Morphology and Nitrogenase Activity of Agar Cultures and Root Nodules Formed by D-Cycloserine-resistant Mutants of *Rhizobium* sp. Strain 32H1

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One-step mutants of *Rhizobium* sp. strain 32H1 resistant to D-cycloserine showed substantial ($\geq 90\%$) loss of asymbiotic (agar culture) nitrogenase activity but only partial (25 to 50%) loss of symbiotic (root nodule) nitrogenase activity. Two- and three-step resistant mutants showed a further decline or complete loss of both forms of nitrogenase activity. Since D-cycloserine inhibited the uptake of alanine by the parent strain and since the mutants possessed a defective transport system for alanine, it is suggested that the mutants are unable to concentrate D-cycloserine. Agar cultures of a one-step resistant mutant contained the three morphological types of *Rhizobium* present in the parent culture. In contrast, only one morphological type of *Rhizobium* was found in cultures of two- and three-step resistant mutants. Root nodules formed by a one-step resistant mutant on cowpea contained fewer bacteroid-filled plant cells than were present in nodules formed by the parent strain. Root nodules formed by two- and three-step resistant mutants contained only a small number of infected plant cells and in many of these rhizobia had not been released from infection threads. It is suggested that changes in the permeability of the *Rhizobium* cell wall, resulting in resistance to D-cycloserine, are responsible for the morphological changes and loss of nitrogenase activity in these mutants.

INTRODUCTION

Rhizobium mutants resistant to antibiotics which interfere with or inhibit bacterial cell wall/cell membrane synthesis [e.g. D-cycloserine, novobiocin, penicillin (Benveniste & Davies, 1973)] often show loss of symbiotic effectiveness [the ability to form effective nitrogen-fixing root nodules on a suitable host legume (Schwinghamer, 1967; MacKenzie & Jordan, 1970; Pankhurst, 1977)]. Because of this, it has been suggested that the physiological and structural integrity of the *Rhizobium* cell wall/cell membrane complex is important for successful nodule development (Pankhurst, 1977).

With the development of methods for the detection of nitrogenase activity in some strains of *Rhizobium* grown in pure culture (Keister, 1975; Pagan *et al.*, 1975; Kurz & La Rue, 1975; McComb *et al.*, 1975), it is now possible to study directly the effects of antibiotic resistance on the nitrogenase activity of a *Rhizobium* strain. In particular, it is possible to determine whether loss of symbiotic effectiveness by antibiotic-resistant mutants of *Rhizobium* is also accompanied by loss of asymbiotic nitrogenase activity.

We have investigated the asymbiotic and symbiotic nitrogenase activity of mutants of *Rhizobium* sp. strain 32H1 which are resistant to D-cycloserine, an inhibitor of peptidoglycan biosynthesis in bacteria (Reitz *et al.*, 1967). Changes in cell wall permeability resulting in decreased uptake of D- and L-alanine by these mutants were found to accompany loss of

both forms of nitrogenase activity and to result in gross changes in the morphology of agar cultures and root nodules formed by the mutants.

METHODS

Mutant isolation. *Rhizobium* sp. strain 32H1, sensitive to D-cycloserine, was grown aerobically in a mannitol/salts/yeast extract (M^+) medium (Pankhurst, 1977) to exponential phase, washed once in sterile 0.01 M-phosphate buffer (pH 7.0) and diluted to give about 5×10^7 bacteria ml^{-1} . Samples (0.1 ml) were plated on the surface of M^+ agar containing different concentrations of D-cycloserine (Sigma). D-Cycloserine was sterilized by filtration through a sintered glass filter (0.3 μm pore size) and added to the agar medium immediately before pouring and using the agar plates. A number of one-step resistant mutants, selected from plates containing the minimum concentration of D-cycloserine which inhibited growth of the parent strain, were streaked on to M^+ agar without D-cycloserine and isolated colonies were transferred to M^+ agar slopes. In a similar manner, a number of two-step and three-step resistant mutants were isolated by plating out a mixed inoculum of, respectively, the one-step or two-step resistant mutants on to M^+ agar containing successively higher concentrations of D-cycloserine. Sensitivity to D-cycloserine was quantified in M^+ broth. The minimum inhibitory concentration was defined as the concentration of D-cycloserine that restricted growth to an A_{600} of 0.1 after 72 h.

