The Expression of Resistance of *Ustilago avenae* to the Sterol Demethylation Inhibitor Triadimenol is an Induced Response

Franzine D. Smith and Wolfram Köller

Research associate and assistant professor, respectively, Department of Plant Pathology, Cornell University, New York State Agricultural Experiment Station, Geneva 14456.

This work was supported in part by a grant from the Cornell Biotechnology Program which is sponsored by the New York State Science Technology Foundation, a consortium of industries, and the U.S. Army Research Office.

Accepted for publication 28 December 1989 (submitted for electronic processing)

ABSTRACT

Smith, F. D., and Köller, W. 1990. The expression of resistance of *Ustilago avenae* to the sterol demethylation inhibitor triadimenol is an induced response. Phytopathology 80: 584-590.

A triadimenol-sensitive wild-type strain and a triadimenol-resistant mutant of *Ustilago avenae* were investigated with regard to their responses to the sterol demethylation inhibitor triadimenol. A fungicide concentration of 2 μg ml^{-1} was fully inhibitory to the sensitive but not the resistant strain after long-term treatments for 48 hr. The initial response to triadimenol treatment was clearly different from the long-term effects. Reproduction of the sensitive strain was halted within 6 hr, and the few cells that continued to emerge over a period of 24 hr were morphologically altered and remained in cell aggregates. The initial pattern of growth inhibition was similar for the resistant mutant. Reproduction of sporidia was blocked subsequent to a 4-hr lag-phase, and new cells remained in aggregates. In contrast to the sensitive strain, the inhibitory effect on both sporidial reproduction and segregation of daughter cells was transient for the resistant mutant, and undisturbed growth resumed 12 hr after treatment with triadimenol. The alteration of sterol profiles during the course of inhibitor treatment reflected the different patterns of morphological responses. During the initial inhibitory phase, C-14 methyl sterols accumulated under the action of triadimenol in both strains. The extent of target saturation, as revealed by lipid labeling with [^{14}C]-acetate, also was similar for both the sensitive and resistant strains. In the sensitive strain, the sterol precursors continued to comprise the pool of free sterols. In contrast, the accumulation of precursors was only transient for the triadimenol-resistant mutant. When growth resumed, authentic desmethyl sterols were synthesized, and the content of precursor sterols declined to levels similar to the nontreated control. The expression of resistance of *U. avenae* to triadimenol appears to involve an inducible system that relieves the cells from an initially pronounced inhibitory action of the sterol demethylation inhibitor.

The group of fungicides acting as sterol demethylation inhibitors is widely used to control a broad spectrum of fungal plant diseases (27,35). In sensitive fungi, these compounds are known to inhibit the C-14 demethylation of lanosterol or, more often, 24-methylenedihydrolanosterol. This inhibitory action leads to a substantial accumulation of C-14 methyl sterol precursors and, subsequently, to the inhibition of growth (3,23,38). Fungal phenotypes resistant to sterol demethylation inhibitors have been described for a wide variety of plant pathogens. These resistant phenotypes were either isolated from natural populations or were obtained by mutagenesis and subsequent selection (2,23). The presence of resistant isolates in field populations of plant pathogens is not surprising, since a wide and continuous sensitivity distribution can be identified in wild-type populations not yet exposed to this class of fungicides (2,24,37). Under selection, this initial pathogen population responds with a quantitative and gradual sensitivity shift toward resistance. As the frequency of resistant phenotypes increases gradually, thresholds of satisfactory disease control can be surpassed (2,24). So far, cases of practical resistance are largely restricted to several powdery mildew diseases and occurred only after widespread and intensive use of sterol

demethylation inhibitors such as triadimefon (2,24,34). Reports of unsatisfactory control of apple scab under experimental orchard conditions (12,15) and the identification of phenotypes of *Penicillium digitatum* (Pers.:Fr.) Sacc. resistant to imazalil (11) might indicate the possibility of slow shifts toward resistance for pathogen populations other than powdery mildews and, thus, more widespread problems in the future.

The prediction of future developments would require a profound knowledge of the pathogenic fitness inherent to phenotypes with increasingly high levels of resistance, a trait closely related to the molecular mechanism of resistance. For example, the structural alteration of the target site leading to a decreased binding affinity of the inhibitor but not to a functional impairment of the target enzyme would not connote a fitness penalty. In contrast, a mechanism of resistance based on energy-consuming reactions could impair the pathogenic fitness of the challenged organism. The mechanism of fungal resistance to sterol demethylation inhibitors remains uncertain, although several models have been suggested in the past. A constitutive and active efflux of the inhibitor has been reported for *Aspergillus nidulans* (Eidam) G. Wint. and *Penicillium italicum* Wehmer (9). More recently, an elevated level of cytochrome P-450 monooxygenase in transformed *Saccharomyces cerevisiae* (4), a decreased binding affinity of the inhibitors to cytochrome P-450 isolated from resistant *Candida albicans* (C. P. Robin) Berkhout (39) and the segregation of the inhibitor into vacuoles of *Ustilago avenae* (Pers. Rostr.) (17) have been discussed as alternative models. An altered requirement for membrane sterols, as suggested as an explanation for the natural insensitivity of *Mucor rouxii* (41), might also be operative in resistance. Most of the suggested mechanisms of resistance would imply a constitutive expression of resistance. Thus, the growth of a fungus resistant to sterol demethylation inhibitors should not be affected at any stage of inhibitor treatment.

The objective of the present study was to investigate the initial response of a laboratory mutant of *Ustilago avenae* ((Pers.) Rostrup) that is resistant to triadimenol. The model system of *U. avenae* has been characterized extensively. The resistant mutant was obtained by UV-mutagenesis and subsequent selection on triadimefon (18), a fungicide closely related to triadimenol (21). The morphology, the sterol composition of the resistant strain, and the main location of sterols in plasma membranes were shown to be unaffected by the mutational change leading to resistance (19). Patterns of cross-resistance suggested a specific resistance to the group of sterol demethylation inhibitors. Several fungicides with target sites different from the C-14 demethylation of sterols, including the morpholines as a second group of sterol biosynthesis inhibitors, were equally effective on both the wild-type strain and the resistant mutant (26). Although a large number of different sterol demethylation inhibitors were shown to be cross-resistant to each other, the resistance factors were greatly variable (25). Triadimenol exhibited the highest level of resistance (25) and, thus, was chosen as the most suitable compound for our studies on the mechanism of resistance.

