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Article

Plant-Pathogenic *Streptomyces* Species Produce Nitric Oxide Synthase-Derived Nitric Oxide in Response to Host Signals

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SUMMARY

Nitric oxide (NO) is a potent intercellular signal for defense, development, and metabolism in animals and plants. In mammals, highly regulated nitric oxide synthases (NOSs) generate NO. NOS homologs exist in some prokaryotes, but direct evidence for NO production by these proteins has been lacking. Here, we demonstrate that a NOS in plant-pathogenic *Streptomyces* species produces diffusible NO. NOS-dependent NO production increased in response to cellobiose, a plant cell wall component, and occurred at the host-pathogen interface, demonstrating induction by host signals. These data document in vivo production of NO by prokaryotic NOSs and implicate pathogen-derived NO in host-pathogen interactions. NO may serve as a signaling molecule in other NOS-containing bacteria, including the medically and environmentally important organisms *Bacillus anthracis*, *Staphylococcus aureus*, and *Deinococcus radiodurans*.

INTRODUCTION

Nitric oxide (NO) is a potent intercellular signal in animals and plants. Because of its small size and lipophylic nature, NO can diffuse rapidly across biological membranes and travel for hundreds of microns in biological systems (Moller et al., 2005; Rubbo et al., 2002). As a free radical, NO reacts with a multitude of biological targets, including heme groups and sulfhydryl groups . Nitrosation, the reaction of NO with cysteine residues in proteins, is thought to be an important posttranslational modification that can modulate protein function (Gow et al., 2004). The mobility and reactivity of NO in biological systems makes it an ideal signaling molecule. In mammals, NO mediates key aspects of blood pressure, hormone release, nerve transmission, and the immune response.

NO is also an important defense and metabolic signaling molecule in plants. The mechanisms by which plants produce NO remain elusive and have been the subject of much controversy (Crawford et al., 2006). NO is generated in response to pathogen infection, leading to the upregulation of many defense-related genes, including phenylalanine ammonia lyase (PAL) and pathogenicity-related 1 (PR-1) gene (Delledonne et al., 1998, 2001). Furthermore, NO functions as a short-range hormonal signaling molecule, affecting physiological processes such as stomatal closure, stress response, and adventitious and lateral root formation (Neill et al., 2003).

Nitric oxide synthases (NOSs) in mammals generate NO in a highly regulated manner. These dimeric proteins contain an N-terminal oxygenase domain that converts arginine into citrulline and the molecular signal NO, as well as a reductase domain that shuttles electrons to the heme of the oxygenase domain (Garcin et al., 2004). Genome sequencing has revealed that some bacteria contain genes coding for truncated NOS proteins; this finding is consistent with reports of NOS-like activities in bacterial extracts. Bacterial NOSs have been found in more than 20 bacterial genera, including *Bacillus*, *Staphylococcus*, and *Deinococcus*. Structural analysis suggests that bacterial NOSs should have the capacity to produce NO and is supported by in vitro production with extracted and purified proteins (Buddha et al., 2004; Wang et al., 2007). Indirect in vivo evidence for NOS-dependent NO production in *B. subtilis*, *Rhodococcus* sp. strain APG1, and other *Streptomyces* species also exists (Cohen and Yamasaki, 2003; Cohen et al., 2005; Gusarov and Nudler, 2005). However, conserved differences between mammalian and bacterial NOSs, the absence of a C-terminal reductase domain, and the lack of direct evidence for the production of NO in vivo cast doubt on the ability of the truncated NOSs that exist in bacteria to produce NO in nature.

We have investigated a NOS in plant-pathogenic streptomycetes for its role in the biosynthesis of thaxtomins, a family of nitrated dipeptide phytotoxins (Figure 1). Through multiple lines of evidence, we demonstrated that this NOS provides the active nitrogen species used in nitration, the first, to our knowledge, example of a biological function for a bacterial NOS (Kers et al., 2004). Thaxtomins play a role in cellulose biosynthesis inhibition, which is essential for plant pathogenicity in most pathogenic streptomycetes (Fry and Loria, 2002; Scheible et al., 2003). While investigating the role of NOS in the nitration of thaxtomin, we obtained evidence suggesting that this NOS was also responsible

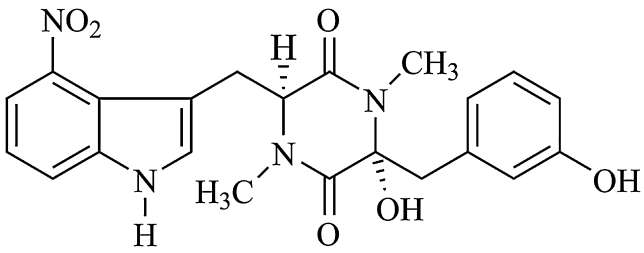

Figure 1. Thaxtomin A

for producing freely diffusible NO (Wach et al., 2005). These data were noteworthy for two reasons: bacterial NOSs had never before, to our knowledge, been demonstrated to release NO in vivo, and production of NO has significant implications for bacterial signaling, as well as for eukaryote-microbe and microbe-microbe interactions. Here, we show that plant-pathogenic *Streptomyces* spp. produce NOS-derived NO at the host-pathogen interface, and that NO synthesis is induced by the plant cell wall component, cellobiose.