Alanine accumulation assay. The amount of [^{14}C]alanine concentrated by cells of strain 32H1 (or mutants) was established using the following procedure. The bacteria were grown in 500 ml M^+ medium until the mid-exponential phase of growth was reached. The cultures were then chilled (4 °C) and harvested by centrifuging at 12000 g for 20 min. The cells were washed once in 0.05 M-potassium phosphate buffer (pH 8.0), and then resuspended in the same buffer to give 100 mg cells (wet wt) ml^{-1} and equilibrated at 25 °C. They were then preloaded with alanine by a procedure similar to that described by Reitz *et al.* (1967): cells were incubated for 30 min at 25 °C in a mixture (total vol. 2 ml) containing 180 mg cells (wet wt), 20 mg mannitol, 2 μmol D- or L-alanine and 90 μmol potassium phosphate buffer (pH 8.0). After preloading, 50 nmol D- or L-[U- ^{14}C]alanine (The Radiochemical Centre, Amersham) was added. Samples (200 μl) were removed at intervals and added to 2 ml 0.05 M-potassium phosphate buffer (pH 8.0) containing 1 mM-D- or L-alanine held in small tubes at 0 °C, to terminate uptake of the ^{14}C -labelled alanine. The tubes were immediately centrifuged at 12000 g for 5 min. The cells were washed with 0.05 M-potassium phosphate buffer (pH 8.0) at 0 to 4 °C, and the pellet was resuspended in 1 ml distilled water and transferred to a vial containing 10 ml toluene/Triton X-100 scintillation fluid (Patterson & Greene, 1965). Radioactivity was measured in a liquid scintillation spectrometer.

Plant nodulation tests. Cultures of strain 32H1 and of the 35 D-cycloserine-resistant mutants derived from strain 32H1 were tested for their ability to nodulate cowpea (*Vigna unguiculata* var. Caloona) using the methods described by Pankhurst (1977).

Nitrogenase activity. Nitrogenase activity was assayed by acetylene reduction (Hardy *et al.*, 1968). Freshly harvested root nodules (100 to 200 mg) were placed in 15 ml bottles fitted with screw caps containing a central hole (3 mm diam.) and lined with a thick (2.5 mm) rubber liner. Air (1.5 ml) was withdrawn from the bottles with a syringe and replaced with acetylene to give an acetylene concentration of 0.1 atm. The bottles were then incubated at 25 °C. Gas samples (0.2 ml) were removed at various times after 30 min and analysed for acetylene and ethylene (C_2H_4) as described by Pankhurst & Craig (1978).

The nitrogenase activities of agar cultures of strain 32H1 and of the D-cycloserine-resistant mutants of strain 32H1 were determined as described by Pankhurst & Craig (1978).

Serology. Rabbit antiserum was prepared for strain 32H1 using the immunization schedule described by Dudman (1971). Antigens [100 to 200 mg bacteria (wet wt) ml^{-1}] for immunodiffusion analyses were prepared by centrifuging cultures which had been grown in M^+ broth and then diluted with saline (0.14 M-NaCl) to reduce their viscosity. Immunodiffusion was done as described by Dudman (1971).

Microscopy. Root nodules were prepared for light and electron microscopy by the routine method of Craig & Williamson (1973). Agar cultures of strain 32H1 and of the D-cycloserine-resistant mutants of strain 32H1 were prepared for microscopy by the method of Pankhurst & Craig (1978).