MATERIALS AND METHODS

Fungal cultures and materials. The origin of strains of *U. avenae* sen and rl used in this study are described elsewhere (19). Triadimenol (chemical purity >95%) was provided by Bayer AG, Leverkusen, West Germany. [1,2-^{14}C]-Acetic acid, sodium salt (2.0 GBq mmol^{-1}) was obtained from NEN Research Products, DuPont. Plates for thin-layer chromatography (TLC) were purchased from Analtech, Newark, DE, for preparative (Silica Gel G; 0.5 mm) or from EM Science, Philadelphia, PA (Merck Silica Gel 60; 0.25 mm), for analytical separations. Millex-HV filters (0.45 μm) were obtained from Millipore Corporation, Bedford, MA. All other chemicals were from Sigma Chemical Company, St. Louis, MO.

Growth of fungal cultures and lipid labeling. Cultures of *U. avenae* maintained on agar slants were transferred to a liquid growth medium described elsewhere (25) and incubated for 36 hr. Sporidia from these actively growing cultures were transferred to fresh medium (20 ml; final cell density 10^5 ml^{-1}). Triadimenol was added from a stock-solution in acteone to a final concentration of 2 μg ml^{-1} at stages as indicated. The final acetone concentration was 0.1% (v/v); control cultures were supplemented with the same amount of acetone. Cultures were shaken at 150 rpm and 20 C. Small samples were withdrawn at time intervals as indicated, and the number of cells was determined in triplicate with a hemacytometer.

For the radioactive labeling of lipids, cultures were precultivated for 15 hr and treated with triadimenol (2μg ml^{-1}) for 8 hr. [^{14}C]-Acetate (750 kBq) then was added to 10-ml portions of these cultures. The labeling of lipids was stopped by heating the cell suspension in a water bath for 30 min, either after 20 min or 15 hr of incubation with the radioactive precursor.

Lipid extraction. Fungal cultures were centrifuged, the sporidial pellet was washed with distilled water, centrifuged, and resuspended in 1 ml of water. After lyophilization, the cells were stirred in 6 ml of chloroform/methanol (2:1, v/v). To avoid sterol oxidation, all organic solvents were flushed with nitrogen and supplemented with butylated hydroxytoluene (BHT) (final concentration = 0.05 [w/v]) before use. The lipid extract was clarified by filtration (Millex-HV filters), and the solvent was evaporated under a stream of nitrogen. The lipids were dissolved in a small volume of chloroform/methanol (2:1) and subjected to further analysis.

Sterol analysis. Desmethyl, 4-methyl, and 4,4-dimethyl sterols were separated by TLC as previously described (25). For sterol analysis by gas-liquid chromatography (GLC), the lipid fractions were separated on scored TLC plates. The scored portion, loaded with a small portion of the lipid extract and a mixture of lanosterol and ergosterol as chromatographic standards, was removed after development, and the sterol fractions were visualized by spraying with 30% ethanolic sulfuric acid and heating at 120 C for 30 min. The regions on the remaining main portion of the TLC plates corresponding to the three sterol fractions were scraped off, eluted with chlorform/methanol (2:1), and evaporated under nitrogen. The dried sterols were dissolved in identical volumes of chloroform/methanol (2:1) and subjected to GLC analysis.

In the case of lipid labelings, the free desmethyl sterols and the sterol esters were detected by proportional counting and eluted with chloroform/methanol (2:1). The free sterols were acetylated

TABLE 1. Fragment ions and their relative intensities of the mass spectra of free sterols identified in cultures of *Ustilago avenae*[a]

	Sterols[b]				
Fragmentation[c]	A	B	C	D	E
M+	396(77)	398(60)	412(33)	426(37)	440(45)
M-15	381(3)	383(7)	397(100)	411(100)	425(100)
M-18	378(4)	380(6)			
M-(15+18)	363(100)	365(100)	379(9)	393(14)	407(37)
M-43			369(11)	383(10)	397(10)
M-59	337(35)	339(38)			
M-(84+15)			313(17)	327(19)	341(15)
M-SC	271(12)	271(8)	287(3)		
M-(SC+2H)	269(9)	269(1)	285(5)	299(3)	313(3)
M-(SC+2H+15)	254(6)	254(2)	271(8)	285(10)	299(4)
M-(SC+18)	253(27)	253(9)			
M-(SC+42)	229(2)	229(3)	245(12)	259(10)	273(8)
M-(SC+56)			231(39)	245(40)	259(30)
M-(SC+42+18)	211(18)	211(13)	227(8)	241(11)	255(10)
RRT[d]	1.07	1.14	1.15	1.18	1.32

[a] Figures in parentheses are the relative intensities of the ions (higher mass range).

[b] A, ergosterol; B, ergosta-5,7-dien(3)ol; C, 14 methylfecosterol; D, obtusifoliol; E, 24-methylenedihydrolanosterol.

[c] SC, sterol side chain; 15, CH_3; 18, H_2O; 43, C_3H_7 (loss of C-25 to C-27); 42, C_3H_6 (loss of C-15 to C-16); 56, C_4H_8 (loss of C-15 to C-17 plus C-32); 59, (loss of C-1 to C-3 in a $\Delta^{5,7}$-sterol); 84, C_6H_{12} (loss of C-23 to C-28 in a 24-methylene sterol).

