

# Construction of Secretion Vectors and Use of Heterologous Signal Sequences for Protein Secretion in *Clavibacter xyli* subsp. *cynodontis*

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We have constructed a vector to assess the ability of heterologous signal sequences to function in the endophytic bacterium *Clavibacter xyli* subsp. *cynodontis*. This secretion reporter contains the *phoA* gene from *Escherichia coli* from which the promoter and signal sequences have been deleted. Signal sequences of streptomycete origin were cloned into the vector and the level of secreted alkaline phosphatase determined enzymatically and by Western blotting. We show that a number of the signal sequences of streptomycete origin function well in *C. xyli* subsp. *cynodontis*. By inoculating corn plants with *C. xyli* subsp. *cynodontis* expressing the *sti2-phoA* fusion, we demonstrated alkaline phosphatase activity in planta. Our data show that *phoA* can be used to detect endophytic bacteria in some locations in planta, and is therefore useful in the study of plant-microbe interactions. Furthermore, our data illustrate how *phoA* fusions can be useful as a reporter for protein secretion activity of *C. xyli* subsp. *cynodontis* in planta.

*Additional keywords:* coryneform-actinomycete, xylem.

*Clavibacter xyli* subsp. *cynodontis* is a xylem-inhabiting endophyte of Bermudagrass (*Cynodon dactylon*). When artificially inoculated, *C. xyli* subsp. *cynodontis* also colonizes the vascular system of corn (*Zea mays* (L.)) at levels up to  $10^9$  CFU per ml of xylem sap (Kostka et al. 1988). This organism is a Gram-positive, high G+C content, coryneform-actinomycete bacterium (Davis et al. 1984) with a single copy 16S rRNA gene that is 89.9% identical to that of *Streptomyces* species (Sathiamoorthy et al. 1991). We have been developing recombinant strains of *C. xyli* subsp. *cynodontis* to deliver biopesticides in corn and other crops and have shown insecticidal activity against the European corn borer in corn plants inoculated with a genetically modified *C. xyli* subsp. *cynodontis* producing the  $\delta$ -endotoxin of *Bacillus thuringiensis* (Lampel et al. 1994). An additional application of endophyte delivery would be possible with *C. xyli* subsp. *cynodontis* strains engineered to secrete heterologous proteins with pesticidal activity into the vascular system of colonized plants. Such an application depends on the availability of functional secretion signal sequences for *C. xyli* subsp. *cynodontis*.

