Research Note

Suppression of Septoria tritici Blotch and Leaf Rust of Wheat by Recombinant Cyanide-Producing Strains of Pseudomonas putida

Moshe A. Flashman,¹ Zahir Eyal,¹ Aviah Zilberstein,¹ Christophe Voisard,² and Dieter Haas²

¹Department of Botany, The George S. Wise Faculty of Life Science, Tel-Aviv University, Tel-Aviv 69978, Israel, ²Department of Microbiology, Swiss Federal Institute of Technology (ETH), Zürich, Switzerland
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Pseudomonas putida strain BK8661, which produces siderophore(s), antibiotic(s), and low levels of hydrogen cyanide (HCN), suppresses growth of Septoria tritici and Puccinia recondita f. sp. tritici in vitro and on wheat leaves. Pleiotropic mutants of strain BK8661 deficient in siderophore and antibiotic synthesis were significantly less effective in suppressing symptoms than was the wild type. HCN overproducing derivatives of the pleiotropic mutants were constructed by the integration of the hcnABC gene cluster from Pseudomonas fluorescens CHAO. In the absence of siderophore(s) and antibiotic(s), HCN production by the overproducing bacterial strains resulted in a small but statistically significant increase in the suppression of symptoms caused by S. tritici and P. recondita f. sp. tritici on wheat seedling leaves.

Additional keywords: biological control, Triticum spp.

Many pseudomonads produce hydrogen cyanide (HCN) from the oxidation of glycine. Fe³⁺ ions and glycine enhance HCN synthesis by Pseudomonas spp. in culture (Castric 1977; Bakker and Schippers 1987). The physiological function of HCN is unknown. However, HCN production by some root-colonizing pseudomonads can contribute to suppression of several soil-borne pathogens (Voisard et al. 1989; Haas et al. 1991; Lam and Gaffney 1993; Cook 1994). An HCN-deficient mutant of P. fluorescens, derived from the HCN-producer strain CHAO, is less effective than is the wild type in suppressing tobacco black root caused by Thielaviopsis basicola (Voisard et al. 1989). This deficiency can be restored by a plasmid carrying a 5.0-kb genomic fragment from strain CHAO. The fragment contains three contiguous open reading frames designated hcnABC, which encode cyanide synthase (Voisard et al. 1989; Labille 1993). Furthermore, three genes, lemA, gacA, and anr, have been shown to be involved in the regulation of cyanogenesis in Pseudomonas spp. The lemA and gacA genes generally control antibiotic and HCN production (Laville et al. 1992; Gaffney et al. 1994; Corbell and Loper 1995), whereas anr is an anaerobic regulator of the arginine deiminase pathway and cyanogenesis (Zimmermann et al. 1991; Voisard et al. 1994).

We have isolated Pseudomonas putida strain BK8661 from wheat leaves. This strain can suppress the growth of the economically important wheat-pathogenic fungi Septoria tritici and Puccinia recondita f. sp. tritici through the production of diffusible antibiotic(s), siderophore(s), and HCN (Flashman and Eyal 1990). A thin-layer chromatography (TLC) inhibition assay was employed to verify the production of diffusible antibiotic(s) in the culture medium (Levy et al. 1989). One of the antibiotics was tentatively identified as pyoluteorin, while HPLC analysis did not provide evidence for the presence of phenazines, salicylic acid, indoleacacetate, monoacetylphloroglucinol, 2,4-diacetylphloroglucinol, and tropolone in an ethyl acetate extract (Flashman 1992). A siderophore produced by BK8661 was isolated and found to be chemically similar to pseudobactin WCS358 isolated from P. putida (Weisbeek et al. 1986). There is extensive evidence that suppressive effects of Pseudomonas spp. on soilborne and foliar pathogens are mediated, in part, by antibiotics and siderophores, whereas disease suppression by the volatile compound HCN has been shown only for the rhizosphere (Weller 1988; Voisard et al. 1989; Pierson and Thomashow 1992; Blakeman 1993). This study shows that bacterial HCN production is actually capable of contributing to the suppression of symptoms caused by two economically important foliar wheat pathogens in the phyllosphere.