[d] Retention time relative to cholesterol (26.9 min).

and analyzed by argentation-TLC as previously described (25). The sterol esters were hydrolyzed and analyzed by TLC according to the procedures described before (22). Quantitative analysis of radioactivity on TLC plates was done by proportional counting (25). Structural analysis of the sterols present in the individual fractions separated by TLC from nontreated and triadimenol-treated strains was done by gas liquid chromatography-mass spectrometry (GC-MS) (30,33) on a Hewlett-Packard 5970B Mass Selective Detector linked to a Hewlett-Packard 5890A capillary gas chromatograph equipped with a fused silica column (SE-54; 30 m × 0.25 mm) (Hewlett-Packard, Avondale, PA). Splitless injection was used, the oven temperature was programmed from 130 to 240 C at 30 C min^{-1}, 240–280 C at 2 C min^{-1}, and the carrier gas (He) flow rate was 31 cm sec^{-1}. The molecular ions and indicative MS-fragments are summarized in Table 1. The individual structures of sterols were deduced from published MS-data (1,30,32,40,42). Quantitative analysis was done with a Hewlett-Packard 5890A instrument equipped with a flame ionization detector. GLC conditions were similar to the GLC-MS analysis, except that an Ultra 2 column (Hewlett-Packard; 25 m × 0.2 mm, 0.33 film thickness) and split injection (split ratio 1:5) were used. Relative amounts were calculated from peak area integrations (Hewlett-Packard 3393A Integrator).

RESULTS

Initial response of a sensitive and a resistant isolate of *U. avenae*. *U. avenae* forms monokaryotic haploid sporidia that replicate in a yeastlike manner in liquid media (28). Under the influence of a sterol demethylation inhibitor such as triadimenol, the segregation of daughter cells is strongly inhibited. Most of the emerging cells remain in cell aggregates and are, furthermore, smaller in size (16,18). The altered cells, although confined in cell aggregates, were counted as individual cells throughout this study. Based on the number of individual cells as the measure for fungal growth, reproduction of the sensitive wild-type strain was almost completely abolished (>95%) at an inhibitor concentration of 2 $\mu g\ ml^{-1}$ after 48 hr of cultivation, when fungal cultures were started with a low cell density of $10^5\ ml^{-1}$ (Fig. 1). In contrast, reproduction of the resistant mutant was hardly affected under these conditions (Fig. 1). This growth pattern indicated a high level of triadimenol resistance and identified a triadimenol concentration of 2 $\mu g\ ml^{-1}$ as an ideal discriminatory dose for the following studies.

To investigate the initial effects of inhibitor treatment, untreated cultures were precultivated until they reached an early exponential growth phase, and then triadimenol was added. The events subsequent to the inhibitor treatment were monitored over a period of 24 hr and, thus, to the late stage of exponential growth. The first effects of triadimenol on sensitive sporidia were observed after 4 hr of treatment, and reproduction was blocked after 6 hr (Fig. 2). This lag-phase corresponds to approximately one cell cycle and has been frequently reported for *Ustilago* spp. after treatment with sterol demethylation inhibitors (3). Surprisingly, the initial response of the resistant mutant was not different from that of the sensitive wild-type strain. Reproduction was completely blocked after 4 hr of incubation with the inhibitor (Fig. 2). However, drastic differences between the two strains became apparent after 10 hr of treatment. Reproduction of the sensitive sporidia remained blocked over the entire investigated period of 24 hr. Although a slight increase in cell numbers was still observed, the morphology of the newly emerging cells was drastically altered. As previously reported before for *U. avenae* treated with sterol demethylation inhibitors (3,16,18), slowly emerging and distorted daughter cells did not segregate from mother cells. We found that the percentage of cells confined in multicellular aggregates containing more than two cells increased continuously for the treated wild-type strain (Fig. 2). In summary, the effect of triadimenol on sensitive sporidia was twofold: The reproduction was inhibited subsequent to a lag-phase representing aproximately one cell cycle, and the separation of slowly emerging cells from their mother cells was prevented.

In contrast to the lasting inhibitory effect of triadimenol on the sensitive strain, the inhibitory phase was transient for the resistant mutant. The initially arrested reproduction of sporidia resumed after 10 hr (Fig. 2). During the interval from 12 to 14 hr, the number of untreated and inhibitor-treated cells increased by a factor of 1.5 and 1.7, respectively. This slight growth stimulation mediated by the inhibitor continued, and the number of sporidia was almost the same in both the treated and nontreated culture after 24 hr of triadimenol treatment (Fig. 2). The initial inhibitory action of triadimenol, followed by a renewed and slightly faster growth of sporidia was also reflected by the

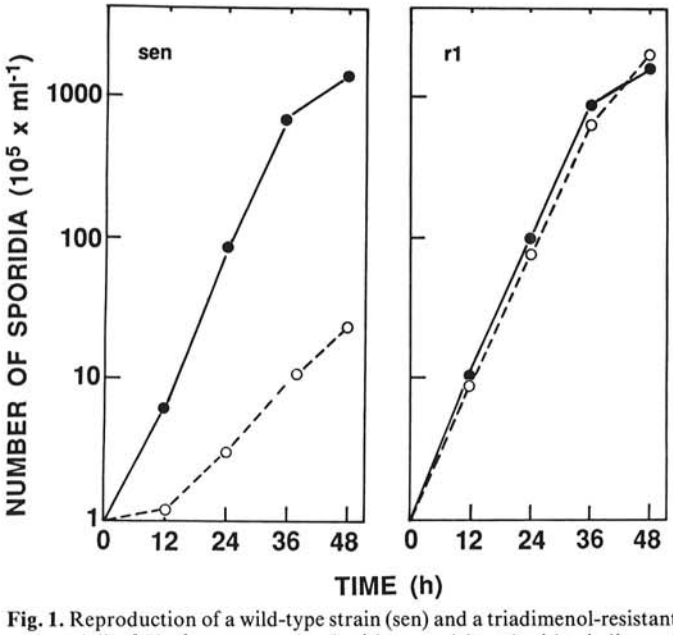

Fig. 1. Reproduction of a wild-type strain (sen) and a triadimenol-resistant mutant (r1) of *Ustilago avenae* (●-●) without and (○-○) with triadimenol at a concentration of 2 $\mu g\ ml^{-1}$. The initial density of sporidia was $10^5 ml^{-1}$. Each point represents the mean of three replicates. The standard errors are smaller than symbols.

