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Nitric Oxide 9 (2003) 1–9
NITRIC OXIDE Biology and Chemistry
www.elsevier.com/locate/yniox

Involvement of nitric oxide synthase in sucrose-enhanced hydrogen peroxide tolerance of *Rhodococcus* sp. strain APG1, a plant-colonizing bacterium

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Received 26 November 2002; received in revised form 20 May 2003

Communicated by Jack Lancaster, Jr.

Abstract

Hydrogen peroxide (H_2O_2) tolerance of *Rhodococcus* sp. strain APG1, previously isolated from the aquatic fern *Azolla pinnata*, was examined in relation to nitric oxide (NO) production by cells cultured on a variety of C sources. Cells inoculated onto *A. pinnata* fronds established a surface-sterilant resistant density of $2–4 \times 10^7$ cells g^{-1} without causing disease. Compared to cultures containing glucose, fructose, mannitol, or glycerol, those provided only with sucrose displayed, on a per C basis, substantially lower (<10%) growth yields and higher resistance to H_2O_2. NO, a positive regulator of catalase synthesis in bacteria, was produced in larger amounts in sucrose-grown cells as evidence by eightfold greater per cell accumulations in the medium of nitrite (NO_2^-), a stable oxidation product of NO. Addition to cells of L-arginine, the substrate for nitric oxide synthase (NOS), stimulated production of NO, detected both by fluorometric reaction with diaminofluorescein-FM diacetate (DAF-FM DA) and by increased levels of NO_2^- in the culture medium. These results suggest that sucrose may enhance H_2O_2 tolerance of *Rhodococcus* APG1 by increasing cellular NO producing capacity. We propose a regulatory role for NOS in promoting tolerance of *Rhodococcus* APG1 to oxidative stress in the phyllosphere.

Keywords: Phyllobacteria; *Rhodococcus*; *Azolla*; Nitric oxide synthase; Hydrogen peroxide tolerance; Plant–microorganism interaction; Endophytic colonization

Bacteria colonize the exterior and interior habitat of leaves, or phyllosphere, nourished by sugars (mostly sucrose) [1] and other products of plant metabolism [2]. Some of these phyllobacteria may benefit their plant host by providing metabolites, consuming wastes [2], or out-competing pathogens [3]. To establish a population on a leaf, a bacterium must be able to overcome several abiotic and biotic stresses [4] including autogenic and plant-derived hydrogen peroxide (H_2O_2), a membrane-diffusible by-product of respiratory and photosynthetic metabolism. Plants under ultraviolet-B stress [5] or pathogen attack [6] increase H_2O_2 production by up-regulating expression of NADPH oxidase, whose product, superoxide ($O_2^{\cdot-}$), is disproportionated to H_2O_2 by superoxide dismutase (SOD). Concentrations of H_2O_2 on plant leaves have been measured between 0.1 and 0.8 μmol g^{-1} [7]. Though H_2O_2 itself is not particularly dangerous, it can react with Fe^{2+} to form the toxic hydroxyl radical ($HO^{\cdot}$), a highly reactive agent of oxidative stress that can cause irreparable damage to biological molecules [8,9]. Thus, to preempt its potential harm, organisms must have efficient mechanisms for destroying H_2O_2. In many bacteria H_2O_2 is decomposed by catalase.

In general, catalase activity increases in stationary phase or growth-arrested bacteria [9–14]. In plant-colonizing *Pseudomonas* species the increase in catalase

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doi:10.1016/S1089-8603(03)00043-0

activity is coincident with a rise in the number of catalase isoforms, from one in logarithmic phase cells to three or four in stationary phase cells [15]. The expression of catalase has been shown in many bacteria to be positively regulated by the transcription factor OxyR [9,16], which becomes activated in cells exposed to H_2O_2 or nitric oxide (NO) via oxidation or nitrosation, respectively, of an OxyR cysteine redox center [9,17–19]. H_2O_2 and NO can also activate SoxR, another transcriptional global regulator of genes involved in oxidative stress tolerance, such as *sodA*, which encodes a manganese SOD [9,20].

NO-related activities of plant or animal-associated bacteria have typically been viewed as responses to NO arising from the host. NO itself can limit bacterial cell growth by lowering respiration through the binding of cytochromes [21] and/or the citric acid cycle enzyme aconitase [22]. However, it is now known that bacteria, like eukaryotes, can catalyze NO production via nitric oxide synthase (NOS) [23–27]. In a process unrelated to dentrification-linked NO formation of some bacteria [28], NOS produces NO by oxidizing L-arginine to L-citrulline. NOS genes from bacteria have been cloned and sequenced and NOS activity has been found in preparations from *Staphylococcus aureus* [25], *Deinococcus radiodurans* [24], *Bacillus subtilis* [23], *Salmonella typhimurium* [26], and from the closely related genera *Rhodococcus* [27] and *Nocardia* [29,30]. Recently, investigations into roles for endogenous NO formation by NOS in bacteria have been initiated with studies on a *Rhodococcus* sp. [27] and a *Nocardia* sp. [31].

Eukaryotes are known to produce NO as a membrane diffusible signal and as a precursor for the microbicidal peroxynitrite ($ONOO^-$) [32,33]. Similar to eukaryotes, in a *Nocardia* sp. NO produced by NOS activates guanylate cyclase to increase synthesis of the intracellular signaling molecule cyclic guanosine 3′,5′-monophosphate (cGMP) [31]. The effects of higher cGMP levels in *Nocardia* are not known but, consistent with a role for NO in adaptation of cells to oxidative stress, exposure of microaerobically grown *Rhizobium japonicum* cells to aerobic conditions substantially increases the intracellular pool of cGMP, consequently inhibiting the synthesis of certain oxygen-sensitive enzymes [34]. In several *Rhodococcus* spp. nitrile hydratases are inactivated in the dark by the binding of NO to a non-heme ferric iron at the catalytic center [35].