RESULTS

NOS-Dependent NO Production

Detection of NO in biological systems is complicated by the chemically complex cellular environment and the short half-life of NO. For selective detection, we used electron spin resonance (ESR) with spin traps that react with NO to give a unique and longer-lived ESR spectrum. The NO scavenger, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (CPTIO), has a native five-peak ESR signal, whereas the NO reaction product, CPTI, produces a seven-peak ESR signal (Akaike and Maeda, 1996). To determine whether NOS-dependent NO production occurs in thaxtomin-producing streptomycetes, 1 mM CPTIO was added to oat bran broth (OBB) cultures of both the wild-type strain *S. turgidiscabies* Car8 and the *nos* deletion strain Car8Δ*nos*. The five-peak CPTIO signal disappeared in cultures of *S. turgidiscabies* Car8 over a period of 40 min after adding the spin trap, whereas Car8Δ*nos* cultures retained a strong CPTIO signal after 40 min, suggesting that NOS-derived NO reacts with the spin trap and is present at relatively high concentrations (Figure 2A). The lack of a seven-peak CPTI signal in Car8 cultures is consistent with the instability of CPTI in biological systems, but it does not directly demonstrate the presence of NO (Akaike and Maeda, 1996).

To further investigate NO production, we repeated ESR experiments by using the spin trap dithiocarboxy-sarcosine (DTCS). DTCS binds iron to form the active spin trap $Fe(DTCS)_2$, which lacks an ESR signal. After NO binds to the $Fe(DTCS)_2$ complex, the spin trap produces a characteristic three-peak signal (Yoshimura and Kotake, 2004). $Fe(DTCS)_2$ was added to OBB-grown cultures of Car8 and Car8Δ*nos* to a final concentration of 1 mM. The spin trap was also added to OBB medium, with or without the addition of the NO donor diethylamine nitric oxide (DEANO). Incubation of $Fe(DTCS)_2$ with Car8 resulted in the appearance of the characteristic three-peak signal of NO-$Fe(DTCS)_2$, identical to that produced by addition of DEANO (Figure 2B). This NO-specific three-peak signal was not observed in samples from Car8Δ*nos* cultures. These data confirmed that NOS-dependent NO is released by thaxtomin-producing streptomycetes in a medium known to induce thaxtomin production. However, the amplitude of the NO-specific signal was well below that of the positive control in which the medium was spiked with 1 μM NO donor (Figure 2B). Determining NO concentrations in biological systems by using dithiocarbamate spin traps is difficult because they are well known for underestimating NO concentration when other biological redox compounds, including excess NO, are present (Pou et al., 1999).

To independently confirm the ESR data, we quantified NO partial pressures in the gas phase by using an ECO PHYSICS CLD 770 AL ppt Chemiluminescence NO analyzer. Partial pressures of NO in the headspace were detected in an open-flow system containing *S. turgidiscabies* Car8, with a sample flow rate of 1 l per min. Car8 and Car8Δ*nos* were grown on oat bran agar supplemented with 0.7% (w/v) cellobiose (OBAC) in a 250 ml Erlenmeyer flask fitted with two Teflon ports mounted through a silicone stopper. The Car8 strain was also grown on OBAC amended with 100 nM L-NAME, a mammalian NOS inhibitor known to inhibit thaxtomin production (Wach et al., 2005). After 3 days, the rate of NO production in the headspace of *S. turgidiscabies* Car8 cultures was 5.23 nmol/hr, whereas NO production in the headspace of Car8Δ*nos* and Car8 cultures containing L-NAME was below the detection limit (Figure 1C), confirming NOS-dependent NO production.

The Rate of NO Production Exceeds That of Thaxtomin Biosynthesis

Since NOS-dependent NO production was easily detected in the gas phase above streptomycete cultures with chemiluminescence, we used this system to further characterize NO production. Thaxtomin biosynthesis is stimulated by cell wall components, particularly cellobiose (Wach et al., 2007), resulting from transcriptional upregulation of the biosynthetic genes by the AraC-family regulator, TxtR (Johnson et al., 2007; Joshi et al., 2007b), raising the question of whether diffusible NO production is also induced by this disaccharide. A 3.9-fold increase in the rate of NO release was observed from Car8 cultures grown on OBAC compared to cultures grown on oat bran agar without cellobiose. Because of the conservation of the thaxtomin biosynthetic pathway and the NOS in other characterized pathogens, we expected that NOS-dependent NO production would also be conserved. Indeed, both *S. scabies* strain 87.22 and *S. acidiscabies* strain 84.104 produced NO at rates similar to *S. turgidiscabies*; rates vary by less than 1.36-fold of Car8 NO production, whereas thaxtomin production varies by up to 2.4-fold of Car8 levels.