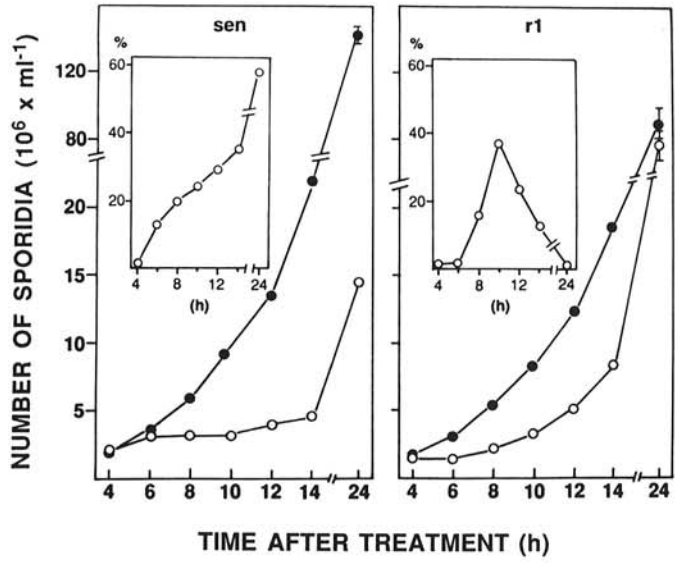

Fig. 2. Reproduction of a wild-type strain (sen) and a triadimenol-resistant mutant (r1) of *Ustilago avenae*. The percentages of total sporidia present in aggregates of more than two cells are shown in the inserts. Sporidia were precultivated for 15 hr (initial cell density was $10^5\ ml^{-1}$) and remained untreated (●-●), or were treated with triadimenol (○-○) at a final concentration of 2 $\mu g\ ml^{-1}$. Each point represents the mean of three replicates. Vertical bars represent the standard errors and are not shown if smaller than symbols.

morphological appearance of treated cells. The percentage of sporidia in aggregates increased during the inhibitory phase and peaked at 10 hr of treatment (Fig. 2). Triadimenol-resistant cells confined in aggregates (Fig. 3) were morphologically similar to those observed for the treated sensitive strain (16,18). However, the percentage of cells in aggregates decreased continuously after reproduction resumed (Fig. 2). After 24 hr of treatment, all sporidia were found as single or budding cells (Fig. 3), a state similar to the nontreated control.

Sterol patterns in response to triadimenol treatment. The sterol precursors 24-methylenedihydrolanosterol, obtusifoliol, and 14-methylfecosterol are known to accumulate in fungal cells under the action of a sterol demethylation inhibitor (3,23,38). Our observation of an initial inhibition followed by renewed growth of the triadimenol-resistant mutant of *U. avenae* prompted us to investigate the relative distribution of sterols present during the inhibitory phase, and at crucial points during the recovery phase of the resistant mutant (Table 2). After 6 hr of triadimenol treatment, which corresponds to the stage of a complete arrest of reproduction (Fig. 2), both the sensitive and the resistant strains were almost depleted of ergosterol, the most prominent sterol (>95%) identified in nontreated cultures of both the sensitive and resistant strain (19). The sterol precursors expected to accumulate, 24-methylenedihydrolanosterol and obtusifoliol, comprised the major free sterols recovered from the treated samples. This sterol pattern did not change over time for the treated sensitive strain. Only 14-methyl fecosterol, an artificial sterol frequently shown to slowly originate from the precursor 24-methylenedihydrolanosterol via two C-4 demethylations (3,23,32,38), was identified as an additional component of the pool of C-14 methyl sterols after 24 hr of treatment.

The situation was drastically different for the resistant mutant. At the onset of renewed growth (10 hr of treatment), the ergosterol content had increased substantially, while the sterol precursors were still detected at the relatively high level of 44.6% (Table 2). The precursors were almost absent after 24 hr, and the sterol pattern present in the treated culture was similar to the nontreated control. The results clearly indicate that at first sterol biosynthesis was equally inhibited in both strains, and that the recovery from the initial phase of growth inhibition by the resistant mutant was accompanied by a renewed appearance of the authentic sterol end products ergosterol, and as the cells approach the late stage of exponential growth, ergosta-5,7-dien(3)ol. The latter sterol, which has also been identified in *U. maydis* (DC.) Corda (10,32), was absent during the phase of full exponential growth.

Radioactive sterol labeling. The analytical data described above were confirmed by the analysis of sterols after the labeling of lipids with [^{14}C]-acetate. Here, the strategy was to treat wild-type and resistant sporidia for 8 hr with triadimenol prior to the labeling. This pretreatment assured that the inhibitory effect was fully established in the wild-type strain, whereas the resistant strain was at the latest stage of growth inhibition (Fig. 2). Both a short pulse-labeling for 20 min and an extended labeling for 15 hr were done following the treatment with triadimenol. Examples for the distribution of radioactivity found in the three sterol fractions separated by TLC according to the number of methyl groups present at the C-4 position are presented in Figure 4.

In nontreated cultures, the brief pulse-labeling trapped a considerable proportion of radioactivity in the dimethyl fraction that contains the C-14 methyl sterols normally accumulating under the action of a sterol demethylation inhibitor. No radioactivity had yet reached the metabolic pool of sterol esters. This distribution indicates that the brief labeling of sterols allowed little time for the full metabolism of labeled sterols and, thus, was suitable to visualize the saturation of the target site with triadimenol. The sterol labeling in the presence of the inhibitor led to a considerably increased amount of radioactivity in the precursor pool, concomitant with a decreased amount of acetate incorporated into the desmethyl sterol fraction comprising the

TABLE 2. Distribution of free sterols in a sensitive (sen) and resistant (rl) strain of *Ustilago avenae* treated with triadimenol at a concentration of 2 $\mu g/ml^{-1}$

			Sterol distribution (%)[a]				
			Desmethyl		14-Methyl		
Strain	Triadimenol	Hours after treatment	ERG	EDI	MDL	OBT	MFE
sen	+	6	1.9	ND[b]	76.9	21.2	ND
	+	10	3.3	ND	84.5	12.2	ND
	+	24	ND	ND	60.5	29.5	10.0
	−	24	51.9	25.3	21.5	1.3	ND
rl	+	6	1.1	ND	66.9	32.0	ND
	+	10	55.4	ND	28.4	15.2	ND
	+	24	90.5	8.8	nd	0.7	ND
	−	24	95.9	3.5	0.1	0.5	ND

[a]ERG = ergosterol; EDI = ergosta-5,7-dienol; MDL = 24-methylenedihydrolanosterol; OBT = obtusifoliol; MFE = 14-methyl fecosterol.
[b]Not detected.