Some *Rhodococcus* spp. form associations with plants ranging from mutualistic to pathogenic [36–40], but none of these have been examined for their tolerance to H_2O_2 or for NOS activity. We have recently isolated an endophytic *Rhodococcus* sp., termed strain APG1, [41] from *Azolla pinnata*, a floating fern native to subtropical and tropical regions [42]. This was, to our knowledge, the first finding of a *Rhodococcus* sp. living in association with an *Azolla* sp. In this report, we have confirmed its ability to non-pathogenically colonize *A. pinnata*. Furthermore, we have cultured *Rhodococcus* APG1 in sucrose-containing medium presupposing that cells might display traits normally reserved for plant-associated survival. Relative to cells grown on other sources of C, sucrose-grown cells were found to reach lower growth yields and show higher tolerance to H_2O_2 that correlated with increased formation of NO.

Experimental procedures

Bacterial culture conditions

A mineral salts medium based on that of Verstraete and Alexander [43] was prepared containing per liter: 4.7 g $(NH_4)_2SO_4$, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.5 g KCl, 0.5 mg each of $CaCl_2$, $CuSO_4 \cdot 5H_2O$, and $ZnSO_4 \cdot 7H_2O$, and 0.1 mg Fe^{3+} provided as 0.5 mg $FeCl_3 \cdot 6H_2O$ or 0.2 ml microelements solution [44]. The medium was made at pH 7 by the addition of 8.2 g KH_2PO_4 and 1.6 g NaOH or, for cultures to be subjected to DAF-FM DA analysis, at pH 7.9 by the addition of 3.48 g KH_2PO_4 and 2.84 g Na_2HPO_4 per liter. Concentrated solutions of C sources were added following autoclaving of the medium to provide a final ratio of 3.5 mol C to 1 mol N. For growth yield comparisons (Fig. 2) exponential phase cells from nutrient broth cultures were centrifuged at 5000*g* for 5 min, inoculated into 30 ml of medium to 0.001 optical density units at 600 nm (OD_{600}), and incubated with 120 rpm shaking at 26 °C.

Rhodococcus APG1 colonization of A. pinnata

Rhodococcus sp. strain APG1 was previously isolated from a healthy $HOCl/H_2O_2$-treated frond of *A. pinnata* that originated from a population in Ginowan, Okinawa [41]. Plants derived from an *A. pinnata* population about 30 km distant in Kin, Okinawa did not show the presence of *Rhodococcus* APG1. Therefore, these plants were used for determining the ability of *Rhodococcus* APG1 to colonize *A. pinnata*.

In separate experiments, cultured stationary phase *Rhodococcus* APG1 cells from nutrient broth or sucrose cultures were centrifuged at 5000*g* and resuspended to 0.014 or 0.03 OD_{600}, respectively, in beakers containing 200 ml sterile water; one OD_{600} unit corresponded to approximately 1.1×10^9 colony-forming units (cfu) per ml. Plants from an outdoor pond confluent with *A. pinnata* were placed in a strainer and rinsed for 5 min under tap water. The plants were then placed in nylon nets and immersed in either the *Rhodococcus* APG1 suspension or in sterile water only as a control and vacuum infiltrated for 5 min. Plants having a measured surface area of 13.85 cm^2 were transferred to 500 ml

Erlenmyer flasks containing 200 ml plant culture medium that was changed weekly. Cultivation conditions were as previously described except for use of 0.5 mM total phosphate in the N-free medium [45]; *A. pinnata* obtains N as ammonium from symbiotic N_2-fixing cyanobacteria in dorsal leaf cavities [42].

To monitor changes in culturable bacterial populations on the leaves of *A. pinnata*, samples were periodically removed, de-rooted, wrapped in a nylon net, rinsed 10 min under a stream of tap water, and treated successively with 15% commercial bleach/0.01% SDS for 1 min followed by three sterile water rinses and 3 min with 3% H_2O_2 followed by two sterile water rinses [41]. The fronds were then weighed to 0.05 g, ground in sterile microfuge tubes, and brought up to 1 ml total volume with sterile water. The ground extract was serially diluted in C-free medium, plated onto nutrient agar (Difco), and incubated at 26 °C in darkness. After 5 days the plates were placed under fluorescent light ($60\ \mu mol\ m^{-2}\ s^{-1}$) for 3 days to induce photochromogenesis; in response to light *Rhodococcus* APG1 shows a sevenfold increase in a carotenoid spectrophotometrically similar to β-carotene [41]. Colonies were identified as *Rhodococcus* APG1 based on time of appearance ($t = 5$ days), morphology (smooth with entire margins), and photochromogenesis. The identity of randomly selected colonies was further confirmed by a positive catalase test and lack lysis following 3% KOH treatment [41].

H_2O_2 degradation and tolerance

The H_2O_2 degradation activity and H_2O_2 resistance of stationary phase *Rhodococcus* APG1 cells were determined based on the methods of Chen et al. [12]. To measure degradation activity, cells were diluted in a cuvette to 0.05 OD_{600} in 50 mM phosphate buffer for a final volume of 960 µl. The reaction was started by adding 40 µl of 3% H_2O_2 and loss of H_2O_2 was monitored by the decline in A_{240} at 25 °C. The units of activity were recorded as $\Delta A_{240}\ min^{-1}\ OD_{600}^{-1}$.

To measure resistance to killing by H_2O_2, cells were harvested by centrifugation at 5000*g*, suspended in C-free medium containing from 0 to 1 M H_2O_2 for 15 min, and then serially diluted in 10-fold increments. Samples were spread onto nutrient agar to determine the decrease in cfu per ml caused by exposure to H_2O_2.

Measurement of NO_2^- and NO

The formation of NO_2^- was monitored by periodic removal of 0.5 ml medium from the culture flask. The sample was centrifuged at 18,000*g* and the supernatant combined with an equal volume of sulfanilamide (1%, w/v in 3 N HCl) followed by *N*-napthylethylenediamine dihydrochloride (0.02%, w/v). After a 15–30 min incubation at room temperature, the A_{540} was measured and NO_2^- concentrations calculated based on a standard curve made from a $NaNO_2$ dilution series. To test for induction of NO_2^- formation, 1 part filter-sterilized aqueous arginine solution (or water only as a control) was added to 4 parts *Rhodococcus* APG1 culture in test tubes.