Table 1. *Minimum inhibitory concentrations of D-cycloserine for strain 32H1 and D-cycloserine-resistant mutants of strain 32H1*

Strain	No. of isolates tested	Minimum concn of D-cycloserine required to inhibit growth* ($\mu\text{g ml}^{-1}$)
32H1 (parent)	—	45
32H1 D-cyc ^r -1	15	90 (75–110)
32H1 D-cyc ^r -2	10	210 (190–230)
32H1 D-cyc ^r -3	10	320 (280–350)

* Average value, with range of values in parentheses.

Table 2. *Symbiotic effectiveness and asymbiotic nitrogenase activity of D-cycloserine-resistant mutants of strain 32H1*

Strain	No. of isolates tested	No. of isolates producing the indicated nodulation response on cowpea*			No. of isolates showing the indicated asymbiotic nitrogenase activity [nmol C ₂ H ₄ h ⁻¹ (culture) ⁻¹]†		
		Effective	Partially effective	Ineffective	> 20	5–20	< 5
32H1 D-cyc ^r -1	15	2	11	2 (2)	3	10	2
32H1 D-cyc ^r -2	10	0	3	7 (4)	0	6	4
32H1 D-cyc ^r -3	10	0	0	10 (7)	0	3	7

* Nodulation response was assessed visually by the appearance of plants (size, yellowness of leaves) and by dry weight measurement of plant shoots. Numbers in parentheses indicate the number of isolates showing activities of < 5 nmol C₂H₄ h⁻¹ (culture)⁻¹ in the asymbiotic assay.

† The cultures were established with an inoculum containing 5×10^7 bacteria ml⁻¹, and measurements were made after 14 d growth at 28 °C.

RESULTS

Minimum inhibitory concentrations of D-cycloserine

Table 1 shows the minimum inhibitory concentrations of D-cycloserine for strain 32H1 and for the one-, two- and three-step D-cycloserine-resistant mutants. The minimum concentrations of D-cycloserine which allowed the selection of these three types of mutants were, respectively, 120, 250 and 350 $\mu\text{g ml}^{-1}$. The incidence of mutants resistant to these concentrations of D-cycloserine was, in each case, about 1.5×10^{-7} .

Symbiotic effectiveness and asymbiotic nitrogenase activity of D-cycloserine-resistant mutants

Of 35 D-cycloserine-resistant mutants of strain 32H1 tested for symbiotic effectiveness on cowpea, 33 showed partial or complete loss of effectiveness (Table 2). All of these mutants also showed a marked decrease in their ability to develop asymbiotic nitrogenase activity, with many (particularly those that were ineffective) developing no activity (Table 2). The proportion of mutants showing complete loss of effectiveness and complete loss of asymbiotic nitrogenase activity increased with increasing resistance to D-cycloserine (Tables 1 and 2). All 35 mutants were serologically indistinguishable from the parent strain 32H1.

On the basis of these results, one mutant from each group (one-, two- and three-step resistant mutants) was selected for further study. These mutants, designated 32H1 D-cyc^r-1/1, 32H1 D-cyc^r-2/3 and 32H1 D-cyc^r-3/1, were chosen to represent the range of symbiotic response and asymbiotic nitrogenase activity obtained. Their specific symbiotic and asymbiotic nitrogenase activities are shown in Table 3.

Table 3. *Nitrogenase activity and morphology of agar cultures and root nodules formed by strain 32H1 and D-cycloserine-resistant mutants of strain 32H1*

	32H1 (parent)	32H1 D-cyc ^r -1/1	32H1 D-cyc ^r -2/3	32H1 D-cyc ^r -3/1
Asymbiotic nitrogenase activity [nmol C ₂ H ₄ h ⁻¹ (mg protein) ⁻¹]*	92.6 ± 5.7	7.0 ± 1.8	0.4 ± 0.1	0.0
Symbiotic nitrogenase activity [nmol C ₂ H ₄ h ⁻¹ (mg nodule fresh wt) ⁻¹]*	21.0 ± 3.1	14.5 ± 3.1	2.4 ± 1.2	0.0
Morphological type of agar culture†	X	Y	Z	Z
Morphological type of root nodule†	x	y	y/z	z

* Each value is the mean of six replicates (± S.E.M.).

