Detoxification of the macrolide toxin brefeldin A by *Bacillus subtilis*

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The macrolide toxin brefeldin A is a determinant of *Alternaria* leaf blight disease in safflower, which causes severe economic losses worldwide. Soilborne bacteria, classified as *Bacillus subtilis* spp., were isolated and shown to readily metabolize brefeldin A in laboratory culture to one major product. This product was identified by high resolution 2D ^{1}H NMR and FAB mass spectroscopies as the acid resulting from hydrolysis of the macrolide ring in brefeldin A. In contrast to brefeldin A, the acid completely lacked phytotoxic activity in the standard leaf bioassay. Detoxification of brefeldin A by the lactonase activity from *Bacillus subtilis* may be exploited in the future to introduce resistance to *Alternaria* leaf blight in safflower.

Brefeldin A detoxification; Brefeldin A acid; Brefeldin A lactonase; Macrolide antibiotic; Macrolide phytotoxin

1. INTRODUCTION

Brefeldin A (BFA), a macrolide antibiotic indentified first from *Eupenicillium brefeldianum* [1] and later from various other fungi [2], has attracted research interest for many years due to its peculiar molecular structure and its antifungal and antiviral activities [3,4]. More recently, a pronounced detrimental effect of BFA on the growth of animal cells and tissues has been discovered and its mechanism is being studied intensively. The latter effect appears to be due to impediment to the flux and processing of glycoproteins form the endoplasmic reticulum to the Golgi complex [5,6]. Furthermore, BFA causes the disintegration of the Golgi complex and the accumulation of secretory proteins in the endoplasmic reticulum [7–13].

BFA has been additionally described as a non-specific albeit selective phytotoxin, which plays a crucial role in the development of disease symptoms in safflower plants infected with the leaf blight pathogen *Alternaria carthami* Chowdury [14]. *Alternaria carthami* is by far the biggest problem in safflower on a worldwide basis, and since safflower is a very valuable dietary oil crop, effective control of the blight disease is desirable [15]. Virulence ratings of various field isolates of *A. carthami* closely correlated with their potential for toxin production in laboratory culture, and BFA was the only toxin produced by *A. carthami* growing in safflower leaves [14]. The determination of toxin distribution in situ by a sensitive radio-immunioassay

revealed significant toxin concentrations in close proximity to the fungal mycelia only and diffusing approx. 2 mm in advance of the leading hyphae [2]. In cell cultures, it has been shown that small concentrations of BFA (down to nM) considerably interfere with the defense response of safflower, i.e. the accumulation of polyacetylenic phytoalexins [16]. These results strongly suggest that BFA provides the niche in live safflower tissue for fungal growth. Detoxification of BFA in vivo would be a suitable countermeasure for protection of the plant by exposing the fungus to the full disease resistance response.

Resistance to macrolide antibiotics, as studied commonly with the commercial antibiotic erythromycin, has been ascribed to modifications of ribosomal subunits with reduced binding affinity [17–19]. A different type of resistance has been elucidated only recently in *Enterobacteriaceae*, where hydrolysis of the macrolide lactone [20–22] or 2'-phosphorylation inactivates the antibiotic [23]. Such a one step enzymatic detoxification applied to BFA would have an enormous impact on the control of *Alternaria* leaf blight disease in safflower. In this paper, we describe the hydrolysis of brefeldin A by a strain of *Bacillus subtilis*.

2. MATERIALS AND METHODS

2.1. *Reagents*
All reagents were of analytical grade. Sodium [1-^{14}C]acetate (2 GBq/mmol) was purchased from Amersham Buchler (Braunschweig, FRG).