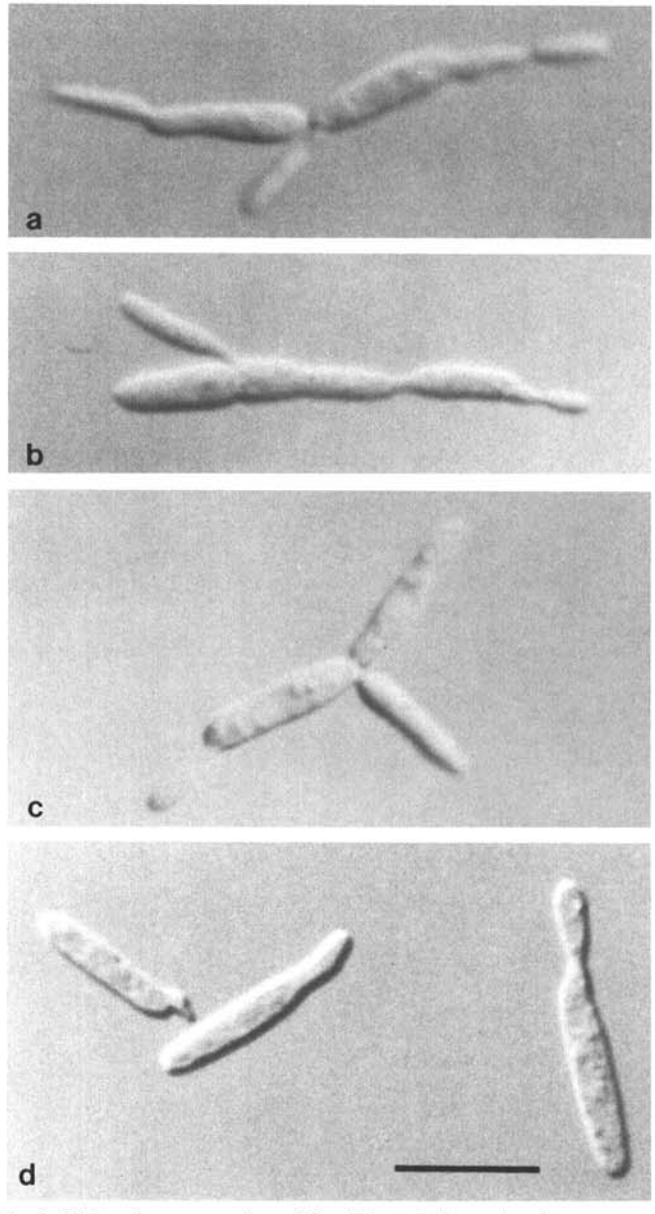

Fig. 3. Light microscopy of sporidia of the triadimenol-resistant mutant (rl) of *Ustilago avenae*. **A–C,** Represents cell aggregates present after 10 hr of treatment with triadimenol (2 $\mu g\ ml^{-1}$). **D,** Shows typical cells present after 24 hr of treatment. Sporidia were photographed with a Zeiss Photomicroscope III with differential interference contrast optics. The bar represents 40 μm.

end products. The accumulation of precursors was substantial for both strains (Fig. 4 and Table 3). Thus, the extent of target site saturation was apparently not diminished in resistant strains after 8 hr of inhibitor treatment.

The result of the extended labeling, which monitors the full metabolism of sterols over the entire period of labeling (15 hr), was strikingly different. Continual accumulation in the precursor pools of 4,4-dimethyl and 4-methyl sterol precursors was only observed with the sensitive but not the resistant strain. The bulk of radioactivity incorporated into the sterols isolated from the treated sensitive strain was recovered in the fraction of desmethyl sterols (Table 3). This fraction contains the end products of sterol synthesis, but also 14-methyl fecosterol. To evaluate the presence of 14-methyl fecosterol as a potential precursor, the desmethyl sterol fractions were acetylated and subjected to argentation-TLC (25). The sterols isolated from the nontreated and inhibitor treated resistant strain comigrated with or close to authentic ergosterol and similar to the sterols isolated from nontreated wild-type cells. A fourth band that migrated faster and contained 80% of the radioactivity was observed only with the wild-type strain treated with triadimenol (data not shown). Most likely, this band can be attributed to 14-methyl fecosterol identified as a major sterol component in these cells (Table 2). To the contrary, the desmethyl sterols recovered from the treated resistant mutant could be attributed to the pool of end products identified by GLC analysis (Table 2). The results of the lipid labeling experiments demonstrated, as did the GLC analysis of free sterols, that the sterol precursors that accumulated during the inhibitory phase in resistant cells were largely absent after growth had resumed.

One potential explanation for the disappearance of sterol precursors could be a rapid metabolic segregation from the pool of free sterols. Sterol esterification has been suggested to provide a mechanism for the sequestering of excess free sterols in *S. cerevisiae* (29) and for the segregation of suboptimal sterol precursors accumulating under the action of a sterol biosynthesis inhibitor with a mode of action different from the sterol demethylation inhibitors (31). Furthermore, sterol esters in yeast were shown to be mainly localized in lipid particles (5) and, thus, would not exert their disturbing effects in membranes. No radioactivity was incorporated into the fraction of sterol esters during the 20-min pulse-labeling of sensitive and triadimenol-resistant sporidia of *U. avenae*. However, considerable radioactivity was recovered in this fraction after the extended labeling for 15 hr (Table 3). To investigate the possibility of a pronounced segregation of free 14-methyl sterol precursors into the pool of sterol esters similar to that in *S. cerevisiae* (31), the esters were hydrolyzed, and the products were reanalyzed by TLC. The result showed that the proportion of 4,4-dimethyl precursors was consistently smaller than that found in the pool of free sterols (Table 3). Thus, the preferential segragation of accumulating precursor sterols into the pool of sterol esters cannot be considered to function as a main course of precursor segregation in the resistant strain.