To assess the potential role of bacterial HCN in suppressing different phylloplane microorganisms and the effect of HCN on wheat, we first tested the effect of exogenously applied KCN in vitro. Test microorganisms were grown in sealed partitioned plates on a sterilized cellophane membrane placed on malt agar (for fungi) or on nutrient agar (for bacteria). The other compartment contained 4 ml of an aqueous KCN solution at concentrations ranging from 5 to 1,000 μM. HCN volatilized from 50 and 125 μM KCN entirely suppressed germination and growth of P. recondita f. sp. tritici and S. tritici, respectively, after incubation at 20°C for 5 days. Inhi-
bition of bacterial inhabitants of the wheat phylloplane such as Bacillus subtilis, Xanthomonas campestris pv. triticif, and X. campestris pv. pelargonii, occurred at 100 μM KCN after incubation at 32°C for 2 days. By contrast, the HCN-producing strains P. aeruginosa LEC1 (Levy et al. 1989) and P. putida Bk8661 were insensitive to 1,000 μM KCN. The germination of seeds of the wheat cultivar Shafir placed in partitioned plates was inhibited by 0.5 mM KCN. Ten-day-old seedlings of Shafir grown in hermetically sealed containers (250 ml) were either sprayed with 10 ml of 0.5 mM KCN, or grown in the presence of 5 ml of 1.0 mM KCN solution placed in an open petri plate. There was no visible effect on plant growth by the two treatments. These results are in agreement with other reports which have demonstrated that HCN is less inhibitory to growth of wheat than to growth of dicotyledonous and other monocotyledous plants (Miller and Conn 1980). Since the sensitivity of S. tritici and P. recondita f. sp. tritici to HCN is higher than that of growing wheat seedlings, HCN levels in the phylloplane environment could be raised to inhibit specifically pathogen development.

Strain Bk8661 is cyanogenic and when growing on plates completely inhibits growth of the two fungal pathogens via the gas phase. However, the HCN amounts produced by strain Bk8661 in vitro are relatively small (Table 1). To increase HCN production by strain Bk8661, the suicide plasmid pME3045 carrying Tnhen, a modified Tn5 with the hcnABC gene cluster of P. fluorescens CHAO (Vosillard et al. 1989, Fig. 1C), was mobilized with the conjugative plasmid R64drrd-11 (Meynell and Datta 1967) from E. coli to strain Bk8661 according to published procedures (Vosillard et al. 1983). Integration of pME3045 was selected on nutrient agar containing kanamycin (Km) at 250 μg/ml and chloramphenicol at 15 μg/ml. Fifty-one kanamycin-resistant colonies were obtained at a frequency of 10^(-4) per E. coli donor. In principle, integration of the hcnABC genes could occur either by Tnhen transposition or via homologous recombination between the native hcn genes of strain Bk8661 and the incoming plasmid pME3045. The insertion of the hcnABC genes into the Bk8661 genome was confirmed by Southern blot analysis using the CHAO hcnABC genes as a probe. The structural genes for HCN production in P. putida Bk8661 have not been characterized. However, they appear to be closely related to the hcnABC genes of strain CHAO, because hybridization occurred under conditions of high stringency. A 5.6-kb HindIII-EcoRI band resulting from the integration of the CHAO hcn genes and a 4.0-kb fragment corresponding to the Bk8661 native hcn genes were detected (Fig. 1A) in the derivative Bk8661::pME3045. This strain overproduced HCN sixfold in vitro (Table 1), whereas siderophore and antibiotic production were not altered in comparison with the parent strain Bk8661 (data not shown). In bioassay controls using wheat seedling leaves infected with S. tritici or P. recondita, strains Bk8661 and Bk8661::pME3045 suppressed disease with similar efficiencies (Table 1). Since antibiotics and siderophores may account for most of the biocontrol properties of P. putida, strain Bk8661 was subjected to Tn5 mutagenesis, by mobilizing the suicide plasmid pSUP2201 (Simon et al. 1983), which contains Tn5, from E. coli to P. putida Bk8661 using the helper plasmid R64drrd-11. Km-resistant colonies were selected as above and screened for loss of siderophore activity. Two mutants obtained (1b6, 1c1) were unable to grow on succinate medium supplemented with the iron chelator ethylenediamine-di-(o-hydroxyphenylacetae) (EDDHA; 100 μg/ml). Addition of 100 μM FeCl3 to the medium restored growth of both mutants, as previously described for pyoverdine-negative mutants of P. aeruginosa (Hohnadel et al. 1986). Strains 1b6 and 1c1 also had lost their ability to inhibit S. tritici on malt agar and in a TLC bioassay (Levy et al. 1989). It is not clear whether the pleiotropic defects of the mutants were due to single Tn5 insertions. Both mutants retained their ability to produce low levels of HCN and their growth rate was similar to that of the wild type.