2.2. *Chromatography*
Thin layer chromatography (TLC) was carried out on silica gel 60 plates (Merck, Darmstadt) in solvents (I) diethyl ether/ethyl acetate/ethanol 12:12:1 (v/v/v) and (II) diethyl ether/acetone 3:1 (v/v) Reversed phase HPLC was performed on Waters equipment (Eschborn, FRG) in line with a UV detector (254 nm) and a Ramona radiomonitor (Raytest, Straubenhardt, FRG). Analytical (Lichrosorb

Abbreviations: BFA, brefeldin A; BFA acid, brefeldin A acid; BMM, bacterial minimal medium; LB, Luria-Bertani medium

RP-18, 5 μm, 250 ×4 mm, Knauer, Bad Homburg, FRG) and preparative columns (Nucleosil RP-18, 7 μm 250 × 20 mm, Macherey & Nagel, Düren, FRG) were used with solvents (III) 40% methanol, 0.1% acetic acid and (IV) 50% methanol, 0.1% acetic acid. Flow rates were 1 ml/min for the analytical and 5 ml/min for the preparative column.

2.3. Isolation of brefeldin A and [^{14}C]brefeldin A

Brefeldin A was isolated from the medium of 5 week old *Alternaria carthami* cultures as described previously [2]. For isolation of [^{14}C]BFA, *A. carthami* was incubated with [1-^{14}C]acetate as the sole carbon source. Twelve-day-old mycelia (500 mg wet weight) were thoroughly washed in sterile 0.9% NaCl, suspended in 10 ml Filner's medium [24] containing 9.25 MBq[1-^{14}C]acetate (2 GBq/mmol) instead of sucrose, and cultivated stationary in daylight for 3 days. [^{14}C]BFA was isolated by extraction of the culture filtrate into chloroform, TLC in solvents I and II and HPLC in solvent III. Total incorporation varied between different experiments (0.1% to 2%), yielding specific incorporations between 50 and 310 MBq/mmol [^{14}C]BFA.

2.4. Isolation and culture of soilborne bacteria

Soil samples were inoculated into 5 ml of minimal medium (BMM) [25] containing 360 μM BFA and cultivated on a rotary shaker for 24 h at 37°C. The culture was then plated on minimal agar containing BFA (360 μM) and incubated at 37°C for up to 7 days. Single colonies growing under these conditions were isolated and cultured in LB medium [26] for the investigation of BFA metabolism. Taxonomic classification of bacteria was performed by the Deutsche Sammlung für Mikroorganismen (Braunschweig, FRG).

2.5. Assay for bacterial BFA metabolism

Aliquots (100 μl) of overnight cultures of soilborne bacteria were inoculated into LB medium or BMM (5 ml) containing [^{14}C] BFA (1632 Bq, 5 Bq/nmol). After 1–7 days at 37°C, the broth was acified with 50 μl of 1 N HCl, extracted with ethyl acetate, and the extract was analyzed by TLC in solvent I and subsequent radioscanning with a TLC linear analyzer (Berthold, Wildbad, FRG).

2.6. Isolation of BFA metabolites

For spectroscopic indentification, BFA metabolites were isolated from four 50 ml LB cultures containing [^{14}C]BFA (16 KBq/μmol) that had been inoculated each with 1 ml of an overnight culture and incubated in a rotary shaker for 72 h at 37°C. The broth was extracted with ethyl acetate and the extract was subfractionated by silica column chromatography (50 ml bed volume) in solvent (A) diethyl ether/ethyl acetate 1:1 (500 ml), followed by 10% ethanol in A (500 ml) and 50% ethanol in A (500 ml) prior to preparative HPLC purification in solvent III.

2.7. Biotest

Phytotoxicity of BFA and its metabolites isolated from bacterial cultures was monitored as desbribed previously [2] using 4-week-old safflower plants. Solutions of BFA or of individual metabolites (20 mM) in ethanol were applied to surface-sterilized leaves that had been lightly scratched with a sterile hypodermic needle. The development of symptoms was followed over a period of 5 days. Pure solvent was used for control biotests.

2.8. Spectroscopy

2.8.1. ^{1}H NMR

1D and 2D COSY ^{1}H NMR spectra were recorded at room temperature on Bruker WM 400 or AM 600 NMR spectrometers locked to the major resonance of the solvent, CD$_3$OD. All 1D and 2D spectra were recorded using the standard Bruker software package. All chemical shifts are given in ppm relative to TMS and couplings in Hz.