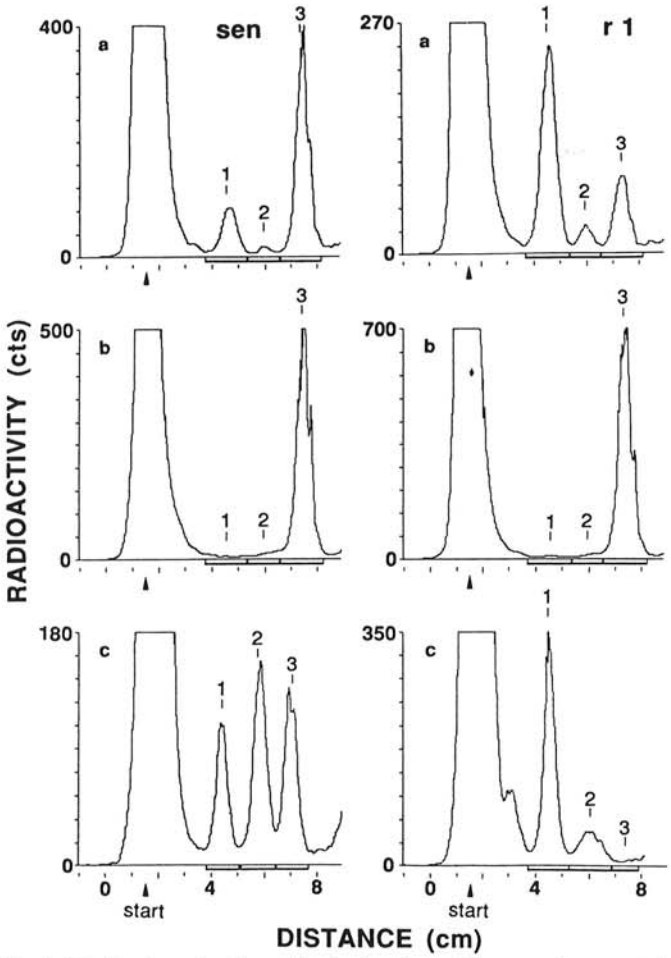

Fig. 4. Distribution of radioactivity in fractions of free sterols separated by thin-layer chromatography. The sterols were isolated from a sensitive wild-type strain (sen) or a triadimenol-resistant mutant (rl) of *Ustilago avenae* grown without (a) or with (b and c) triadimenol at a concentration of 2μg ml^{-1} under conditions described in Materials and Methods. Lipids were labeled with [^{14}C]-acetate for 20 min (a and b) or 15 hr (c) (1): 4-desmethyl sterols; (2): α-methyl sterols; (3): 4,4-dimethyl sterols. Radioactivity is expressed as accumulated counts (cts).

DISCUSSION

The expression of triadimenol-resistance by the mutant of *U. avenae* investigated in this study is an induced response rather than a constitutive trait. This conclusion is based on several lines of evidence. At a dose of triadimenol (2 μg ml^{-1}) inhibitory to the sensitive wild-type strain, the reproduction of the resistant mutant was also severely blocked following inhibitor treatment. This initial inhibitory phase was not different from that observed for the sensitive cells. It was accompanied by an altered morphology, by the accumulation of C-14 methyl sterol precursors and the depletion of the ergosterol pool, and by a high degree of saturation of the target site with inhibitor. In sharp contrast to the sensitive wild-type strain, however, the inhibitory phase was only transient for the resistant mutant, and undisturbed growth resumed. The recovery phase was accompanied by the proportional decrease of morphologically altered cells, and by the relief of inhibitor treated cells from the high content of sterol precursors than accumulated during the initial inhibitory phase. An induced expression of resistance has not been reported previously for a site-specific fungicide used in agriculture (8). Although not investigated in this study, the induced response implies, most likely, the activation of normally repressed

TABLE 3. Radioactivity distribution in sterol fractions separated by TLC after labeling of lipids with [^{14}C]-acetate

Strain	Triadimenol	Radioactivity distribution in sterols (%) 4-Desmethyl	4-Methyl	4,4-Dimethyl	Ester[a]
Pulse-labeling (20 min)					
sen	−	21.7	4.5	73.8	ND[b]
sen	+	3.1	4.2	92.7	ND
rl	−	66.3	8.5	25.2	ND
rl	+	3.9	2.9	93.2	ND
Extended labeling (15 hr)					
sen	−	52.5	23.5	13.4	10.6 (3.6)
sen	+	23.3[c]	35.0	29.9	11.8 (23.7)
rl	−	55.2	24.5	6.8	13.5 (3.4)
rl	+	63.8	17.3	6.2	12.7 (3.5)

[a] Figures in parentheses represent the proportion (%) of 4,4-dimethyl sterol precursor present in the sterol ester fraction.
[b] Not detected.
[c] Mostly 14-methyl fecosterol.

"resistance genes," either induced by the inhibitor as such, or by the precursor sterols that initially accumulate.

In principal, a recovery from growth inhibition by triadimenol has also been described for the triadimenol-sensitive strain of *U. avenae*. Hippe and Giesen (18) reported that the initially inhibited sensitive cells recovered after 4 days of cultivation. Under their experimental condition (higher incubation temperature), the stationary growth phase was reached after 24 hr of cultivation, and the cell mass of the nontreated culture started to decline after 2 days. Thus, a recovery from growth inhibition occurred only after an excessive period of inhibitor treatment. Moreover and in striking contrast to the induced expression of resistance described in our study, the sensitive sporidia that finally escaped the inhibitory action of triadimenol remained in cell aggregates and did not revert to the yeastlike pattern of reproduction (18). The authors concluded that the growth recovery resembled an adaptational response rather than the expression of resistance. As frequently shown, a mutational and stable fungicide resistance is different from an adaptational tolerance (14,36). This is clearly the case for the sensitive strain of *U. avenae* described by others (18) and the triadimenol-resistant mutant characterized in this study. Both the time frame of growth recovery and the morphology of cells resuming growth demonstrate a fundamental difference between the two phenotypes of *U. avenae*.