Since diffusible NO is produced under conditions that also stimulate thaxtomin production, and thaxtomin biosynthesis is a sink for NOS-derived NO, we were interested in comparing the release of NO to the production of thaxtomin over time by using chemiluminescence. Car8 cultures were grown on OBAC in Erlenmeyer flasks over the course of several days. The rate of NO production was measured every 6 hr between 48 and 144 hr after inoculation and was integrated to determine total NO release into the headspace. At 12 hr intervals, cultures were harvested and thaxtomin concentration in the culture medium was determined. Car8Δ*nos* cultures were grown under

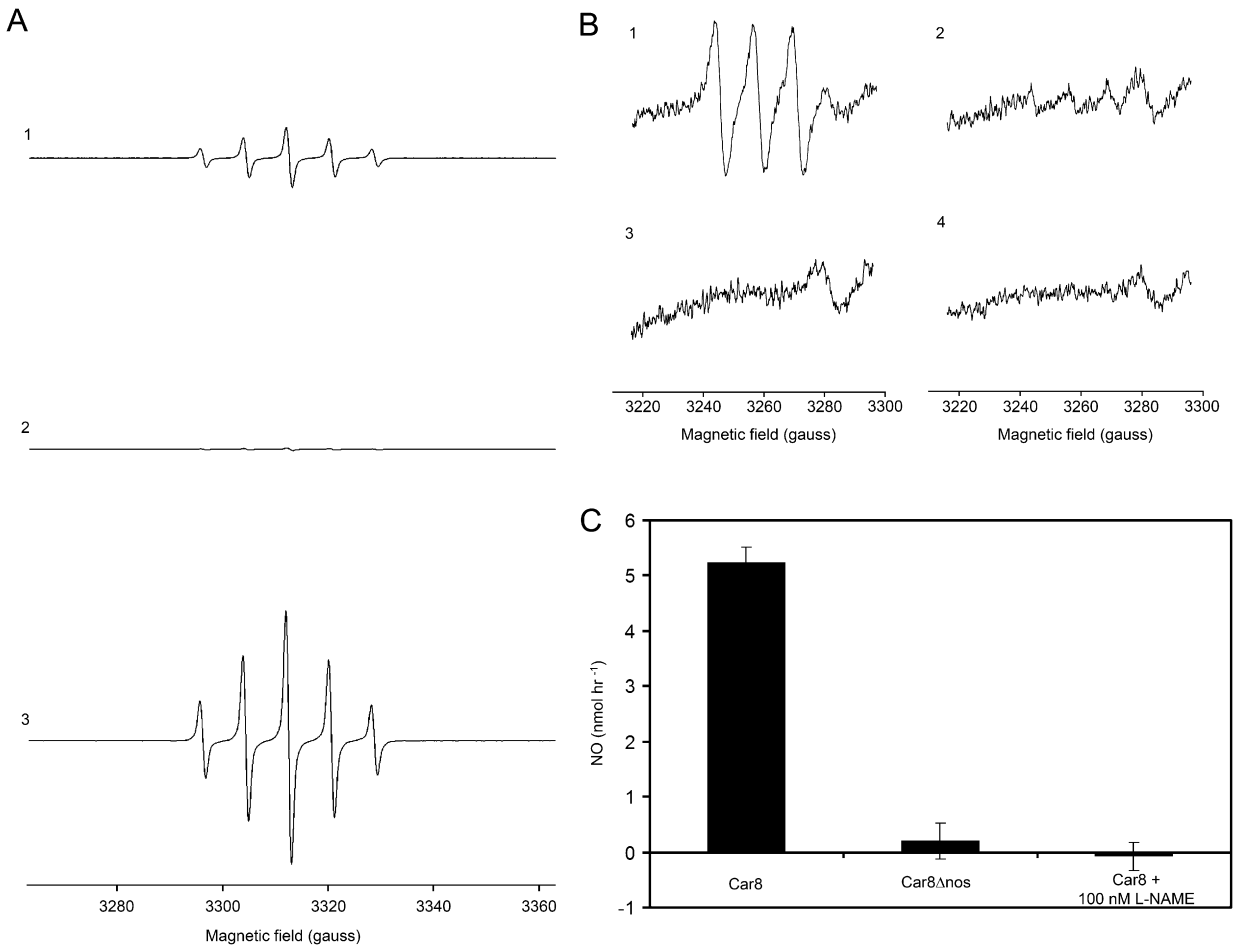

Figure 2. Evidence for NOS-Dependent NO Production

(A) ESR spectra of 1 mM CPTIO from OBB-grown cultures of *S. turgidiscabies* strain Car8 after (1) 20 min and (2) 40 min, and (3) Car8Δ*nos* after 40 min.

(B) ESR spectra of $Fe(DTCS)_2$ after a 30 min incubation in 3-day-old OBB cultures: (1) 1 μM DEANO, (2) *S. turgidiscabies* strain Car8, (3) uninoculated, (4) *S. turgidiscabies* strain Car8Δ*nos*.