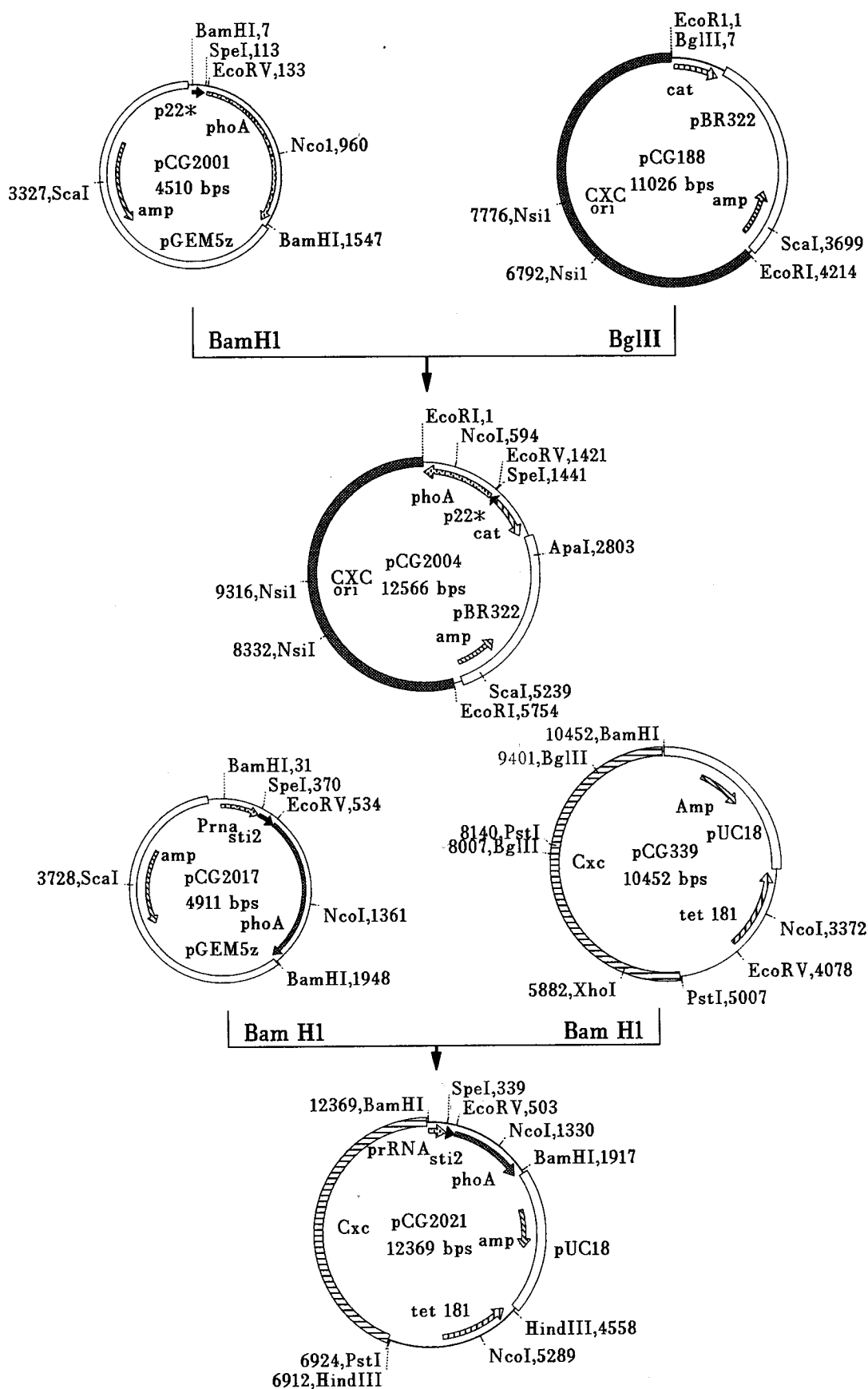
In *Escherichia coli*, *phoA* encodes the secreted protein, alkaline phosphatase (AP; EC3.1.3.1). Hoffman and Wright (1985) developed a reporter system based on translational fusions to *phoA* to study protein secretion in *E. coli*. Nagarajan et al. (1992) and Payne and Jackson (1991) have also used *phoA* protein fusions to study protein secretion in *Bacillus subtilis*. The advantages of AP as a reporter for secretion are: (i) enzyme activity can be assayed spectrophotometrically; (ii) activity can be detected on plates containing the chromogenic substrate 5-bromo-4-chloro-3-indolyl phosphate (XP); (iii) the enzyme is stable and can tolerate N-terminal extensions; and (iv) activity is absolutely dependent upon secretion from the cytoplasm. No published studies are available on protein secretion in *C. xyli* subsp. *cynodontis*. In this study, we describe construction of *phoA* fusion vectors for *C. xyli* subsp. *cynodontis* and the screening of signal sequences of streptomycete origin for protein secretion in *C. xyli* subsp. *cynodontis*. The streptomycete signal sequences chosen were all shown in the literature to direct secretion of the corresponding gene product (see references cited in Materials and Methods). We focused on those of streptomycete origin because of the strong homology in 16S rRNA genes and similar high G+C content between *C. xyli* subsp. *cynodontis* and streptomyces species (Sathiamoorthy et al. 1991). We show that the signal sequences are able to direct the secretion of AP from *C. xyli* subsp. *cynodontis* grown in culture and in planta.

## RESULTS

### Construction of the secretion probe vectors.

The plasmid pCG2001, a derivative of pGEM5Zf, contains the secretion-probe as a *Bam*HI cassette. The *phoA* gene in pCG2001, generated by polymerase chain reaction (PCR), lacks the native promoter and signal sequence but encompasses the DNA encoding the mature portion of the secreted protein. Upstream of *phoA* is the P22 promoter, derived from the *Bacillus*  $\phi$ 29 phage (H. E. Schnepf and H. R. Whiteley, unpublished results). Plasmid pCG2001 was digested with *Bam*HI. The *phoA*-containing DNA was ligated into the unique *Bgl*III site of pCG188 (Taylor et al. 1993), a self-replicating plasmid in *C. xyli* subsp. *cynodontis*. The resulting vector, pCG2004, is a *C. xyli* subsp. *cynodontis*-*E. coli* shuttle vector (Fig. 1A). The various signal sequences amplified by PCR were cloned between the unique *Spe*I and *Eco*RV