The molecular mechanism leading to the recovery of the resistant cells from growth inhibition, and the identity of "resistance genes" presumably induced during the inhibitory phase remains to be elucidated. However, our results clearly indicate the absence of a constitutive system conferring resistance to sterol demethylation inhibitors and, thus, are not in agreement with most of the resistance mechanisms proposed in the literature. For example, a constitutive molecular mechanism of resistance would be operative in case of a mutational change of the target site leading to a decreased binding affinity of the inhibitor, a mechanism verified for the benzimidazoles and phenylamides (6,7,8,13). A similar decreased binding affinity of sterol demethylation inhibitors to their target site has so far only been reported for resistant *Candida albicans* (20). However, the differences regarding the in vitro sensitivities of respective sterol demethylases were not great, and there was no direct correlation between the in vitro and in vivo sensitivities. Both the induced nature of resistance expression and the full inhibition of sterol synthesis during the initial inhibitory phase exclude this possibility for the resistant mutant of *U. avenae* investigated in this study.

A constitutive expression of resistance is not restricted to a mutational change of the target site. The active efflux mechanism (9) and an altered sterol requirement in membranes (41) would also mandate a constitutive response. The compensation of higher inhibitor concentrations by an increased level of target sites (4) could reflect an induced response, if the transcription and translation of the sterol demethylase would be drastically increased during the inhibitory phase. Similarly, the segregation of triadimenol into vacuoles as suggested by Hippe (17) might involve the induction of a specific tonoplast transport system. However, other alternatives exist. Preliminary results indicate an altered lipid metabolism during the recovery phase of the resistant mutant. Work is in progress to investigate the biosynthesis but also the catabolism of sterols and other lipids under these conditions.

LITERATURE CITED

1. Baloch, R. I., Mercer, E. I., Wiggins, T. E., and Baldwin, B. C. 1984. Inhibition of ergosterol biosynthesis in *Saccharomyces cerevisiae* and *Ustilago maydis* by tridemorph, fenpropimorph and fenpropidin. Phytochemistry 23:2219-2216.
2. Brent, K. J., and Hollomon, D. W. 1988. Risk of resistance against sterol biosynthesis inhibitors. Pages 332-346 in: Sterol Biosynthesis Inhibitors—Pharmaceutical and Agrochemical Aspects. D. Berg and M. Plempel, eds. Ellis Horwood Ltd., Chichester.
3. Buchenauer, H. 1987. Mechanism of action of triazolyl fungicides and related compounds. Pages 205-232 in: Modern Selective Fungicides—Properties, Applications and Mechanisms of Actions. H. Lyr, ed. John Wiley & Sons, Inc., New York.
4. Chen, C., Turi, T. G., Sanglard, D., and Loper, J. C. 1987 Isolation of the *Candida tropicalis* gene for P450 lanosterol demethylase and its expression in *Saccharomyces cerevisiae*. Biochem. Biophys. Res. Commun. 146: 1311-1317.
5. Clausen, M. K., Christiansen, K., Jensen, P. K., and Behnke, O. 1974. Isolation of lipid particles from baker's yeast. FEBS Lett. 43:176-179.
6. Davidse, L. C. 1987. Biochemical aspects of benzimidazole fungicides—action and resistance. Pages 245-257 in: Modern Selective Fungicides—Properties, Applications and Mechanisms of Actions. H. Lyr, ed. John Wiley & Sons, Inc., New York.
7. Davidse, L. C. 1987. Biochemical aspects of phenylamide fungicides—action and resistance. Pages 275-282 in: Modern Selective Fungicides—Properties, Applications and Mechanisms of Actions. H. Lyr, ed. John Wiley & Sons, Inc., New York.
8. Dekker, J. 1985. The development of resistance to fungicides. Prog. Pestic. Biochem. Toxicol. 4:165-218.
9. De Waard, M., and Van Nistelrooy, J. G. M. 1987. Inhibitors of energy-dependent efflux of the fungicide fenarimol by *Aspergillus nidulans*. Exp. Mycol. 11:1-10.
10. Ebert, E., Gaudin, J., Muecke, W., Ramsteiner, K., and Fuhrer, H. 1983. Inhibition of ergosterol biosynthesis by etaconazole in *Ustilago maydis*. Z. Naturforsch. 38c:28-34.
11. Eckert, J. W. 1987. *Penicillium digitatum* biotypes with reduced sensitivity to imazalil. (Abstr.) Phytopathology 77:1728.
12. Fiaccadori, R., Gielink, A. J., and Dekker, J. 1987. Sensitivity to inhibitors of sterol biosynthesis in isolates of *Venturia inaequalis* from Italian and Dutch orchards. Neth. J. Plant Pathol. 93:285-287.
13. Georgopoulos, S. G. 1987. The genetics of fungicide resistance. Pages 53-62 in: Modern Selective Fungicides—Properties, Applications and Mechanisms of Action. H. Lyr, ed. John Wiley & Sons, Inc., New York.
14. Georgopoulos, S. G., and Zaracovitis, C. 1967. Tolerance of fungi to organic fungicides. Annu. Rev. Phytopathol. 5:109-130.
15. Hildebrand, P. D., Lockhart, C. L., Newbery, R. J., and Ross, R. G. 1989. Resistance of *Venturia inaequalis* to bitertanol and other demethylation-inhibiting fungicides. Can. J. Plant Pathol. 10:311-316.
16. Hippe, S. 1984. Ultrastructural changes induced by the systemic fungicides triadimefon, nuarimol, and imazalil nitrate in sporidia of *Ustilago avenae*. Pestic. Sci. 15:10-214.
17. Hippe, S. 1987. Combined application of low temperature preparation and electron microscopic autoradiography for the localization of systemic fungicides. Histochemistry 87:309-315.
18. Hippe, S., and Giesen, U. 1988. The effect of triadimenol on the cytology and growth of sensitive and resistant strains of *Ustilago avenae*. Ann. Appl. Biol. 112:70-90.
19. Hippe, S., and Koller, W. 1986. Ultrastructure and sterol composition of laboratory strains of *Ustilago avenae* resistant to triazole fungicides. Pestic. Biochem. Physiol. 26:209-219.
20. Hitchcock, C. A., Barrett-Bee, K. J., and Russell, N. J. 1987. Inhibition of 14 α-sterol demethylase activity in *Candida albicans* Darlington does not correlate with resistance to azole. J. Med. Vet. Mycol. 25:329-333.
21. Köller, W. 1987. Isomers of sterol synthesis inhibitors: Fungicidal effects and plant growth regulator activities. Pestic. Sci. 18:129-147.
22. Köller, W. 1987. Plant growth regulator activities of stereochemical isomers of triadimenol. Physiol. Plant. 71:309-315.
23. Köller, W. 1988. Sterol demethylation inhibitors: Mechanism of action and resistance. Pages 79-88 in: Fungicide Resistance in North America. C. J. Delp, ed. American Phytopathological Society, St. Paul, MN.
24. Köller, W., and Scheinpflug, W. 1987. Fungal resistance to sterol biosynthesis inhibitors: A new challenge. Plant Dis. 71:1066-1074.
25. Köller, W., and Wubben, J. P. 1989. Variable resistance factors of fungicides acting as sterol demethylation inhibitors. Pestic. Sci. 26:133-145.
26. Krämer, W., Berg, D., and Köller, W. 1987. Chemical synthesis and fungicidal resistance. Pages 291-305 in: Combating Resistance to Xenobiotics—Biological and Chemical Approaches. M. G. Ford, D. W. Hollomon, B. P. S. Khambay, and R. M. Sawicki, eds. Ellis Horwood Ltd., Chichester.
27. Kuck, K. H., and Scheinpflug, H. 1986. Biology of sterol-biosynthesis inhibiting fungicides. Pages 65-96 in: Chemistry of Plant Protection, Vol. 1. G. Haug and H. Hoffmann, eds. Springer-Verlag, Berlin.
28. Nielsen, J. 1988. *Ustilago* spp., smuts. Pages 483-490 in: Advances in Plant Pathology, Vol. 6. D. S. Ingram and P. H. Williams, eds. Academic Press, London.
29. Parks, L. W., Lewis, T. A., Low, C., and Haeckler, K. 1987. Effect of intracellular sterol concentration on sterol esterification. Pages 57-61 in: The Metabolism, Structure, and Function of Plant Lipids. P. K. Stumpf and W. D. Nes, eds. Plenum Press, New York.