† See text and Figs 1 and 2 for a description of the types of agar culture and root nodule morphology.

Morphology of agar cultures formed by D-cycloserine-resistant mutants

In view of the distinctive morphology of nitrogenase-active agar cultures of strain 32H1 (Pankhurst & Craig, 1978), it was of interest to examine the morphology of agar cultures of the three D-cycloserine-resistant mutants of strain 32H1. Sections of 14-d-old agar cultures grown at 28 °C were therefore prepared and examined by light and electron microscopy. Three types of culture morphology were found: type X (Table 3, Fig. 1*a*), formed by the parent strain 32H1; type Y (Table 3, Fig. 1*b*), formed by mutant 32H1 D-cyc^r-1/1; and type Z (Table 3, Fig. 1*c*), formed by mutants 32H1 D-cyc^r-2/3 and 32H1 D-cyc^r-3/1.

Cultures of mutant 32H1 D-cyc^r-1/1 contained a large zone of colonies with type 1 rhizobia (Pankhurst & Craig, 1978) (Fig. 1*b*). Type 2 rhizobia, which are believed to be the only rhizobia in the parent 32H1 culture to contain nitrogenase (Pankhurst & Craig, 1978) (Fig. 1*a*), were found only in small numbers in this culture. They were present at the junction of the type 1 and type 3 rhizobia (Pankhurst & Craig, 1978) (Fig. 1*b*) but did not form a distinct 'band' of cells passing through colonies at a constant depth in the agar as seen in the parent 32H1 culture (Fig. 1*a*).

Cultures of mutants 32H1 D-cyc^r-2/3 and 32H1 D-cyc^r-3/1, which showed little or no asymbiotic nitrogenase activity (culture type Z, Table 3), contained small colonies of rhizobia whose morphology was the same in colonies near the surface of the soft agar layer as in those near the bottom of the soft agar layer (Fig. 1*c*). The morphology of these rhizobia was classified as intermediate between that of typical type 1 rhizobia (with large deposits of poly-β-hydroxybutyrate) and that of typical type 3 rhizobia (with prominent nuclear material) (Pankhurst & Craig, 1978).

Morphology of root nodules formed by D-cycloserine-resistant mutants

The morphology of root nodules formed by strain 32H1 and by the three D-cycloserine-resistant mutants on cowpea (*Vigna unguiculata*) was also compared. Nodules formed by strain 32H1 (nodule type x; Table 3, Fig. 2*a*) contained a central zone of large plant cells filled with bacteroids. Nodules formed by mutant 32H1 D-cyc^r-1/1 (nodule type y; Table 3, Fig. 2*b*) also contained a central zone of infected plant cells. However, the number of these cells and the number of bacteroids within them was greatly decreased (Fig. 2*b*). Many starch grains were found in the infected and uninfected cells of these nodules (Fig. 2*b*). Nodules formed by mutants 32H1 D-cyc^r-2/3 and 32H1 D-cyc^r-3/1 (nodule type z; Table 3, Fig. 2*c*) were ineffective and showed little or no nitrogenase activity (Table 3). In some of these nodules infected plant cells containing small numbers of bacteroids were found but, in

Fig. 1. Sections through the soft agar layer of 14-d-old cultures of *Rhizobium* sp. strain 32H1 and of two D-cycloserine-resistant mutants of strain 32H1. (a) Culture of strain 32H1 (culture type X) showing individual colonies containing rhizobia designated as type 1 (R1), type 2 (R2) and type 3 (R3) (Pankhurst & Craig, 1978). (b) Culture of mutant 32H1 D-cyc⁻1/1 (culture type Y) showing colonies containing type 1 (R1) and type 3 (R3) rhizobia, but no band of type 2 rhizobia (cf. a). (c) Culture of mutant 32H1 D-cyc⁻3/1 (culture type Z) showing uniform distribution of colonies containing rhizobia classified as intermediate in morphology between type 1 and type 3 rhizobia (Pankhurst & Craig, 1978).