2.8.2 FAB MS

Negative ion FAB mass spectra were recorded on a Kratos MS 50

3. RESULTS AND DISCUSSION

Some bacterial colonies isolated from local soil showed growth in the presence of a BFA concentration (360 μM), which usually inhibits the growth of either *E. coli* or plant cells completely. Growth was always rather sluggish in the presence of BFA and the bacteria initially tended to form clumps at the bottom of the culture vessel which disintegrated to fine suspensions with time. One of these bacterial strains (BG3) was isolated and selected for further study on the bais of its extraordinary metabolic capacity for BFA. Growth and metabolic character of this strain were stable over extended periods of culture (several months) in the presence of BFA. Strain BG3 was identified as *Bacillus subtilis*.

Bacillus subtilis BG3 converted within 3 days almost all of the radioactive BFA added to the culture in LB medium to a single, extractable metabolite, which upon chromatography appeared to be more hydrophilic than BFA. After incubation for more than 3 days, this metabolite was converted further to even more hydrophilic compounds which were not analyzed. The first metabolite was isolated on a preparative scale from the broth of four cultures using BFA of low specific radioactivity (5 MBq/mmol) as the substrate. In these experiments, more than 50% of the BFA added was re-isolated form the culture by silica column chromatography (fraction eluted with 10% ethanol) while the metabolite was eluted in the presence of 50% ethanol. The metabolite was purified by preparative HPLC (Rt 53.8 min) and, for control, analytical HPLC of this compound revealed only one radiosignal correlating with one large UV (254 nm) absorption signal (RT 10.5 min). The pure metabolite (0.7 mg) was subjected to spectroscopic examination. In eighter the presence or absence of BFA, an insignificant decrease in the pH of the cultures was noted (from pH 7.4 to pH 6.8 within the growth period of 3 days), which has no effect on BFA stability.

The structure of the metabolite (Fig. 1) was readily deduced from the ^{1}H NMR data on comparison with that of BFA (Table I). The ^{1}H assignment of both BFA and the metabolite was established from the cross peaks in the 2D COSY spectra. The most pronounced chemical shift differences between the two compounds are found for H-2, H-3 and H-15. In the latter the high field shift of 1.1 ppm is characteristic of the loss of an acyl group at C-15 and is only comparable with the opening of the ring system. In both compounds the double bonds have vicinal coupling constants (15–16
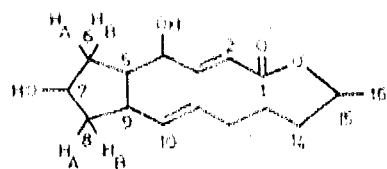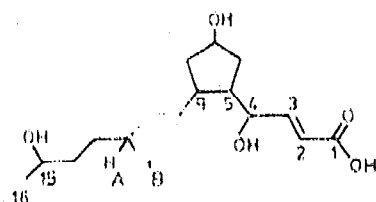
Fig. 1. Hydrolysis of brefeldin A to brefeldin A acid catalyzed by soilborne *Bacillus subtilis*.

Table I

^{1}H NMR data of brefeldin A and the corresponding acid in CD₃OD.

H	Multiplicity[a]	Brefeldin A acid[b] δ(ppm)	Multiplicity[a]	Brefeldin A[c] δ(ppm)
2	bd	6.02	dd	5.86
3	dd	6.81	dd	7.50
4	m	4.24	ddd	4.08
5	m	2.01	m	1.89
6A	ddd	1.88	m	2.06
6B	ddd	1.61	m	1.85
7	m	4.23	p	4.26
8A	ddd	2.18	ddd	2.17
8B	m	1.43	dddd	1.49
9	m	2.48	p	2.43
10	dd	5.44	dd	5.32
11	dt	5.48	ddd	5.80
12	m	2.07	(12A)m	2.06
			(12B)m	1.89
13	m	1.45	(13A)m	1.91
			(13B)m	0.94
14	m	1.51	(14A)m	1.79
			(14B)m	1.62
15	m	3.76	m	4.84
16	d	1.18	d	1.28