(C) Chemiluminescence NO measurements from the headspace of *S. turgidiscabies* cultures grown on OBAC with or without the NOS inhibitor L-NAME. Data are presented as mean ± SD.

identical conditions, but they were measured every 12 hr for NO production and every 48 hr for thaxtomin accumulation. NO was detected in the Car8 culture headspace at approximately the same time as thaxtomin was detected in the medium (Figure 3). The production rate of NO was dynamic, with a maximum change in rate of 2.6-fold during the course of thaxtomin biosynthesis; NO production peaked at about 5.15 nmol/hr at 96 hr, then decreased to a rate of 3.2 nmol/hr until a precipitous drop in production at 144 hr (Figures 3A and 3B). In contrast, the thaxtomin concentration increased linearly at 6.7 nmol/hr from 60 to 120 hr after inoculation, after which it remained relatively stable (Figure 3C). As expected, NO and thaxtomin levels in Car8Δ*nos* cultures were consistently at or near zero.

To determine if the disparity in NO production and thaxtomin accumulation was due to degradation of thaxtomin over time, we evaluated thaxtomin concentrations in *S. turgidiscabies* Car8Δ*nos* cultures that were amended with thaxtomin (10 μg) at 120 hr, the point at which the thaxtomin concentration stabilized. Thaxtomin concentrations were stable in Car8Δ*nos* cultures until 168 hr (Table 1), verifying the integrity of the thaxtomin measurements in the time course assay.

NOS-Dependent NO Production at the Host-Pathogen Interface

Cellulose synthesis occurs in expanding plant tissues, such as root elongation zones. During cellulose synthesis, cellobiose and other cello-oligosaccharides are produced by auxin-induced plant cellulases (Ohmiya et al., 2000, 2003; Shani et al., 2006). Interestingly, scab-causing streptomycetes only infect expanding plant tissue. We have recently demonstrated that cello-oligosaccharides are released from susceptible host tissue and that this release appears to be stimulated by thaxtomin (Johnson et al., 2007). Taken together, these data suggest that NOS-derived NO might be present at the host-pathogen interface. To test this hypothesis, intracellular NO production by *S. turgidiscabies* was monitored with DAF-FM DA, a fluorescein-based dye that is taken up by cells, during root colonization. Roots of 2-week-old radish seedlings grown in sterile half-strength liquid Murashige

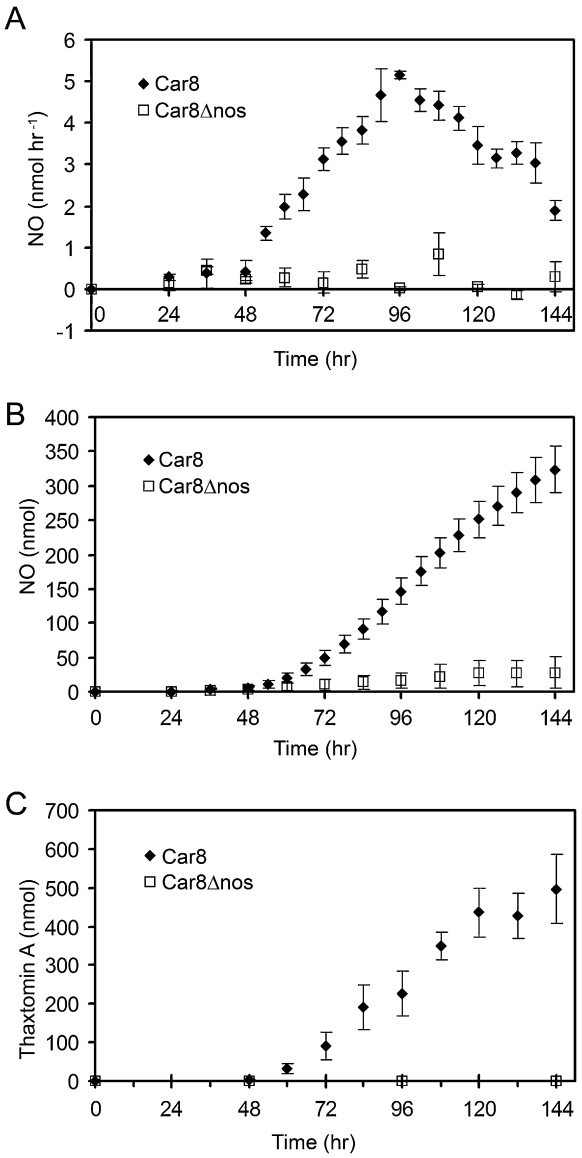

Figure 3. Comparison of NO Production to Thaxtomin A Accumulation Over Time

(A) NO production rate in the culture headspace.

(B) Total NO released to the culture headspace calculated by integrating the rate of release.

(C) Accumulation of thaxtomin A in the culture medium in Car8 (solid diamonds) and Car8Δ*nos* (open squares).