Fig. 2. Transverse sections of 28-d-old cowpea root nodules formed by *Rhizobium* sp. strain 32H1 and by two D-cycloserine-resistant mutants of strain 32H1. (a) Infected plant cells in a nodule formed by strain 32H1 (nodule type x). These cells have a central nucleus (N) and are filled with bacteroids (B). Many uninfected plant cells (UC) are present. (b) Infected plant cells in a nodule formed by mutant 32H1 D-cyc⁻1/1 (nodule type y). These cells contain a small number of bacteroids (B) and many starch grains (S). (c) Infected plant cells in a nodule formed by mutant 32H1 D-cyc⁻3/1 (nodule type z). These cells are located in the centre of a zone of 'meristematic activity' in the nodule. They contain infection threads (IT) but no rhizobia free in their cytoplasm.

general, the infected plant cells contained infection threads only and had no rhizobia free in their cytoplasm. The latter cells were usually located near the centre of a zone of 'meristematic activity' in the nodule (Fig. 2*c*).

Resistance to D-cycloserine

In view of the differences in the morphology of agar cultures and root nodules formed by strain 32H1 and the three D-cycloserine-resistant mutants, it was of interest to investigate the possible mode of resistance of the mutants to D-cycloserine. D-Cycloserine is reported to be concentrated by the alanine transport system in *Streptococcus faecalis* (Reitz *et al.*, 1967) and *Escherichia coli* (Curtiss *et al.*, 1965; Wargel *et al.*, 1971). We therefore examined the uptake of D- or L-[¹⁴C]alanine by strain 32H1 and the three D-cycloserine-resistant mutants. All three mutants showed decreased rates of uptake of both D- and L-alanine compared with the parent strain (Table 4). With mutant 32H1 D-cyc^r-1/1, the initial rates of D- and L-alanine uptake were 50% and 40% of the parent values, respectively. With mutants 32H1 D-cyc^r-2/3 and 32H1 D-cyc^r-3/1, the uptake rates were even lower, being about 50% of those for mutant 32H1 D-cyc^r-1/1. In all experiments, the amount of D- or L-alanine accumulated was proportional to time (within the limits of a 15 min assay). To minimize possible secondary reactions, the initial rates of uptake (presented in Table 4) were established during short time intervals (2 min) in preloaded cells (see Methods). D-Alanine (1 mM) decreased the uptake of L-[¹⁴C]alanine (1 mM) and L-alanine (1 mM) decreased the uptake of D-[¹⁴C]alanine (1 mM), indicating that D-alanine and L-alanine enter strain 32H1 by the same transport system. In a similar fashion, D-cycloserine (1 mM) decreased the uptake of both D-[¹⁴C]alanine and L-[¹⁴C]alanine (Table 4).

DISCUSSION

Acquisition of D-cycloserine resistance by *Rhizobium* sp. strain 32H1 was associated with loss of the ability of this strain to develop both asymbiotic (agar culture) and symbiotic (root nodule) nitrogenase activity. The extent of this loss was associated with the level of resistance to the antibiotic. One-step resistant mutants showed substantial ($\geq 90\%$) loss of asymbiotic nitrogenase activity but only partial (25 to 50%) loss of symbiotic nitrogenase activity. Two- and three-step resistant mutants, which were four and seven times more resistant to D-cycloserine than the parent strain (Table 1), showed a further decline or a complete loss of both forms of nitrogenase activity.