30. Patterson, G. W. 1984. Chemical and physical methods in the analysis of plant sterols. Pages 293-323 in: Isopentenoids in Plants—Biochemistry and Function. W. D. Nes, G. Fuller and L.-S. Tsai, eds. Marcel Dekker, New York.
31. Pereira, R., Holmlund, C. E., and Whittaker, N. 1983. The effect of AY-9944 on yeast sterol and sterol ester metabolism. Lipids 18:545-552.
32. Ragsdale, N. N. 1975. Specific effects of triarimol on sterol biosynthesis in *Ustilago maydis*. Biochim. Biophys. Acta 380:81-96.
33. Rahier, A., and Benveniste, P. 1989. Mass spectral identification of phytosterols. Pages 223-250 in: Analysis of sterols and other biologically significant steroids. W. D. Nes, and E. J. Parish, eds. Academic Press, San Diego.
34. Scheinpflug, H. 1988. Resistance management strategies for using DMI fungicides. Pages 93-94 in: Fungicide Resistance in North America. C. J. Delp, ed. American Phytopathological Society, St. Paul.
35. Scheinpflug, H., and Kuck, K. H. 1987. Sterol biosynthesis inhibiting piperazine, pyridine, pyrimidine and azole fungicides. Pages 173-204 in: Modern Selective Fungicides—Properties, Applications and Mechanisms of Action. H. Lyr, ed. John Wiley & Sons, Inc., New York.
36. Scholer, H. J., and Polak, A. 1984. Resistance to systemic antifungal agents. Pages 394-460 in: Antimicrobial Drug Resistance. L. E. Bryan, ed. Academic Press, New York.
37. Smith, F. D., Parker, D. M., and Köller, W. 1989. Baseline sensitivity of three populations of *Venturia inaequalis* to flusilazole. (Abstr.) Phytopathology 80:1171.
38. Vanden Bossche, H. 1988. Mode of action of pyridine, pyrimidine and azole antifungals. Pages 79-119 in: Sterol Biosynthesis Inhibitors—Pharmaceutical and Agrochemical Aspects. D. Berg and M. Plempel, eds. Ellis Horwood Ltd., Chichester.
39. Vanden Bossche, H., Marichal, P., Gorrens, J., Bellens, D., Verhoeven, H., Coene, M.-C., Lauwers, W., and Janssen, P. A. J. 1988. Interaction of azole derivatives with cytochrome P-450 isozymes in yeast, fungi, plants and mammalian cells. Pestic. Sci. 21:289-306.
40. Vanden Bossche, H., Willemsens, G., Cools, W., Lauwers, W. F. J., and LeJeune, L. 1978. Biochemical effects of miconazole on fungi. II. Inhibition of ergosterol biosynthesis in *Candida albicans*. Chem.-Biol. Interact. 21:59-78.
41. Weete, J. D., and Wise, M. L. 1987. Effects of triazoles on fungi. V. Response by a naturally tolerant species, *Mucor rouxii*. Exp. Mycol. 11:214-222.
42. Ziogas, B. N., Sisler, H. D., and Lusby, W. R. 1983. Sterol content and other characteristics of pimaricin-resistant mutants of *Aspergillus nidulans*. Pestic. Biochem. Physiol. 20:320-329.