NO production was measured as fluorescence from a triazole product (DAF-FM T) formed in the reaction of NO and O_2 with 3-amino-4-(*N*-methylamino)-2′,7′-difluorofluorescein diacetate (DAF-FM DA; Daiichi Pure Chemicals, Tokyo) [46]. One ml *Rhodococcus* APG1 cells (0.1 OD_{600}) cultured in pH 7.9 medium was combined in a cuvette with 1 ml of an assay buffer containing: 10 mM Hepes/KOH (pH 8.2), 7.5 mM K_2SO_4, 10 µM $CaCl_2$, and 6 µM EDTA. The cuvette was placed in a fluorescence spectrophotometer (RF-5300PC, Shimadzu, Kyoto, Japan) and the solution equilibrated to 30 °C with constant magnetic bar stirring. The NO detection reaction was initiated by addition of 8 µl of 20 µM DAF-FM DA. To determine L-arginine-induced NO formation, 220 µl of a solution containing 0–200 mM L-arginine in 5% ethanol was added to the cell suspension. The excitation and emission wavelength settings on the spectrophotometer were 500 and 515 nm, respectively. Cells for use in a negative control were killed by heating for 30 s in a microwave oven.

Results

Colonization of A. pinnata by Rhodococcus APG1

Rinsed plants from a population of *A. pinnata* that did not show signs of colonization by *Rhodococcus* APG1 were inoculated by vacuum infiltration with stationary phase *Rhodococcus* APG1 cells and incubated under laboratory conditions. Inoculated and non-inoculated control plants were found to have similar growth yields and morphology. From a starting surface area of 13.85 cm^2, after seven days the control plants grew to $31.0 \pm 2.8\ cm^2$ (mean ± SE, $n = 9$) while those inoculated with *Rhodococcus* APG1 reached $34.7 \pm 1.4\ cm^2$ (mean ± SE, $n = 9$).

To monitor populations of bacteria in close association with the leaves, plants were occasionally removed, the fronds subjected to a $HOCl/H_2O_2$ treatment, and bacteria isolated from ground tissue as described in Experimental procedures. *Rhodococcus* APG1 established a stable population density of $2–4 \times 10^7$ cfu g^{-1} in inoculated plants (Fig. 1) and were not found in control plants. Other bacteria were detected from $HOCl/H_2O_2$-treated tissue up to 2 weeks after inoculation but not thereafter (Fig. 1), raising the possibility that they were out-competed by *Rhodococcus* APG1.

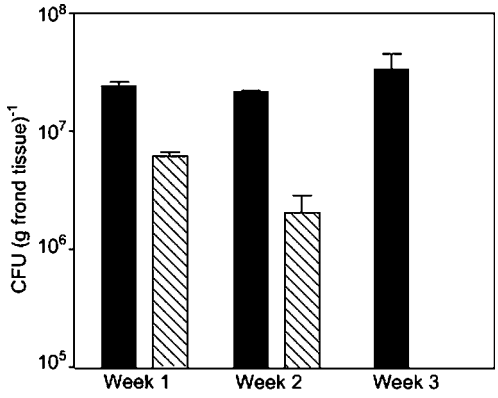

Fig. 1. Epiphytic colonization of *Azolla pinnata* by *Rhodococcus* APG1. Bars represent average numbers of *Rhodococcus* APG1 (black) and other bacteria (hatched) that survived surface sterilization of fronds with bleach and H_2O_2. Error bars indicate the range of variation between duplicate countings of bacteria isolated from fronds. The baseline is set at the approximate limit of detection, 10^5 cfu (g fresh frond weight)$^{-1}$.

Comparative C source-dependent growth yields

Fig. 2 shows representative growth curves of *Rhodococcus* APG1 on a variety of C sources in a mineral salts medium containing a high concentration of ammonium (71 mM) and low concentration of iron (2.2 μM). The yield of cells cultured in sucrose medium was lower than that of cells in media containing equivalent moles of C provided as glucose, fructose, mannitol, or glycerol (Fig. 2). This is of particular interest, since sucrose is likely to be the predominant C source available to *Azolla*-associated bacteria [47]. Growth rates of cells were difficult to determine by optical density measurements due to their tendency to clump during exponential phase.

A role for the sucrose molecule itself in the growth yield inhibition was inferred from our finding of a high yield of cells in medium containing an equal proportion of glucose and fructose, the monomeric components of sucrose, as the sole source of C source in the medium (Fig. 2). However, any inhibitory function for sucrose can apparently be overridden, since the combination of sucrose and mannitol in the culture medium did not reduce the growth yield compared to that in medium with mannitol as the sole C source (data not shown).

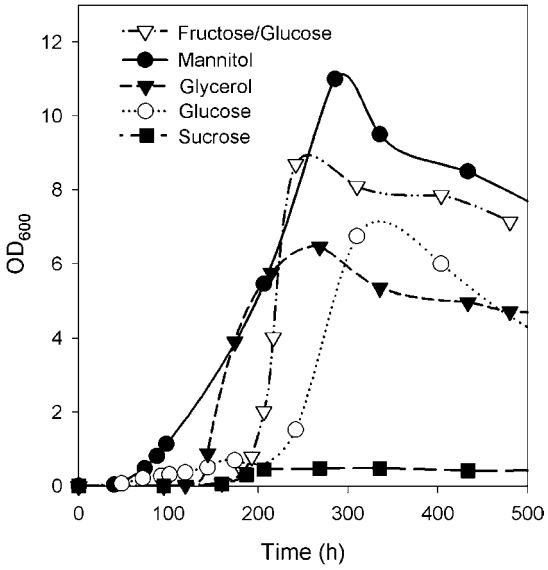

Fig. 2. Growth of *Rhodococcus* APG1 in liquid mineral salts media containing equivalent concentrations of C provided as 0.75% glucose, a combination of 0.375% glucose and 0.375% fructose, 0.61% glycerol, 0.76% mannitol or 0.7125% sucrose. For each growth condition the experiment was repeated at least three times with similar results.