Data are presented as mean ± SD.

and Skoog (MS) plant growth medium (pH 6.3) were inoculated with *S. turgidiscabies* Car8 or Car8Δ*nos* or were not inoculated. Three to six days after inoculation, roots were stained with DAF-FM DA and were observed with confocal fluorescent microscopy. There was extensive epiphytic colonization of roots by both Car8 and Car8Δ*nos*, but only Car8 hyphae were producing NO, as indicated by fluorescence (Figures 4A–4D). Hyphae of Car8Δ*nos* (Figures 4E and 4F) were similar in appearance to the unstained control (Figures 4G and 4H). Interestingly, the location of the Car8 hyphae relative to the root greatly influenced NO production. Hyphae of Car8 associated with root tips and elongation zones almost always fluoresced (Figure 4A), and hyphae associated with root hairs frequently produced NO (Figure 4C). In contrast, NO production by hyphae colonizing other root surfaces was variable (Figures 4B and 4D). Surprisingly, the pattern of NO production within some hyphae suggested that the quantity of NO production also varied among hyphal compartments (Figure 4, arrows). Uninoculated roots showed no sign of bacterial or fungal growth, and root tissue fluorescence was not significantly different from that of inoculated roots (data not shown).

Table 1. Thaxtomin A Content, μg/5 ml Culture, of *S. turgidiscabies* Cultures Grown in OBB after 7 Days Spiked with 10 μg Thaxtomin at 5 Days

	− ThxA	+ ThxA
Car8	38.45 ± 7.75[a]	55.84 ± 2.74[a]
Car8Δ*nos*	ND[b]	11.53 ± 0.95[a]
OBB	ND[b]	9.05 ± 0.65[a]

[a] Standard deviation n = 3.

[b] ND = not detected; detection limit = 0.3 μg.

DISCUSSION

Data presented here provide the first, to our knowledge, direct evidence for NO production by a bacterial NOS in vivo. Indirect data from *Bacillus subtilis* and from other soil-dwelling *Streptomyces* suggest NOS-dependent NO production in these bacteria as well (Cohen et al., 2005; Gusarov and Nudler, 2005), consistent with the structural similarity among bacterial NOSs. Our previous work demonstrated that stNOS produces the nitrogen species that nitrates thaxtomin and suggested, but did not confirm, that NO was this species (Kers et al., 2004). Here, we prove, through both ESR and chemiluminescence, that NOS-derived NO is produced in vivo, supporting the hypothesis that NO is the nitrogen species responsible for nitration of thaxtomin. Just as important is the discovery that NO is produced in excess of that required for thaxtomin nitration. Because the generation of NO by NOSs is potentially cytotoxic and spatially uncontrollable after synthesis, we expected that there would be tight coupling of NO generation and thaxtomin nitration. However, data presented here are consistent with previous results on the effects of NO scavengers and NO donors on thaxtomin production (Wach et al., 2005).

Thaxtomin accumulation and NO production over time suggest that the generation of NO significantly outpaces thaxtomin production (Figure 3), as the total NO production is the sum of that incorporated into thaxtomin and released as free NO. Our estimate of total NO release into the headspace is lower than total thaxtomin accumulation on a molar basis; however, NO released from streptomycete mycelium must travel through a highly reactive intracellular environment, an aerobic aqueous environment, and a phase transition to enter the culture headspace. Therefore, it is safe to assume that NO production and NO concentrations at the host-pathogen interface will be significantly higher than headspace measurements suggest.

Extracellular NO increased in response to cellobiose (see Results), a cell wall component, suggesting that the stNOS is upregulated in the presence of plant tissue. The NO-specific fluorescent