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**Fig. 1. A,** Construction of *Clavibacter xyli* subsp. *cynodontis* secretion probe vector pCG2004. The *phoA* gene, promoter, antibiotic resistance markers, and origins of plasmid replication are as indicated. pCG2004 contains unique *Spe*I and *Eco*RV sites for cloning of polymerase chain reaction-generated signal sequences (described in text). **B,** Construction of *C. xyli* subsp. *cynodontis* integrative plasmid pCG2021 containing the rRNA promoter from *C. xyli* subsp. *cynodontis*, and the signal sequence from *sti2* fused to *phoA*. *cat* denotes the gene coding for chloramphenicol acetyl-transferase.

sites of pCG2004. Plasmids containing the hybrid gene fusions are shown in Figure 2.

*Clavibacter xyli* subsp. *cynodontis* strains containing *phoA* fusions derived from the signal sequences of *sti2*, *chiC*, *snpA*, *dagA*, and *aml* showed high levels of AP activity in culture supernatants, while those derived from *phoA* signal sequence gave 10-fold lower levels of activity, and there was no detectable activity from *melC*. The highest AP activity was observed in *chiC-phoA* fusion strains. AP activity was not detected in the culture supernatants of the host strain MDM1.6 and strains containing *phoA* fusions that were devoid of a signal sequence (Fig. 2).

### Immunodetection of AP.

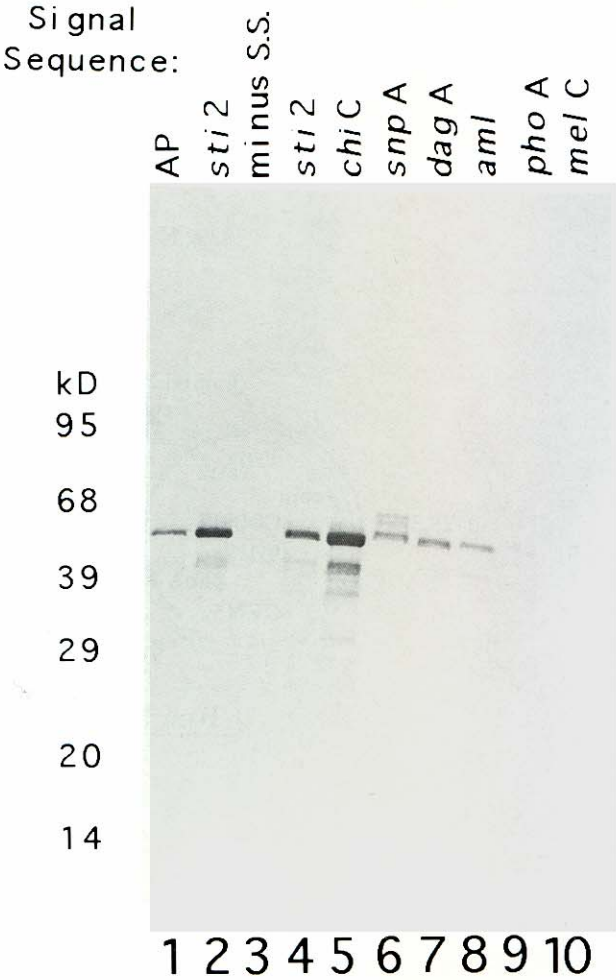
Culture supernatants from *C. xyli* subsp. *cynodontis* recombinants containing *phoA* fusion plasmids were concentrated by (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> precipitation and analyzed by Western blotting. As shown in Figure 3, a major protein band of the expected size of the mature AP was detected in most of the samples. Strains MDQ1.1705 (pCG2318, *sti2*), MDQ1.1759 (pCG2030, *chiC*), and MDQ1.1698 (pCG1145, *snpA*) produce the 50-kDa mature AP. In agreement with the AP enzyme activity, the strongest antigenic signal for AP was seen in the strain containing the *chiC* signal sequence followed by strains containing the *sti2* signal sequence. Multiple cross-reacting bands were seen with the strain containing the *snpA* signal sequence, possibly resulting from cleavage at the “pre” and “pro” processing sites (Lampel et al. 1992). AP was also produced in strains MDQ1.1696 (pCG499, *aml*) and