Sucrose-enhanced H_2O_2 tolerance

Since *Rhodococcus* APG1 cells are likely to be challenged by exposure to H_2O_2 in planta, we examined cultured cells for tolerance to H_2O_2. The H_2O_2 degrading activity of nutrient broth-grown cells increased from 0.14 ± 0.03 U ($n = 5$) in exponential phase to 0.48 ± 0.01 U ($n = 4$) in stationary phase (means ± SE). Compared to stationary-phase cells cultured in mineral salts medium with other C sources, those provided with only sucrose had sixfold higher H_2O_2-degrading activity (Fig. 3) and were less susceptible to killing by H_2O_2 (Table 1). Following a 15 min treatment with 250 mM H_2O_2, the surviving numbers of glycerol-, fructose/glucose-, and mannitol-grown cells were only 7.3, 6.8, and 0.2% of sucrose-grown cells, respectively (Table 1). Cells did not survive a 15 min treatment with 1 M H_2O_2 regardless of the C source utilized for growth.

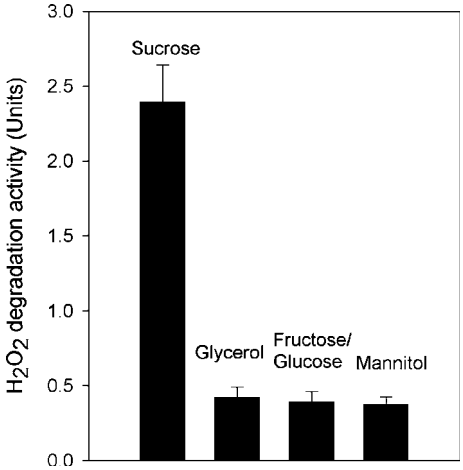

Fig. 3. C source-dependent degradation of H_2O_2 by *Rhodococcus* APG1 stationary phase cells. Values are means of at least three independent measurements. Bars, standard error.

Table 1
C source-dependent tolerance to H_2O_2 by *Rhodococcus* APG1 stationary phase cells

C source	Percentage of cells surviving treatment		
	10 mM H_2O_2	100 mM H_2O_2	250 mM H_2O_2
Sucrose	73.1 ± 4.6	66.8 ± 5.3	32.5 ± 1.5
Glycerol	100 ± 2.9	50.6 ± 19.5	2.4 ± 0.4
Fructose/glucose	88.0 ± 8.6	79.8 ± 6.2	2.2 ± 0.5
Mannitol	87.8 ± 5.2	8.3 ± 0.4	0.07 ± 0.02

Cells were sampled 24–48 h following the end of exponential phase growth. Values indicate percentage of surviving cfu per ml after a 15 min treatment with H_2O_2 relative to the cfu per ml from the untreated source culture from two independent experiments (means ± range of variation). C source concentrations as in Fig. 2.

NO production by NOS

NO is known to activate the synthesis of H_2O_2 degrading enzymes in bacteria [9] and can inhibit bacterial growth [21,22], two phenomena which we found to be associated with *Rhodococcus* APG1 cells cultured in sucrose medium. Therefore, we examined the effect of sucrose relative to other C sources on NO production in *Rhodococcus* APG1 cultures. NOS activity has been detected in crude homogenates of *Rhodococcus* sp. strain R312 by the formation of NO_2^- (a stable oxidation product of NO) coincident with the conversion of L-arginine to L-citrulline [27]. In all *Rhodococcus* APG1 mineral salts medium cultures NO_2^- accumulation began in exponential phase and peaked several days into stationary phase (Fig. 4, inset). On a cell density basis, sucrose cultures showed the highest level of NO_2^- accumulation (Fig. 4). An increase in NO_2^- accumulation was stimulated by supplementation of cultures with L-arginine (the substrate for NOS), but not with D-arginine (Fig. 5), L-glutamate or L-citrulline (data not shown), indicating that the NO_2^- was ultimately derived from NOS activity.

Direct evidence of NO production by *Rhodococcus* APG1 was obtained by use of the NO-specific detection agent DAF-FM DA, which upon reacting with NO under aerobic conditions, produces the fluorescent product DAF-FM T (Fig. 6, inset) [46]. Addition of L-arginine to sucrose-grown *Rhodococcus* APG1 cells resulted in dose-dependent increases in NO production above the basal level of uninduced cells (Fig. 6), whereas D-arginine showed no effect (Fig. 5, inset). Heat-killed control cells did not display detectable basal NO production and released barely detectable levels of NO following addition of 15 mM L-arginine (Fig. 6). Known inhibitors of mammalian NOSs can inhibit the activity of bacterial NOSs in crude homogenates or purified preparations but not of intact *S. aureus* cells [48]. Accordingly, we found that treatment of *Rhodococcus* APG1 cells with 250 μM *N*-nitro-arginine, aminoguanidine, 7-nitroindazole, or *S*-methylisothiocitrulline had no effect on NO production (data not shown).

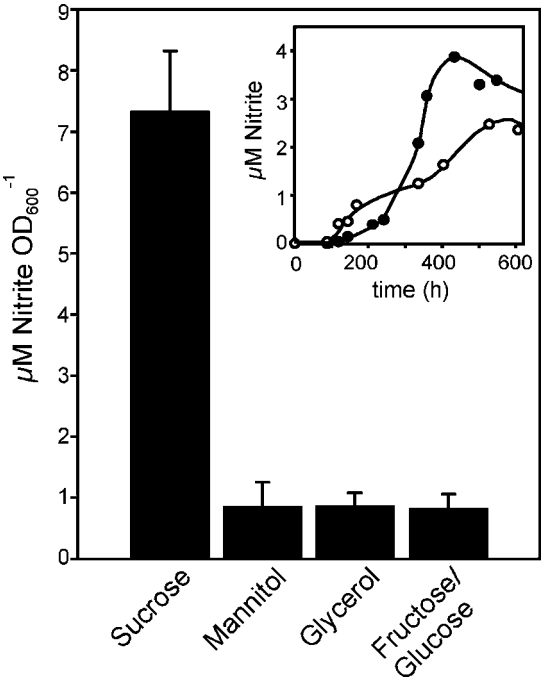

Fig. 4. Maximal nitrite accumulations per unit of cell density in stationary phase *Rhodococcus* APG1 cultures containing different C sources. Values are the means of at least three independent measurements. Bars, standard error. *Inset*: time course of nitrite concentrations (not normalized) in sucrose (open circles) and mannitol (closed circles) cultures.