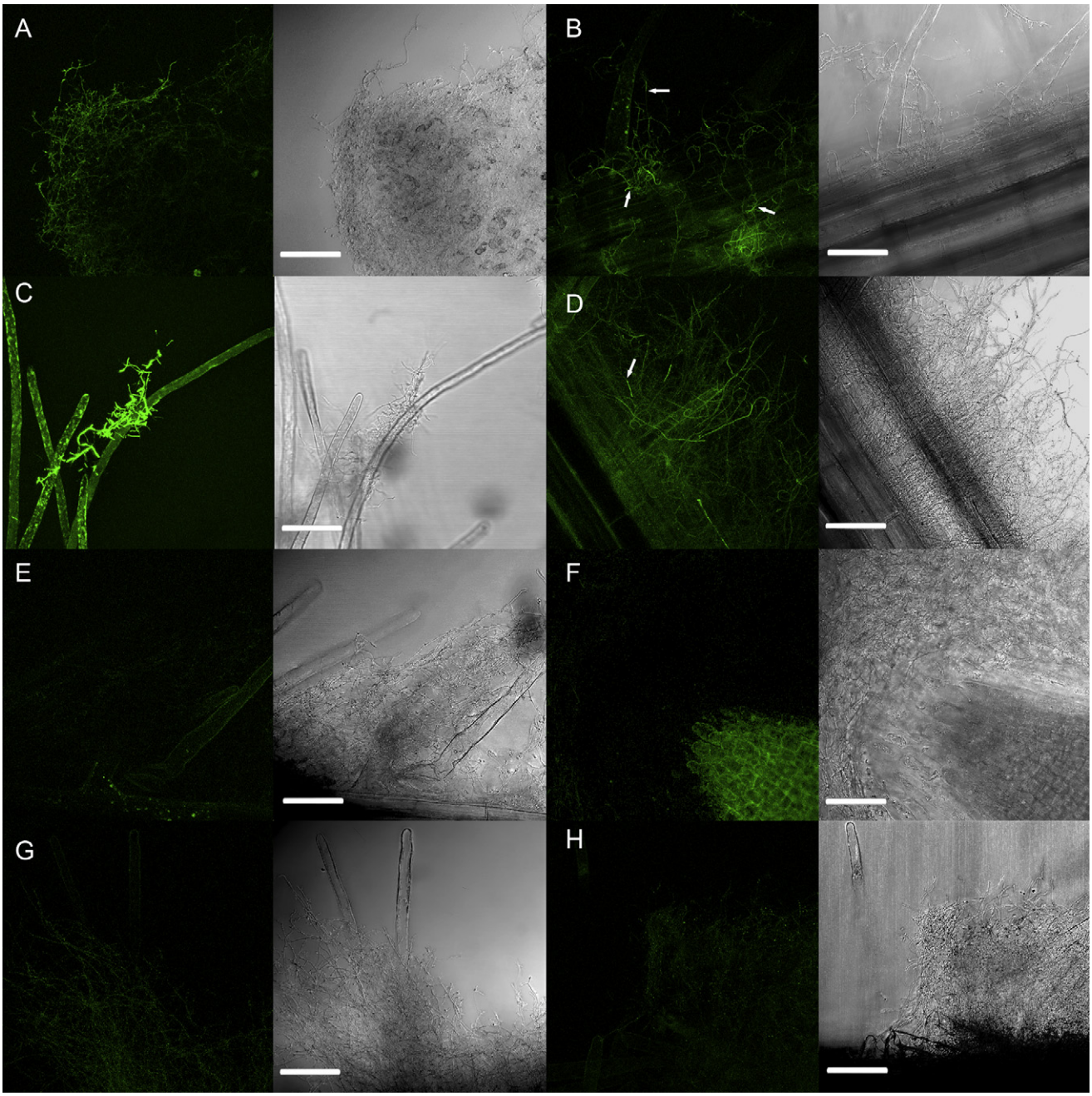

Figure 4. Visualization of NO Production by *S. turgidiscabies* in Radish Seedlings with the NO-Reactive Dye, DAF-FM DA, by Using Confocal Fluorescence Microscopy

(A–F) NO production by: wild-type strain Car8 colonizing (A) root tip, (B) root surface, (C) root hairs, and (D) root surface; and Car8Δ*nos* colonizing (E) root surface and root hairs and (F) root tip; or the unstained controls of (G) Car8 and (H) Car8Δ*nos*. Arrows designate hyphae with compartmentalized NO production. Scale bars are 50 μm.

dye, DAF-FM DA, demonstrated that NOS-derived NO was present in *S. turgidiscabies* hyphae colonizing roots. However, the variability of NO production in hyphae colonizing roots indicated that specific root surface environments might either activate or suppress NO production, an aspect that warrants further study. Association of NO production with hyphae colonizing root hairs and the root meristematic region is interesting, because these are both sites of plant cell wall expansion and infection by thaxtomin-producing streptomycetes (Scheible et al., 2003). It is possible that the process of cell wall expansion is linked to the production of plant signals that stimulate both thaxtomin and NO production.

Although diffusible NO appears to be a byproduct of thaxtomin production in plant-pathogenic streptomycetes, it may play additional roles in plant-microbe interactions. For example, NO

might serve as an intercellular signaling molecule for coordinating the infection process in this filamentous pathogen. NO appears to have a signaling role in some bacteria, as it upregulates reactive oxygen defenses in *B. subtilis* and causes biofilm dispersal in *Pseudomonas aeriginosa* (Barraud et al., 2006; Gusarov and Nudler, 2005). NO might also protect plant-pathogenic bacteria from reactive oxygen species (ROSs) generated in plants in expanding tissues during normal cell growth and in response to microbial attack (Carol and Dolan, 2006). Given the requirement of expanding tissue for infection, it is expected that plant-pathogenic streptomycetes would encounter ROSs early in the infection process. NO might provide protection by reacting with organic peroxyl radicals, notably in lipid membranes, preventing peroxidation chain reactions (Moller et al., 2005; Rubbo et al., 2002).

