.42 ± 0.24	2.2 ± 0.2
	2.5	1.43 ± 0.18	2.3 ± 0.3
	5	1.08 ± 0.14	1.8 ± 0.2
	10	0.54 ± 0.06	1.3 ± 0.1
	25	0	0
SPF 410	0	1.39 ± 0.20	2.0 ± 0.2
	2.5	1.40 ± 0.21	2.1 ± 0.2
	5	1.40 ± 0.23	2.0 ± 0.2
	10	1.38 ± 0.19	2.0 ± 0.2
	25	1.36 ± 0.16	1.9 ± 0.2

* Expressed as µmol acetylene reduced h⁻¹ (mg protein)⁻¹.

were 35 and 60 µg ml⁻¹, respectively. Growth curves in the presence of Captan in MSP liquid medium indicated that 15 µg Captan ml⁻¹ appreciably reduced the growth rate of the culture, while 30 µg ml⁻¹ completely inhibited growth (data not shown).

Ability of natural metabolites to remove the growth inhibition by fungicides. Among the natural metabolites assayed, only cysteine and GSH were able to remove growth inhibition by Captan, Folpet and Captafol. The concentration of GSH or cysteine required for removing the inhibition was 1.5 times that of the fungicides (Table 1).

Isolation and characterization of mutants resistant to Captan. Thirteen spontaneous mutants resistant to Captan were selected by plating cells of *A. brasilense* Cd on MSP medium containing 50 µg Captan ml⁻¹. The frequency of resistant mutants was about 5 × 10⁻⁷. One of the mutants, designated SPF 410, was able to grow on solid MSP in the presence of 100 µg Captan ml⁻¹. This mutant also appeared resistant to 125 µg Folpet ml⁻¹ and 150 µg Captafol ml⁻¹.

Addition of Captan (2.5–10 µg ml⁻¹) did not affect the survival or the nitrogenase activity of the mutant, while the survival of the parental strain was reduced (Table 2).

Determination of GSH content and GSH metabolizing enzyme activity in the parental and mutant strain SPF 410. The GSH concentration in the mutant SPF 410 was 1121 ± 245.8 nmol per g of cells compared to 11.0 ± 3.1 nmol per g of cells in the parent strain (values are the means ± SD of three experiments, each with two replicates). GSH transferase activity was 10-times higher in

Table 3. Activities of GSH-metabolizing enzymes in *A. brasilense* parent strain (Cd) and Captan-resistant mutant (SPF 410)

Values are the mean $\pm$ SD of three experiments with two replicates per experiment.

Strain	Enzyme activity				GSH reductase†
	GSH transferase*	GSH peroxidase†			
		Total (a)	Selenium dependent (b)	Selenium independent (a - b)	
Cd	0.45 ± 0.07	11.0 ± 2.6	15.0 ± 9.0	0	108 ± 4
SPF 410	4.50 ± 0.80	3.2 ± 0.8	0	3.2	105 ± 12

* Activity expressed as nmol GSH conjugated min⁻¹ (mg protein)⁻¹.

† Activity expressed as nmol NADPH oxidized min⁻¹ (mg protein)⁻¹.

the mutant than in the parent strain. (Table 3). Furthermore, the mutant completely lacked the ability to reduce H₂O₂, while the selenium-independent GSH peroxidase activity of the mutant was 3.2 nmol min⁻¹ (mg protein)⁻¹ whereas it was totally absent from the parent strain. GSH reductase activities of the two strains were comparable.

DISCUSSION

Captan is harmful for symbiotic nitrogen-fixing bacteria (Rennie *et al.*, 1985). We found that 25 μ g Captan ml⁻¹ irreversibly blocked the growth of *A. brasilense*. Captan appeared to be the more toxic compound among the three substituted phthalimide fungicides assayed, in agreement with the data obtained for *R. trifolii* (Ruiz-Sainz *et al.*, 1984). Both GSH and cysteine were capable of antagonizing the toxic effect of Captan, Folpet and Captafol. The detoxification mechanism was probably the same for the three fungicides assayed. The content of GSH found in *A. brasilense* was lower than that reported by Fahey *et al.* (1978) for other Gram-negative bacteria.

The availability of Captan-resistant mutants, besides making compatible the combined use of fungicide and soil or seed-applied bacterial inoculation (Rennie, 1986), offers the possibility of investigating the mechanism of resistance to the fungicide. One of the mutants isolated, SPF 410, was able to grow and fix nitrogen in the presence of high levels of Captan (100 μ g ml⁻¹) and showed cross-resistance towards Folpet and Captafol. The involvement of GSH in the detoxification process was supported by the high intracellular concentration of GSH found in SPF 410. Compared to the parent strain, the mutant SPF 410 showed a notably higher GSH transferase activity and had a selenium-independent GSH peroxidase activity (Table 3). As found by Lawrence *et al.* (1978) and Prohaska (1980) the selenium-independent GSH peroxidase activity is identifiable with some form(s) of GSH transferase and therefore similar isoenzyme form(s) may be induced in our mutant. However, our data do not exclude that the 10-fold increase in the GSH transferase activity might be due to a 10-fold increase in enzyme concentration. The role of GSH transferase in catalysing the reaction of GSH with Captan, leading to less toxic metabolites, has been discussed by Somerville (1986).

Surprisingly, it was also found that the mutant completely lacked the selenium-dependent GSH peroxidase activity. Thus the total peroxide reducing power of the cell, estimated by using CHP as a substrate, was considerably reduced in spite of the contribution made by the selenium-independent GSH peroxidase activity. In contrast, the GSH reductase activity of the mutant was the same as that of the parent strain; consequently, the capacity of the cell to regenerate GSH from the GSSG produced by the GSH peroxidase reaction was unchanged.

In conclusion, the high levels of GSH found in the mutant suggest an involvement of this thiol in the detoxification process, and the rise in the GSH transferase activity in the mutant is probably part of the mechanism conferring resistance to Captan. We cannot explain the decrease in GSH peroxidase activity and the role of this enzyme in the resistance to Captan. Further investigation is needed to elucidate the function of these cellular components in the mechanism of Captan resistance.

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