Two mechanisms of resistance to D-cycloserine are known for bacteria. The first, reported for D-cycloserine-resistant mutants of *Escherichia coli* (Curtiss *et al.*, 1965; Wargel *et al.*, 1971) and *Streptococcus faecalis* (Reitz *et al.*, 1967) involves a decrease in the permeability of the cell wall to the antibiotic due to defective functioning of the D- and L-alanine transport system. The second, found in some D-cycloserine-resistant mutants of *S. faecalis* (Reitz *et al.*, 1967), involves an increase in the activities of two enzymes selectively inhibited by D-cycloserine, namely alanine racemase and D-alanylalanine synthetase. Although only the former mechanism was investigated in this work, all three D-cycloserine-resistant mutants tested showed a significant decrease in their ability to concentrate D- and L-alanine. As D-cycloserine inhibited the uptake of both D- and L-alanine in the parent strain, resistance to D-cycloserine is probably due to the inability of the mutants to concentrate the antibiotic.

The progressive loss of both forms of nitrogenase activity with increasing resistance to D-cycloserine was also reflected in the morphology of the agar cultures and root nodules developed by the mutants (Table 3). Agar cultures of the one-step resistant mutant 32H1 D-cyc^r-1/1 contained the three morphological types of rhizobia present in the parent strain culture (Pankhurst & Craig, 1978). In contrast, agar cultures of the two- and three-step

Table 4. *Initial rates of accumulation of D-[¹⁴C]alanine and L-[¹⁴C]alanine by strain 32H1 and D-cycloserine-resistant mutants of strain 32H1*

The alanine accumulation assay was done with 1 mM-D-[¹⁴C]alanine or 1 mM-L-[¹⁴C]alanine, and 180 mg bacteria (wet wt). Other additions were made to the assay system as indicated.

Additions to assay system	[¹⁴ C]Alanine accumulated [pmol min ⁻¹ (mg protein) ⁻¹]			
	32H1 (parent)	32H1 D-cyc ^r -1/1	32H1 D-cyc ^r -2/3	32H1 D-cyc ^r -3/1
D-[¹⁴ C]Alanine				
None (complete system)	360	183	84	77
Sodium azide (30 mM)	57	—	—	—
D-Cycloserine (1 mM)	124	—	—	—
L-Alanine (1 mM)	98	—	—	—
L-[¹⁴ C]Alanine				
None (complete system)	345	138	65	73
Sodium azide (30 mM)	35	—	—	—
D-Cycloserine (1 mM)	151	—	—	—
D-Alanine (1 mM)	110	—	—	—

—, Not determined.

resistant mutants, which had little or no nitrogenase activity, contained only one morphological type. These observations were paralleled by a decrease in the proportion of infected plant cells containing bacteroids in nodules formed by the one-step resistant mutant and by failure of rhizobia to be released from infection threads in nodules formed by the two- and three-step resistant mutants. As changes in the cell wall/cell membrane complex are thought to occur when rhizobia are transformed into nitrogen-fixing bacteroids in the nodule (Bergersen, 1974; van Brussel *et al.*, 1977) and when strain 32H1 transforms into a pleiomorphic 'putative nitrogen-fixing form' in agar culture (Pankhurst & Craig, 1978), it seems likely that, if resistance to D-cycloserine involves a change in the permeability of the *Rhizobium* cell wall, this could interfere with these transformations and affect the development of nitrogenase activity.

Simultaneous loss of asymbiotic and symbiotic nitrogenase activity has also been reported for two out of five mutants of *R. japonicum* strain 61A76 (Maier & Brill, 1976) and for two out of 40 nitrate reductase deficient mutants of strain 32H1 (Pagan *et al.*, 1977). The mutants isolated by Maier & Brill (1976) may be defective in nitrogenase synthesis, but those isolated by Pagan *et al.* (1977) show that disruption of other processes within *Rhizobium* may also affect its ability to develop nitrogenase activity. In a similar fashion, we suggest that changes in the permeability of the *Rhizobium* cell wall resulting in resistance to D-cycloserine alter the ability of *Rhizobium* to develop nitrogenase activity.

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