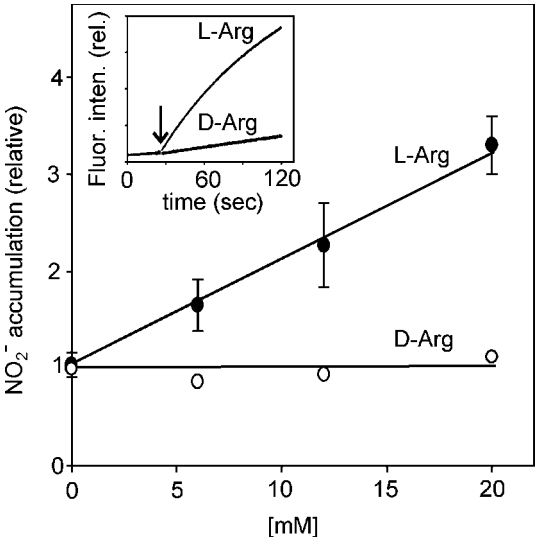

Fig. 5. Accumulations of nitrite in early stationary phase *Rhodococcus* APG1 cultures supplemented with L-arginine or D-arginine following overnight incubation. Values are plotted relative to the nitrite concentration per OD_{600} in the source culture at $t = 0$. L-Arg data points represent the average of two independent experiments with the error bars indicating the range of values. D-Arg data points are the results of a single experiment. *Inset*: change in fluorescence intensity from DAF-FM T in a suspension of sucrose-grown *Rhodococcus* APG1 cells following addition of 15 mM L- or D-arginine with 0.5% ethanol, at the time indicated by the arrow.

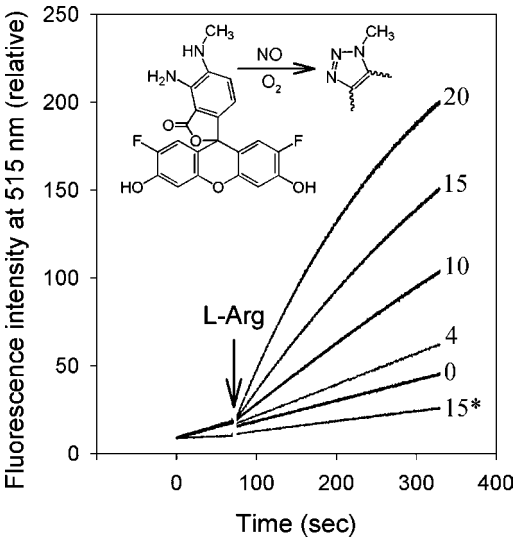

Fig. 6. L-Arginine-stimulated *Rhodococcus* APG1 NO production as detected by DAF-FM T fluorescence. Numbers to the right of the plots give the concentration in mM of L-arginine added to the cell suspensions, along with 0.5% ethanol, at the time indicated by the arrow. The asterisk (*) signifies a suspension containing heat-killed cells. *Inset*: a scheme based on Itoh et al. [46] depicting the formation of the triazole ring in DAF-FM T by the reaction of non-fluorescent DAF-FM with NO and O_2.

Discussion

Colonization of plants by Rhodococcus spp.

Rhodococcus sp. strain APG1 was originally isolated from a healthy frond of the subtropical fern *A. pinnata* [41]. Adaptations of tropical and subtropical phyllobacteria are of particular interest, since their plant hosts are subject to relatively high light irradiance and temperatures, potential causes of oxidative stress for the hosts. Studies of phyllobacteria, however, have focused primarily on those found on temperate agricultural crops [3,49]. In this report, we have confirmed the ability of *Rhodococcus* APG1 to non-pathogenically colonize *A. pinnata* and have demonstrated sucrose-enhanced H_2O_2 tolerance, a trait likely to have relevance in the plant-associated state.

Owing to a combination of tolerance and avoidance, some phyllobacteria can survive surface-sterilant treatment of their host leaves [4]. Bacteria inside dorsal leaf cavities of all *Azolla* species (section *Azolla*) are largely sheltered from surface-sterilants. But for *A. pinnata* (section *Rhizosperma*), whose cavity pores are more than twice in diameter [50], cavity residents are not protected [51]. Although we did not determine localization of the bacteria on *A. pinnata* fronds, *Rhodococcus* spp. are known to exist as endophytes of plants. Cells of pathogenic and non-pathogenic strains of *Rhodococcus fascians* are protected from surface sterilants by entering into the intercellular spaces of *Arabidopsis thaliana* or tobacco leaves [38] and *Rhodococcus luteus* has been found among xylem-inhabiting bacterial communities of grapevine [40]. Thus, it is likely that intracellular colonization accounts for the survival of *Rhodococcus* APG1 cells following surface-sterilant treatment of the fronds.

Mechanisms for sucrose-specific growth control

Sucrose could serve as an ideal plant-specific cue for bacterial immigrants, since it is the most common extractable sugar in the leaves of most plants, including *Azolla* [47]. Growth of *Rhodococcus* APG1 on sucrose in culture resulted in cells reaching stationary phase at a much lower density than would be expected based on the growth yield of cells grown on a combination of glucose and fructose. Stationary phase dark/sucrose-grown cells of *Rhodococcus* APG1 also show higher tolerance to light exposure that appears to correlate with faster photo-induction of carotenoid formation [41] (M.F.C., unpublished data). Concentrating resources on stress tolerance mechanisms, rather than on maximizing growth, may better serve the long-term interest of the bacterium in planta. The mechanism by which sucrose exerts regulation over growth yield and stress tolerance is unknown but clues may be found from studies on other bacteria, especially those within the family Corynebacterineae, which includes *Rhodococcus*, *Nocardia*, *Mycobacterium*, and *Corynebacterium*.

In *Corynebacterium glutamicum* sucrose uptake occurs via a phosphoenolpyruvate-dependent::sucrose phosphotransferase system (PTS^{suc}) [52]. Inside *C. glutamicum* sucrose is cleaved by invertase into glucose and fructose but, since the bacterium lacks fructokinase, the fructose must first be exported and brought back into the cell by group translocation in order to be metabolized [52]. The extreme differences we observed between the sucrose and glucose/fructose growth yields could possibly be explained to some extent if a similar mechanism for sucrose metabolism is operational and subject to regulation in *Rhodococcus* APG1.