To introduce the hcnABC gene cluster from strain CHAO into the pleiotropic mutants, we cloned the 4.7-kb Ω-Hg

### Table 1. HCN production and suppression of symptoms of Septoria tritici blotch and leaf rust on wheat seedling leaves by Pseudomonas putida strains

<table>
<thead>
<tr>
<th>P. putida strains</th>
<th>Phenotypes</th>
<th>HCN production in vitro (μM)</th>
<th>% pycnidial coverage</th>
<th>% Reduction</th>
<th>Uredia/cm²</th>
<th>% Reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bk8661</td>
<td></td>
<td>13</td>
<td>20 f</td>
<td>71</td>
<td>&lt;1 e</td>
<td>&gt;94</td>
</tr>
<tr>
<td>2c1 (Bk8661::Tn5)</td>
<td></td>
<td>10</td>
<td>19 f</td>
<td>73</td>
<td>&lt;1 e</td>
<td>&gt;94</td>
</tr>
<tr>
<td>Bk8661::pME3045</td>
<td></td>
<td>72</td>
<td>16 g</td>
<td>77</td>
<td>&lt;1 e</td>
<td>&gt;94</td>
</tr>
<tr>
<td>IB6 (Bk8661::Tn5)</td>
<td></td>
<td>9</td>
<td>51 c</td>
<td>27</td>
<td>8 c</td>
<td>53</td>
</tr>
<tr>
<td>IB3::pME3044-Hg</td>
<td></td>
<td>97</td>
<td>38 d</td>
<td>41</td>
<td>5 d</td>
<td>70</td>
</tr>
<tr>
<td>IC1 (Bk8661::Tn5)</td>
<td></td>
<td>15</td>
<td>57 b</td>
<td>19</td>
<td>10 b</td>
<td>41</td>
</tr>
<tr>
<td>IC1::pME3044-Hg</td>
<td></td>
<td>85</td>
<td>32 e</td>
<td>64</td>
<td>8 c</td>
<td>53</td>
</tr>
<tr>
<td>(control no bacteria)</td>
<td>0</td>
<td>70 a</td>
<td>...</td>
<td>...</td>
<td>17 a</td>
<td>...</td>
</tr>
</tbody>
</table>

* Phenotypes are abbreviated as follows: Sid, fluorescent siderophore; An, antibiotic compound(s); Hcn, hydrogen cyanide; Hcn*, overproduction of HCN.

* P. putida strains were grown in minimal medium C (Castric 1977) at 30°C for 4 days and HCN production in medium was measured by the method of Gewitz et al. (1976).

* Twenty-five plants (per three replicates) of 10-day-old seedlings of the wheat cv. Shafir were sprayed with 10-ml suspensions of stationary-phase bacterial cells (10^8 cfu/ml in water) which had been grown in LB medium for 16 h. Three hours later, 15 ml of S. tritici conidial suspension (10^7 spores/ml in liquid yeast-extract-sucrose medium (Eyal et al. 1987)), or 10-ml ureidiospore suspension of P. recondita f. sp. tritici (0.2 mg/ml in water) was applied to plants. Plants in Septoria tritici blotch trials were placed at 20°C under continuous misting for 48 h, and pycnidial coverage assessed according to a standard scale 21 days after inoculation (Eyal et al. 1987). Plants in leaf rust trials were placed in humidity chambers for 24 h at 20°C. The number of leaf rust uredia/cm² was assessed 12 days after inoculation. Reductions (%) in symptoms were calculated relative to the control treatments.

* Means followed by the same letter are not statistically significant at P < 0.05 as assessed by Duncan's multiple range test.

* Control wheat seedlings inoculated with spores of S. tritici and P. recondita f. sp. tritici without bacteria.
fragment conferring resistance to mercury (Fellay et al. 1987) next to the hcnABC genes on the CoEl derivative pME3042 (Voisard et al. 1989). The resulting suicide plasmid pME3044-Hg (Fig. 1B) was transferred from E. coli harboring R64dreII to the pleiotropic Tn5 mutants 1B6 and 1C1 of P. putida BK8661 and to the BK8661::Tn5 strain 2C1. The latter was used as a control since it possessed a Tn5 insertion that did not affect antibiotic or siderophore production. Hg-resistant (Hg') transconjugants appeared at a frequency of 8 x 10^4 per donor. They were analyzed by Southern analysis using the hcnABC cluster from strain CHA0 as a probe. A 4.0-kb HindIII-EcoRI band was revealed which represented the native hcn genes of the recipient strain BK8661 plus a new 5.6-kb fragment indicative of the inserted hcnABC cluster from strain CHA0 (Fig. 1A). It is likely that the entire plasmid pME3044-Hg (Fig. 1B) has integrated into the mutants via homologous recombination. This event may have occurred in one of two regions of homology: in the Km resistance determinant and IS50 sequences carried by the chromosomal Tn5 insertion and pME3044-Hg, or in the hcn genes which occur in the BK8661 chromosome and in pME3044-Hg.