Streptomyces-derived NO could also affect plant metabolism, as this molecule functions as a short-range hormonal signal influencing many physiological processes, including lateral root development, senescence, seed germination, stomatal closure, and stress responses (Neill et al., 2003). For example, an increase in the density of lateral roots, resulting from increases in NO levels in plants, could serve to increase infection sites for these pathogens. NO is also a critical signaling molecule in both local and systemic plant defense responses; introduction of exogenous NO could serve to manipulate plant defense responses (Delledonne et al., 2001; Neill et al., 2003). Furthermore, NO is formed in *Medicago truncatula-Sinorhizobium meliloti* nodules by the plant in the early stages of this symbiotic interaction. This finding combined with data presented here suggests that NO is more than just an inducer of the hypersensitive response in plant-microbe interactions (Baudouin et al., 2006).

Production of NO in mammals is highly regulated, and the mechanisms by which these NOSs are regulated is an area of active research (Aktan, 2004; Kone et al., 2003). In contrast, almost nothing is known about NOS regulation in bacteria. Thaxtomin A biosynthesis in *S. turgidiscabies* involves an operon containing the nonribosomal peptide synthetase (NRPS) modules, *txtA* and *txtB*, responsible for producing the dipeptide backbone (Healy et al., 2000), and a P450 monooxygenase, *txtC*, that adds two hydroxyl groups (Healy et al., 2002). Interestingly, the *nos* gene lies about 5 kb upstream of this operon, allowing for independent transcriptional regulation.

Based on data presented here, demonstrating that NOS produces NO, and our previous results demonstrating the ability of NO donors to complement thaxtomin production in a *nos* deletion strain (Wach et al., 2005), the NOS is not directly responsible for the nitration of thaxtomin. Although the NOS has been demonstrated to be the source of reactive nitrogen species for nitration (Kers et al., 2004), it is likely that the site-specific nitration of either tryptophan or the dipeptide backbone is performed by another enzyme. Since there is an unusual P450 monooxygenase encoded directly upstream of the stNOS in *S. scabies*, *S. turgidiscabies*, and *S. acidiscabies*, we are investigating the role of this protein in the specific nitration event required for thaxtomin biosynthesis. It is intriguing that the *Deinococcus radiodurans* NOS can nitrate small amounts of tryptophan in vitro (Buddha et al., 2004); however, there is no evidence that this reaction is involved in biosynthesis of a secondary metabolite.

Bacterial NOSs occur in the genomes of some actinobacteria and firmicutes. Based on the structural conservation of bacterial NOSs characterized to date, it is likely that other NOS-containing bacteria also have the capacity to produce NO. The lack of a reductase domain in bacterial NOSs was resolved in a recent study demonstrating the ability of two *B. subtilis* flavodoxins (YkuN and YkuP) to support NO synthesis from either L-arginine or the intermediate product, *N*-hydroxyarginine, in vitro (Wang et al., 2007). It is unclear whether other bacterial NOSs participate in the biosynthesis of secondary metabolites. Thaxtomin production is limited to the relatively few streptomycetes that are pathogens of plants. In silico analysis of DNA sequences adjacent to bacterial NOSs does not reveal an obvious function for these enzymes. Thaxtomin-producing streptomycetes are the only NOS-containing bacteria in which the *nos* gene is adjacent to or within a secondary metabolite biosynthetic pathway; these pathways are often, but not always, clustered in prokaryotes. It is possible that NO production is the sole function of some, perhaps most, bacterial NOSs and that scab-causing streptomycetes evolved the ability to use some of this NO for thaxtomin biosynthesis relatively recently. Production of NOS-derived NO by bacteria raises many interesting questions, including those regarding mechanisms of transcriptional and posttranscriptional regulation, interacting proteins, participation in signaling pathways, NOS localization, phylogenetic distribution, and ecological significance. Since some of the NOS-containing bacteria, such as *B. anthracis* and *Streptococcus aureus*, are important pathogens of humans, the work presented here has implications for host-pathogen interactions across kingdoms.

SIGNIFICANCE

Here, we demonstrate that NOS-like proteins in bacteria are functional NOSs, in spite of the lack of both a fused reductase domain and certain motifs conserved in mammalian NOSs. Because NO has been well described as a defense and physiological signaling molecule in plants, the production of NO in response to host signals by plant-pathogenic streptomycetes closely interacting with plant tissues suggests that they have the capability of interfering with plant signaling pathways. Animals are also known to use NO for both physiological signaling and pathogen defense. To date, the role of NO in host-microbe interactions has been seen as a unidirectional interaction, with the host producing NO and the pathogen evolving defenses against the highly reactive free radical and its byproducts. The presence of bacterial NOSs in other Gram-positive pathogens suggests that the role of NO in the host-pathogen interaction is more complex than previously thought. The data presented here suggest that pathogen-derived NO warrants investigation in other pathogenic systems, such as the human pathogens *B. anthracis* and *S. aureus*. It is likely that NO signaling in bacteria serves a purpose other than reactive nitrogen defense, including protein modifications such as *S*-nitrosylation.