The well-studied PTS^{suc} of *B. subtilis* uses sucrose-specific signal transduction to regulate expression of genes involved in sucrose metabolism [53]. In facultative plant-colonizing bacteria like *Rhodococcus* APG1, there would be adaptive logic in extending the sucrose regulatory network to other genes such as those involved in stress tolerance. Expression of PTS^{suc} components are generally subject to catabolite repression which may explain the observed loss of a sucrose effect on *Rhodococcus* APG1 in the presence of the readily catabolized carbon source mannitol.

Possible regulatory roles for NO

NOS genes and/or activity have been reported from several bacteria, including a *Rhodococcus* sp. [27], but

our results are the first to directly show in vivo production of NO by NOS in bacteria. This is an important point because, although NO_2^- formed in NOS-containing cells or suspensions is usually a product of the spontaneous oxidation of NO, it is also known that NOS can directly generate NO_2^- under certain conditions [24,54].

Our data showing sucrose-stimulated NO production by *Rhodococcus* APG1 implies that the activity of NOS may be connected in some manner with the putative sucrose regulatory network. The only reported link between sucrose and NO metabolism we have found is in the pond snail *Lymnea stagnalis* where sucrose elicits a positive feeding response by stimulating the NOS activity of buccal ganglia through an unknown mechanism [55]. (This finding may hold additional relevance to the *Azolla–Rhodococcus* relationship, since *Lymnea swinhoei* is known to display species-selective feeding on *Azolla* plants [56].) NO released during growth of *Rhodococcus* APG1 on sucrose could conceivably explain lower growth yields if NO levels are high enough to inhibit enzymes involved in energy metabolism [21,22], but this possibility requires further study.

Autogenic NO production by *Rhodococcus* APG1 may prime cells for oxidative stress by activating transcription factors like OxyR and SoxR [18,19,21,28,35] and by activating guanylate cyclase to increase synthesis of cGMP [31,33]. Conversely, at high enough levels NO can directly inhibit catalase activity [57]. Thus, the effects of NO on bacteria are likely to be dependent on a variety of factors including the concentration and rate of NO production, as well as the oxidation state, growth phase, and density of the cells. Such competing influences of NO may explain the highly variable H_2O_2 degradation activities of cells treated with L-arginine or the NO donor NOC5 (M.F.C., unpublished results).

NO production by phyllobacteria in the leaf habitat may help ward off potential competitors by activating antimicrobial responses of the host. NO can increase H_2O_2 levels in leaves [33] and both H_2O_2 and NO can trigger signaling pathways in plants that lead to the production of protective phenolics [6,58,59]. *R. fascians*-infected tobacco leaves synthesize greater amounts of certain phenolic compounds that are not inhibitory to *R. fascians* cells [60] but may inhibit the growth of other microbes [61,62].

Several studies have found NOS-like activity in plants, but to our knowledge no plant NOS has been purified nor has a plant NOS gene been found. Moreover, extracts of several plants have one or more proteins that cross-react with antibodies raised against mammalian NOSs but the majority have sizes within the range observed for bacterial NOSs (40–100 kDa), not mammalian NOSs (130–185 kDa) [6,59]. Combined with our results showing NOS activity by an endophytic bacterium, these observations imply that care should be taken to demonstrate axenic plant tissue sources in studies of putative plant NOS.

Acknowledgments

We thank Dr. Mark Mazzola for a critical reading of the manuscript and Emi Yamamoto for technical assistance. M.F.C. was supported by a fellowship from the Japanese Society for the Promotion of Science (JSPS).

References

[1] J. Mercier, S.E. Lindow, Role of leaf surface sugars in colonization of plants by bacterial epiphytes, Appl. Environ. Microbiol. 66 (2000) 369–374.
[2] M.A. Holland, R.L.G. Long, J.C. Polacco, *Methylobacterium* spp.: phylloplane bacteria involved in cross-talk with the plant host?, in: S.E. Lindow, E.I. Hecht-Poinar, V.J. Elliott (Eds.), Phyllosphere Microbiology, APS Press, St. Paul, Minnesota, 2002.
[3] C.E. Morris, L.L. Kinkel, Fifty years of phyllosphere microbiology: significant contributions to research in related fields, in: S.E. Lindow, E.I. Hecht-Poinar, V.J. Elliott (Eds.), Phyllosphere Microbiology, APS Press, St. Paul, Minnesota, 2002.
[4] G.A. Beattie, S.E. Lindow, Bacterial colonization of leaves: a spectrum of strategies, Phytopathology 89 (1999) 353–359.
[5] S.A.H. Mackerness, C.F. John, B. Jordan, B. Thomas, Early signaling components in ultraviolet-B responses: distinct roles for different reactive oxygen species and nitric oxide, FEBS Lett. 489 (2001) 237–242.
[6] J. Durner, D.F. Klessig, Nitric oxide as a signal in plants, Curr. Opin. Plant Biol. 2 (1999) 369–374.
[7] J. Liu, B. Lu, L.L. Xu, An improved method for the determination of hydrogen peroxide in leaves, Prog. Biochem. Biophys. 27 (2000) 548–551.
[8] Y. Sakihama, M.F. Cohen, S.C. Grace, H. Yamasaki, Plant phenolic antioxidant and prooxidant activities: phenolics-induced oxidative damage mediated by metals in plants, Toxicology 177 (2002) 67–80.
[9] G. Storz, J.A. Imlay, Oxidative stress, Curr. Opin. Microbiol. 2 (1999) 188–194.
[10] B.L. Beaman, C.M. Black, F. Doughty, L. Beaman, Role of superoxide dismutase and catalase as determinants of pathogenicity of *Nocardia asteroides*: importance in resistance to microbicidal activities of human polymorphonuclear neutrophils, Infect. Immun. 47 (1985) 135–141.
[11] S. Dukan, T. Nystrom, Oxidative stress defense and deterioration of growth-arrested *Escherichia coli* cells, J. Biol. Chem. 274 (1999) 26027–26032.
[12] L. Chen, L. Keramati, J.D. Helmann, Coordinate regulation of *Bacillus subtilis* peroxide stress genes by hydrogen peroxide and metal ions, Proc. Natl. Acad. Sci. USA 92 (1995) 8190–8194.
[13] H.M. Hassan, I. Fridovich, Regulation of the synthesis of catalase and peroxidase in *Escherichia coli*, J. Biol. Chem. 253 (1978) 6420–6445.
[14] H.M. Steinman, F. Fareed, L. Weinstein, Catalase-peroxidase of *Caulobacter crescentus*: function and role in stationary-phase survival, J. Bacteriol. 179 (1997) 6831–6836.
[15] J. Katsuwon, A.J. Anderson, Response of plant-colonizing pseudomonas to hydrogen peroxide, Appl. Environ. Microbiol. 55 (1989) 2985–2989.