Strain #	Plasmid	Signal Sequence	DNA sequence across the junction of gene fusion	AP activity (mu/ml)
MDM1.6	-	-	-	ND
MDQ1.1756	pCG2004	-	-	ND
MDQ1.1705	pCG2318	<i>sti2</i>	GCG CTC GCC ↓ ACC CCG GAT ATC ala leu ala thr pro asp ile	5.5
MDQ1.1759	pCG2030	<i>chiC</i>	GCC CAG GCG ↓ GCC ACG GAT ATC ala gln ala ala thr asp ile	28.5
MDQ1.1698	pCG1145	<i>snpA</i>	AAG AGC ACG ↓ GCG GCC GAT ATC lys ser thr ala ala asp ile	3.3
MDQ1.1781	pCG1170	<i>dagA</i>	GCT CAT GCC ↓ GCA GAC GAT ATC ala his ala ala asp asp ile	3.2
MDQ1.1696	pCG499	<i>aml</i>	GCC GCT GCC ↓ GCC CCG GAT ATC ala ala ala ala pro asp ile	1.3
MDQ1.1704	pCG2314	<i>phoA</i>	ACA AAA GCC ↓ GAT ATC thr lys ala asp ile	3.6 x 10 <sup>-1</sup>
MDQ1.1778	pCG1169	<i>melC</i>	GCC CGC GCG ↓ GAC GAT ATC ala arg ala asp asp ile	ND
MDQ1.1688	(pCG2021, integrated)	<i>sti2</i>	GCG CTC GCC ↓ ACC CCG GAT ATC ala leu ala thr pro asp ile	13.2

**Fig. 2.** Extracellular alkaline phosphatase (AP) activities of fusion strains and DNA sequence across the junction of gene fusion as confirmed by DNA sequence analysis. AP enzymatic activity was assayed as described by Nagarajan et al. (1992). The predicted processing site is indicated by ↓. The codon(s) between the processing site and the *EcoRV* site come from the respective secreted protein. A schematic representation of the signal sequence-*phoA* fusion derived from the secretion probe vector pCG2004 is shown. Symbols: ▨, P22 promoter; □ leader sequences; ■, signal sequence (s.s.); ▤, the mature *phoA* gene; ND, not detectable (the change in absorbance during the assay was ≤ 0.002).

MDQ1.1781 (pCG1170, *dagA*), and was barely detectable in MDQ1.1778 (pCG1169, *melC*) and MDQ1.1704 (pCG2314, *phoA*).

The relationship between growth and AP production was investigated for strains MDQ1.1705 (pCG2318, *sti2*) and MDQ1.1698 (pCG1145, *snpA*) using detection by Western blotting. As expected for these strains in which *phoA* expression is promoted by the P22 promoter that is constitutive in *C. xyli* subsp. *cynodontis*, AP accumulated coincidentally with increased cell density (data not shown). Comparison of Western band intensities with the bands from known amounts of purified *E. coli* AP standards indicates that in the *C. xyli* subsp. *cynodontis* supernatants tested, 1 mg of AP corresponds to about 30 to 50 millinunits of AP activity in the assay used.



**Fig. 3.** Western blot analysis of *phoA*-specific polypeptides encoded by the fusion plasmids. (Sample preparations and Western blotting performed as described in Materials and Methods.) All *Clavibacter xyli* subsp. *cynodontis* transformants except MDQ1.1688 are derived from pCG2004 (Fig. 1A). MDQ1.1688 is the *C. xyli* subsp. *cynodontis* recombinant of integrative plasmid pCG2021 (Fig. 1B). Lanes: 1, 100 ng of purified *Escherichia coli* alkaline phosphatase; 2, MDQ1.1688/*sti2*; 3, MDQ1.1756/no signal sequence; 4, MDQ1.1705/*sti2*; 5, MDQ1.1759/*chiC*; 6, MDQ1.1698/*snpA*; 7, MDQ1.1781/*dagA*; 8, MDQ1.1696/*aml*; 9, MDQ1.1704/*phoA*; 10, MDQ1.1778/*melC*. Amount of protein in each lane corresponds to 400 μl of the culture supernatant.