[a]The abbreviations used are: b = broad, d = doublet, m = multiplet, p = pentet and t = triplet. [b]The coupling constants measured from the 1D[1]H NMR spectrum of brefeldin A acid are as follows: (H2-H3) 15.6, (H3-H4) 5.0, (H6A-H6B) 13.3, (H6A-H5) (H6A-H7) 6.9 9.2, (H6B-H5) (H6B-H7) 4.0 8.4, (H8A-H8B) 13.6, (H8A-H7) (H8A-H9) 6.0 8.1, (H9-H10) 8.4, (H10-H11) 15.2, (H11-H12) 6.5, (H15-H16) 6.2. [c] The coupling constants measured from the 1D ^{1}H NMR spectrum of brefeldin A are as follows: (H2-H3) 15.5, (H2-H4) 2.0, (H3-H4) 3.0, (H4-H5) 9.5, (H6-H7) ~ 4.8, (H7-H8A) 5.3, (H7-H8B) 5.5, (H8A-H8B) 13.5 (H8A-H9) 8.8, (H8B-H9) 7.7, (H8B-) 1.0, (H9-H10) 9.6, (H10-H11) 15.1, (H11-H12A,B) 10.3 4.6, (H15-H16) 6.2.

The proton responsible for the long-range coupling to H8B of 1.0 Hz cannot be un ambigously determined.

Hz) indicative of E-configurations. Similarly, the chemical shifts and the indentification of the appropriate cross peaks in the 2D COSY spectrum of the metabolite (Table I) indicate the pentacyclic ring has remained intact. Finally the structure (Fig. 1) was confirmed by the observation of a deprotonated molecular ion at m/z 297 in the negative ion FAB MS of the metabolite, which is comparable with the addition of a molecule of water BFA (m/z 279). In summary, *Bacillus subtilis* BG3 prossesses the enzymatic capacity for hydroysis of BFA to BFA acid. This capacity for hydrolysis is remarkable, because neither strong acid (1 N HCl) nor strong base hydrolyzed BFA under laboratory conditions (data not shown), probably due to the 2,3-double bond.

The hydrolysis of the lactone-ring of BFA explains the increase in hydrophilic character observed for the metabolite. The hydrolysis would certainly have some effect on uptake and systemic transport of BFA in plants, if it could be exploited for the protection of plants. However, hydrolysis might in addition change the biological activity of BFA, as has been shown by loss of the antibiotic activity of erythromycin upon hydrolysis in *E. coli* [21]. The phytotoxic potential of BFA acid was therefore assayed in a simple and conclusive safflower leaf biotest, which had originally served in the isolation of BFA from *A. carthami* as a phytotoxin [14]. BFA acid was applied over a range of concentrations (40, 100 and 200 nmol) to detached safflower leaves and also to the leaf surface of intact plants, and the leaves were visually examined for a period of 5 days. None of the leaves treated showed discoloration or necrosis, whereas brown and necrotic spots typical for the leaf blight disease developed on all the control leaves treated with equivalent amounts of BFA.

Since the safflower leaves are wounded prior to the bioassays, our results strongly suggest that hydrolysis destroys the phytotoxic activity of BFA rather than merely inhibiting its translocation in plants. Safflower and a variety of other plants appear to posses very little enzyme activity for hydrolysis of BFA [27], and attempts over several years to adapt respective cell cultures to this toxin failed (data not shown). Soilborne *B. subtilis*, however, appears to be a convenient source for such enzyme activity, which has already been determined in the cell-free bacterial extracts (data not shown), as well as for DNA coding for the protein. Cloning of the gene and its use for transformation of safflower to confer resistance to BFA and to *A. carthami* [14] now appears to be a valuable alternative to traditional breeding for resistance which has turned out rather difficult in safflower.

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