[16] U.A. Ochsner, M.L. Vasil, E. Alsabbagh, K. Parvatiyar, D.J. Hassett, Role of the *Pseudomonas aeruginosa oxyR-recG* operon in oxidative stress defense and DNA repair: OxyR-dependent regulation of *katB-ankB*, *ahpB*, and *ahpC-ahpF*, J. Bacteriol. 182 (2000) 4533–4544.

[17] X.-B. Ji, T.C. Hollocher, Mechanism for nitrosation of 2,3-diaminonaphthalene by *Escherichia coli*: enzymatic production of NO followed by O_2-dependent chemical nitrosation, Appl. Environ. Microbiol. 54 (1988) 1791–1794.

[18] S.O. Kim, K. Merchant, R. Nudelman, W.F. Beyer, T. Keng, J. DeAngelo, A. Hausladen, J.S. Stamler, OxyR: a molecular code for redox-related signaling, Cell 109 (2002) 383–396.

[19] H.E. Marshall, K. Merchant, J.S. Stamler, Nitrosation and oxidation in the regulation of gene expression, FASEB J. 14 (2000) 1889–1900.

[20] P.J. Pomposiello, B. Demple, Redox-operated genetic switches: the SoxR and OxyR transcription factors, Trends Biotechnol. 19 (2001) 109–114.

[21] H. Yu, E.F. Sato, K. Nagata, M. Nishikawa, M. Kashiba, T. Arakawa, K. Kobayashi, T. Tamura, M. Inoue, Oxygen-dependent regulation of the respiration and growth of *Escherichia coli* by nitric oxide, FEBS Lett. 409 (1997) 161–165.

[22] P.R. Gardner, G. Costantino, A.L. Salzman, Constitutive and adaptive detoxification of nitric oxide in *Escherichia coli*. Role of nitric oxide dioxygenase in the protection of aconitase, J. Biol. Chem. 273 (1998) 26528–26533.

[23] S. Adak, K.S. Aulak, D.J. Stuehr, Direct evidence for nitric oxide production by a nitric-oxide synthase-like protein from *Bacillus subtilis*, J. Biol. Chem. 277 (2002) 16167–16171.

[24] S. Adak, A.M. Bilwes, K. Panda, D. Hosfield, K.S. Aulak, J.F. McDonald, J.A. Tainer, E.D. Getzoff, B.R. Crane, D.J. Stuehr, Cloning, expression, and characterization of a nitric oxide synthase protein from *Deinococcus radiodurans*, Proc. Natl. Acad. Sci. USA 99 (2002) 107–112.

[25] W.S. Choi, M.S. Chang, J.W. Han, S.Y. Hong, H.W. Lee, Identification of nitric oxide synthase in *Staphylococcus aureus*, Biochem. Biophys. Res. Commun. 237 (1997) 554–558.

[26] D.W. Choi, H.Y. Oh, S.Y. Hong, J.W. Han, H.W. Lee, Identification and characterization of nitric oxide synthase in *Salmonella typhimurium*, Arch. Pharm. Res. 23 (2000) 407–412.

[27] M.A. Sari, C. Moali, J.L. Boucher, M. Jaouen, D. Mansuy, Detection of a nitric oxide synthase possibly involved in the regulation of the *Rhodococcus* sp R312 nitrile hydratase, Biochem. Biophys. Res. Commun. 250 (1998) 364–368.

[28] N.J. Watmough, G. Butland, M.R. Cheesman, J.W. Moir, D.J. Richardson, S. Spiro, Nitric oxide in bacteria: synthesis and consumption, Biochim. Biophys. Acta 1411 (1999) 456–474.

[29] Y. Chen, J.P. Rosazza, A bacterial nitric oxide synthase from a *Nocardia* species, Biochem. Biophys. Res. Commun. 203 (1994) 1251–1258.

[30] Y. Chen, J.P. Rosazza, Purification and characterization of nitric oxide synthase (NOS_{Noc}) from a *Nocardia* species, J. Bacteriol. 177 (1995) 5122–5128.

[31] J.K. Son, J.P.N. Rosazza, Cyclic guanosine-3′,5′-monophosphate and biopteridine biosynthesis in *Nocardia* sp., J. Bacteriol. 182 (2000) 3644–3648.

[32] H. Yamasaki, Nitrite-dependent nitric oxide production pathway: implications for involvement of active nitrogen species in photoinhibition in vivo, Philos. Trans. R. Soc. Lond. B 355 (2000) 1477–1488.

[33] D. Wendehenne, A. Pugin, D.F. Klessig, J. Durner, Nitric oxide: comparative synthesis and signaling in animal and plant cells, Trends Plant Sci. 6 (2001) 177–183.

[34] S.T. Lim, H. Hennecke, D.B. Scott, Effect of cyclic guanosine 3′,5′-monophosphate on nitrogen fixation in *Rhizobium japonicum*, J. Bacteriol. 139 (1979) 256–263.

[35] M. Kobayashi, S. Shimizu, Nitrile hydrolases, Curr. Opin. Chem. Biol. 4 (2000) 95–102.

[36] E.A. Tsavkelova, T.A. Cherdyntseva, E.S. Lobakova, G.L. Kolomeitseva, A.I. Netrusov, Microbiota of the orchid rhizoplane, Mikrobiologiia 70 (2001) 567–573.

[37] D. Vereecke, S. Burssens, C. Simon-Mateo, D. Inze, M. Van Montagu, K. Goethals, M. Jaziri, The *Rhodococcus fascians*–plant interaction: morphological traits and biotechnological applications, Planta 210 (2000) 241–251.