### Histochemical staining of in planta *phoA* activity.

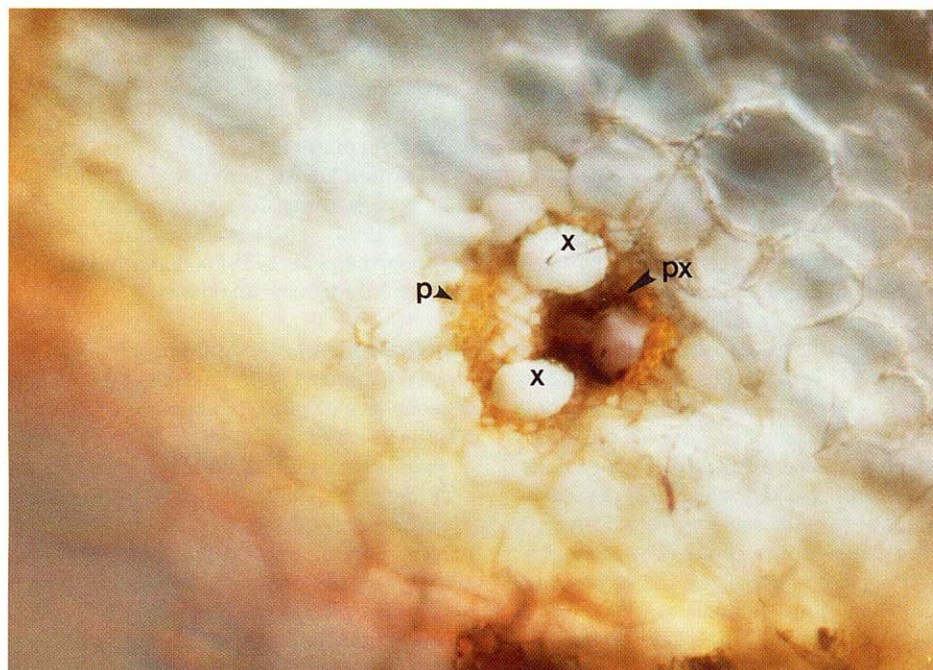
The *C. xyli* subsp. *cynodontis* self-replicating plasmid, pCG188, is unstable in planta (data not shown). In order to determine if *phoA* was expressed in planta, an integrative analog of pCG2004 was constructed. The secretion cassette, consisting of the *C. xyli* subsp. *cynodontis* rRNA promoter (Sathyamoorthy et al. 1991) and the *sti2* leader and signal sequence fused to *phoA*, was isolated from pCG2017. This cassette is analogous to the secretion cassette shown in Figure 2 except that the P22 promoter was replaced by the rRNA promoter. This *Bam*HI fragment was cloned into the unique *Bam*HI site of a *C. xyli* subsp. *cynodontis*-integrative plasmid, pCG339 (Lampel et al. 1994), resulting in plasmid pCG2021. This plasmid contains the ColEI replicon that functions in *E. coli* but not in *C. xyli* subsp. *cynodontis*, a tetracycline resistance marker that functions in *C. xyli* subsp. *cynodontis*, and a fragment of the *C. xyli* subsp. *cynodontis* chromosome that allows integration by homologous recombination of the plasmid into the chromosome. Plasmid pCG2021 was introduced into *C. xyli* subsp. *cynodontis* strain MDM1.6 by electrotransformation using tetracycline selection. The resulting recombinant, MDQ1.1688, was stab-inoculated into corn plants in the greenhouse for the assay of AP activity in planta. Corn plants stab-inoculated with *C. xyli* subsp. *cynodontis* strain MDM1.6 and the recombinant MDM1.1688 (pCG2021, *sti2*) were well colonized after 6 weeks, as determined by microscopic quantitation of populations in expressed plant sap. Thin cross-sections of the lower stem portion of the corn plants were stained for AP activity. AP activity was observed in all the vascular bundles in the stem cross-sections, as shown by the development of brownish color around the xylem wall of the corn colonized with recombinant strain MDM1.1688 (Fig. 4), and not by

strain MDM1.6 (data not shown). AP activity was not detected in the phloem or in other tissue surrounding the vascular bundles.