The pleiotropic mutants 1B6 and 1C1, and the control strain 2C1 produced low, wild-type levels of HCN (Table 1). The derivatives with the inserted hcnABC cluster produced 5 to 10 times more HCN on minimal medium C (Table 1). The addition of 300 μM FeCl₃ or 40 mM glycine to the medium further enhanced HCN production about twofold in the overproducing strains (data not shown). These results indicate that the hcnABC cluster of P. fluorescens CHA0 is more efficient in synthesizing HCN than are the endogenous P. putida BK8661 hcn genes.

Under greenhouse conditions (20 ± 2°C, 80% relative humidity, and supplemental light), all BK8661 derivatives manifested a similar ability to colonize wheat seedling leaves, reaching a level of 10^8 CFU/cm² at 10 days after bacterial inoculation. The suppression capabilities of the mutants and the wild type were assessed on wheat leaves inoculated with S. tritici or P. recondita f. sp. tritici. Septoria tritici blotch of wheat was effectively suppressed by the wild type, in that pycnidal coverage was reduced by 71%. The siderophore- and antibiotic-negative mutants 1B6 and 1C1 provided only 27% and 19% protection, respectively (Table 1), indicating that bacterial antibiotic and/or siderophore production are instrumental to biocontrol of S. tritici. The insertion of the hcnABC genes into strains 1B6 and 1C1 improved the biocontrol abilities by 19% to 35%, compared to the abilities of the parental pleiotropic mutants (Table 1). As noted above, in the BK8661 wild-type background, insertion of hcnABC genes improved suppression of S. tritici only marginally (Table 1). The production of uredia of P. recondita f. sp. tritici on wheat leaves was suppressed by strains BK8661, and BK8661::pME3045 to >90%. The pleiotropic mutants 1B6 and 1C1 were significantly less effective (Table 1). The suppressive ability of the HCN overproducer 1B6::ME3044-Hg was improved by 17% over that of the parental strain 1B6 (Table 1) and by 48% upon addition of 300 μM FeCl₃ (data not shown). However, the HCN overproducing derivatives of the pleiotropic mutants were less effective in controlling S. tritici and P. recondita f. sp. tritici development on wheat leaves than was the original strain BK8661.

The results reported here support the hypothesis that HCN produced by certain pseudomonads can be involved in the

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Fig. 1. A, Verification of the integration of the *Pseudomonas fluorescens* hcnABC gene cluster into *P. putida* (BK8661::pME3045), its pleiotropic Tn5 insertion mutants (1C1::pME3044-Hg; 1B6::pME3044-Hg), and the control strain (2C1::pME3044-Hg). Chromosomal DNA was isolated by the procedure of Chesney et al. (1979). HindIII/EcoRI-digested chromosomal DNA was run on a 0.6% agarose gel at 60 V for 16 h, transferred to a nitrocellulose membrane, and hybridized with a 32P-labeled hcnABC probe from *P. fluorescens* for 15 h at 65°C. After hybridization the blot was washed under high stringency conditions; in the final washing the blot was transferred to 0.1 x SSC and 0.3% SDS solution at 65°C for 2 h (Sambrook et al. 1989). a, 6.8-kb fragment carrying the CHA0 hcnABC genes; b, 5.6-kb fragment carrying the inserted CHA0 hcnABC genes in BK8661 strains; c, 4.0-kb fragment with BK8661 native hcn genes. B, Suicide plasmid pME3044-Hg. The 4.7-kb Ω-Hg fragment conferring resistance to mercury (■) (Fellay et al. 1987) was inserted into the HindIII site next to the hcnABC genes, on the CoEl derivative pME3042 (Voisard et al. 1989); the open boxes designate the Tn5 inverted repeats; B, BamHI; E, EcoRI; H, HindIII; S, SmaI. Plasmid isolation by alkaline lysis, restriction, DNA fragment purification from low-melting agarose, ligation, and transformation to *E. coli*, were all done by standard methods (Sambrook et al. 1989). C, Suicide plasmid pME3045 (after Voisard et al. 1989).
suppression of foliar pathogens on the phylloplane. HCN, in its gaseous phase or dissolved in water, may directly inhibit pathogens on the leaf, in stomatal cavities and intracellular spaces, thus contributing to suppression of disease. Therefore, one should consider HCN production as a factor in biocontrol of foliar pathogens by antagonistic pseudomonads. It is also clear from this study that antibiotics and siderophores can have an important role in the multifactorial interactions of the biocontrol agent with pathogens on the phylloplane.

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LITERATURE CITED