[38] K. Cornelis, T. Ritsema, J. Nijsse, M. Holsters, K. Goethals, M. Jaziri, The plant pathogen *Rhodococcus fascians* colonizes the exterior and interior of the aerial parts of plants, Mol. Plant–Microbe Interact. 14 (2001) 599–608.

[39] A.A. Belimov, V.I. Safronova, T.A. Sergeyeva, T.N. Egorova, V.A. Matveyeva, V.E. Tsyganov, A.Y. Borisov, I.A. Tikhonovich, C. Kluge, Preisfeld, et al., Characterization of plant growth promoting rhizobacteria isolated from polluted soils and containing 1-aminocyclopropane-1-carboxylate deaminase, Can. J. Microbiol. 47 (2001) 642–652.

[40] C.R. Bell, G.A. Dickie, W.L.G. Harvey, J.W.Y.F. Chan, Endophytic bacteria in grapevine, Can. J. Microbiol. 41 (1995) 46–53.

[41] M.F. Cohen, T. Meziane, H. Yamasaki, A photocarotenogenic *Rhodococcus* sp. isolated from the symbiotic fern *Azolla*, in: M. Sugiura (Ed.), (Endo)Symbiosis and Eukaryotic Organelles, Logos Verlag, Berlin (2003) (in press).

[42] G.A. Peters, J.C. Meeks, The *Azolla–Anabaena* symbiosis: basic biology, Annu. Rev. Plant Physiol. Plant Mol. Biol. 40 (1989) 193–210.

[43] W. Verstraete, M. Alexander, Heterotrophic nitrification by *Arthrobacter* sp, J. Bacteriol. 110 (1972) 955–961.

[44] M.B. Allen, D.I. Arnon, Studies on nitrogen-fixing blue-green algae. I. Growth and nitrogen fixation by *Anabaena cylindrica* Lemm, Plant Physiol. 30 (1955) 366–372.

[45] M.F. Cohen, J. Williams, H. Yamasaki, Biodegradation of diesel fuel by an *Azolla*-derived bacterial consortium, J. Environ. Sci. Health A37 (2002) 1593–1606.

[46] Y. Itoh, F.H. Ma, H. Hoshi, M. Oka, K. Noda, Y. Ukai, H. Kojima, T. Nagano, N. Toda, Determination and bioimaging method for nitric oxide in biological specimens by diaminofluorescein fluorometry, Anal. Biochem. 287 (2000) 203–209.

[47] D. Kaplan, G.A. Peters, The *Azolla–Anabaena azollae* relationship. XIV. Chemical composition of the association and soluble carbohydrates of the association, endophyte-free *Azolla*, and the freshly isolated endophyte, Symbiosis 24 (1998) 35–50.

[48] W.S. Choi, D.W. Seo, M.S. Chang, J.W. Han, S.Y. Hong, W.K. Paik, H.W. Lee, Methylesters of L-arginine and *N*-nitro-L-arginine induce nitric oxide synthase in *Staphylococcus aureus*, Biochem. Biophys. Res. Commun. 246 (1998) 431–435.

[49] J. Hallmann, A. Quadt-Hallmann, W.F. Mahaffee, J.W. Kloepper, Bacterial endophytes in agricultural crops, Can. J. Microbiol. 43 (1997) 895–914.

[50] P. Veys, A. Lejeune, C. van Hove, The pore of the leaf cavity of *Azolla*: interspecific morphological differences and continuity between cavity envelopes, Symbiosis 29 (2000) 33–47.

[51] M.J. Petro, J.E. Gates, Distribution of *Arthrobacter* sp. in the leaf cavities of four species of the N-fixing *Azolla* fern, Symbiosis 3 (1987) 41–48.

[52] H. Dominguez, N. Lindley, Complete sucrose metabolism requires fructose phosphotransferase activity in *Corynebacterium glutamicum* to ensure phosphorylation of liberated fructose, Appl. Environ. Microbiol. 62 (1996) 3878–3880.

[53] P. Tortosa, S. Aymerich, C. Lindner, M.H. Saier Jr., J. Reizer, D. Le Coq, Multiple phosphorylation of SacY, a *Bacillus subtilis* transcriptional antiterminator negatively controlled by the phosphotransferase system, J. Biol. Chem. 272 (1997) 17230–17237.

[54] S. Adak, Q. Wang, D.J. Stuehr, Arginine conversion to nitroxide by tetrahydrobiopterin-free neuronal nitric-oxide synthase. Implications for mechanism, J. Biol. Chem. 275 (2000) 33554–33561.
[55] S. Kobayashi, H. Sadamoto, H. Ogawa, Y. Kitamura, K. Oka, K. Tanishita, E. Ito, Nitric oxide generation around buccal ganglia accompanying feeding behavior in the pond snail, *Lymnaea stagnalis*, Neurosci. Res. 38 (2000) 27–34.
[56] M.F. Cohen, T. Meziane, M. Tsuchiya, H. Yamasaki, Feeding deterrence of *Azolla* in relation to deoxyanthocyanin and fatty acid composition, Aquat. Bot. 74 (2002) 181–187.
[57] D. Clark, J. Durner, D.A. Navarre, D.F. Klessig, Nitric oxide inhibition of tobacco catalase and ascorbate peroxidase, Mol. Plant–Microbe Interact. 13 (2000) 1380–1384.
[58] G.P. Bolwell, Role of active oxygen species and NO in plant defence responses, Curr. Opin. Plant Biol. 2 (1999) 287–294.
[59] M.V. Beligni, L. Lamattina, Nitric oxide in plants: the history is just beginning, Plant Cell Environ. 24 (2001) 267–278.
[60] D. Vereecke, E. Messens, K. Klarskov, A. DeBruyn, M. VanMontagu, K. Goethals, Patterns of phenolic compounds in leafy galls of tobacco, Planta 201 (1997) 342–348.
[61] H.M. Appel, Phenolics in ecological interactions: the importance of oxidation, J. Chem. Ecol. 19 (1993) 1521–1552.
[62] F.C. Fischer, H. van Doorne, M.I. Lim, A.B. Svendsen, Bacteriostatic activity of some coumarin derivatives, Phytochemistry 15 (1976) 1078–1079.