EXPERIMENTAL PROCEDURES

Growth Media and Bacterial Strains

Streptomyces turgidiscabies strain Car8 and the *nos* deletion derivative have been previously described (Kers et al., 2004). For routine cultivation of *Streptomyces* strains, we used The International *Streptomyces* Project Medium

2 (ISP2) and ISP4 (BD Biosciences) and grew cultures at 28°C for up to a week. All growth of *Streptomyces* spp. for NO detection was done in oat bran broth (OBB) (Johnson et al., 2007) or agar supplemented with 0.7% cellobiose (OBAC). Liquid cultures were incubated at room temperature (23°C–25°C) with moderate shaking (~120 rpm) for the indicated times.

NO Detection by CPTIO

Five milliliter liquid cultures of *S. turgidiscabies* Car8 and Car8Δ*nos* cultures were grown in OBB in six-well tissue culture plates. At~3 days, upon first observation of thaxtomin production, as evidenced by a yellow coloration in the medium, 50 μl of a 100 mM CPTIO (Invitrogen, Carlsbad, California) stock solution was added to each culture for a final concentration of 1 mM. The cultures were then incubated for 20 or 40 min, and cell supernatant was collected by centrifugation at 4°C at 5000 rpm. The culture supernatants were placed on ice until analysis by ESR. ESR spectra were obtained on a Bruker EMX spectrometer (Bruker, Billerica, MA) at a frequency of 9.4 GHz under standard conditions. The field sweeps were calibrated with a Bruker ER 035 Gaussmeter. The microwave frequency was monitored with a frequency counter. The measurements were carried out at room temperature in glass capillaries at a microwave power of 6.3 mW and a modulation amplitude of 0.5 G.

NO Detection by Dithiocarbamate Spin Traps

Five milliliter liquid cultures of *S. turgidiscabies* Car8 and Car8Δ*nos* cultures were grown in OBB. At~3 days, upon first observation of thaxtomin production, a DTCS (Calbiochem, La Jolla, California) solution was added to the culture medium to a final concentration of 1 mM $Fe(DTCS)_2$ complex. DTCS solutions were prepared as 250 mM of the appropriate spin trap and 50 mM $FeSO_4$ for a 50 mM final concentration of the $Fe(DTCS)_2$ complex. The cultures with spin trap added were then incubated at room temperature for 30 min before harvesting. Cultures were centrifuged, and the supernatant was collected and placed on ice until ESR detection. ESR spectra were obtained on a Bruker EMX spectrometer (Bruker, Billerica, MA) at a frequency of 9.4 GHz under standard conditions. The field sweeps were calibrated with a Bruker ER 035 Gaussmeter. The microwave frequency was monitored with a frequency counter. The measurements were carried out at room temperature in glass capillaries at a microwave power of 0.32 mW (28 dB) and a modulation amplitude of 2G.

NO Detection in Headspace by Chemiluminescence

Each of the test strains was grown on 20 ml OBAC in 250 ml Erlenmeyer flasks at room temperature. Flasks were inoculated with 10^6 spores in 1 ml water and were spread to cover the entire agar surface. For NO detection, the culture flasks were connected to an ECO PHYSICS CLD 770 AL ppt Chemiluminescence NO analyzer (Ecophysics Inc., Switzerland) by using a silicone stopper with Teflon tubing (PFTA) for inflow of zeroed air and outflow to the NO analyzer. The analyzer was calibrated daily by using sequential dilution of a certified standard (Scott Specialty Gases, Irvine, CA). The instrument was tuned for linearity in the 0–5 ppb range at an integration time of 600 s and a flow rate of 1 l min^{-1}. Total NO release during the time course was calculated by using the trapezoid method of integration.

Thaxtomin Analysis

For thaxtomin extraction from agar-based medium, the agar was broken into~1 cm^2 pieces. Agar samples were extracted three times with an equal volume of methanol. The extract was evaporated to dryness and was dissolved in 5 ml methanol. The extract was then diluted 4:1 in water and was passed through Alltech (Deerfield, Illinois) Extract-Clean solid-phase extraction cartridges (C18; 200 mg), and thaxtomin was quantified as previously described for liquid cultures (Wach et al., 2005).

NO Detection in Radish Seedlings by Confocal Microscopy

Radish seedlings were grown in half-strength liquid MS medium (pH 6.3) as previously described (Joshi et al., 2007a). Seedlings were inoculated with 10^6 spores in 100 μl 20% glycerol. After 3–6 days of incubation with moderate shaking (100 rpm) at 22°C with a 16 hr light period, root segments were removed to slides. Roots were incubated for 2 min in 10 μM DAF-FM DA (Invitrogen, Carlsbad, CA) in 50 mM phosphate buffer, (pH 7.0) and were then rinsed with phosphate buffer and observed with a Leica TCS SP2 microscope (Leica Microsystems). DAF-FM T was visualized by using a four-line argon laser with an excitation wavelength of 488 nm and an emission wavelength of 500–600 nm. Fluoresence Z series images were flattened to 2D maximum projections by using Leica Confocal Software. Bright field Z series images were flattened into 2D composite images by using Helicon Focus 4.01.1 Pro software (Helicon Soft Ltd., Kharkov, Ukraine) for visualization of all focal planes in a single image.

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