### DISCUSSION

This report describes an efficient system for probing streptomyces signal sequences that function in *C. xyli* subsp. *cynodontis*. *phoA* fusions have also proven useful in the study of protein secretion in *E. coli* (Hoffman and Wright 1985) and *Bacillus subtilis* (Nagarajan et al. 1992; Payne and Jackson 1991). Nagarajan and co-workers constructed *phoA* fusions by creating an *Eco*RV site just before the coding region for the mature *phoA* and joining heterologous signal sequences at this *Eco*RV site. They demonstrated efficient secretion of *phoA* from *B. subtilis*. Similarly, our secretion probe plasmid contains a promoter recognized by *C. xyli* subsp. *cynodontis* and the *E. coli phoA* gene that lacks its signal sequence. The promoters used are phage promoter P22 (H. E. Schnepf and H. R. Whiteley, unpublished results) and the rRNA promoter from *C. xyli* subsp. *cynodontis* (Sathyamoorthy et al. 1991). Unlike the native *phoA* promoter that is repressed by phosphate, these promoters are expressed constitutively. Using this system, we have screened seven heterologous signal sequences, and have shown that *phoA* from *E. coli* can be used as a reporter for secretion in *C. xyli* subsp. *cynodontis*.

It is not known if *C. xyli* subsp. *cynodontis* synthesizes AP. However, under the conditions described here, there is no background from endogenous AP. Wild-type *C. xyli* subsp. *cynodontis* contains a yellow pigment and forms yellow colonies. We used strain MDM1.6, a spontaneous white mutant of *C. xyli* subsp. *cynodontis*, as the host for the plasmid



**Fig. 4.** Histochemical staining for in planta activity of alkaline phosphatase (AP) in strain MDM1.1688/*sti2*. A cross-section of corn stem was stained for AP activity with substrate NBT-BCIP. Development of brown color, corresponding to active AP, was visible on the wall of the protoxylem in the vascular bundle. x = xylem; px = protoxylem; p = phloem. Magnification is  $\times 400$ .

constructs. Unlike *E. coli* strains expressing *phoA* that arise as blue colonies on indicator plates containing XP, the *C. xyli* subsp. *cynodontis* recombinants show a delay before turning blue. It is possible that the slow growth rate of *C. xyli* subsp. *cynodontis* (doubling time 6 h versus 20 min in *E. coli*) contributes to the delay in color change.

*Clavibacter xyli* subsp. *cynodontis* is a Gram-positive, high G+C organism. We have sequenced the lone 16S and 5S rRNA genes and have shown that they are most similar to those of *Streptomyces* species, which are high G+C Gram-positive actinomycetes (Sathyamoorthy et al. 1991). We have shown here that AP is efficiently secreted from *C. xyli* subsp. *cynodontis* when *phoA* is fused to the signal sequences from *chiC*, *sti2*, *snpA*, *dagA*, and *aml* (Fig. 2). Multiple bands were seen in the Western blot with the *snpA-phoA* fusion (Fig. 3). The signal sequence of *snpA* is 81 amino acids, considerably longer than the other signal sequences used (which range from 20 to 30 amino acids), and contains "pre" and "pro" regions. Therefore, the multiple bands in the Western blot are possibly the result of cleavage at multiple processing sites. Fusion of *phoA* to the signal sequence of *melC* showed no detectable enzyme activity. The only non-streptomycete signal sequence tested was the native *phoA* signal sequence from *E. coli*. The data suggest that this does not function efficiently in *C. xyli* subsp. *cynodontis*. The chimeric *C. xyli* subsp. *cynodontis*-*E. coli* plasmid pCG2004 also allowed us to examine whether the signal sequences of streptomycete origin functioned in *E. coli*. All apparently did, since their respective *E. coli* transformants were blue in the presence of XP (data not shown).

Our results show that AP can be efficiently exported by *C. xyli* subsp. *cynodontis* with five different *Streptomyces* signal sequences. The amount of extracellular AP produced in a complex medium was estimated to be in the range of 0.5 to 1 mg/ml in *C. xyli* subsp. *cynodontis* strain MDQ1.1759 based on a comparison with a known amount of purified AP on a Western blot.

We have demonstrated for the first time that AP is produced and secreted by *C. xyli* subsp. *cynodontis* strain MDQ1.1688 (pCG2021, *sti2*) in planta. Microscopic examination of corn stem sections revealed that AP activity was concentrated only around the wall of the protoxylem (Fig. 4), which is thought to be nonfunctional in a mature plant. Electron micrographs show the presence of *C. xyli* subsp. *cynodontis* adhering to the walls of both the xylem and protoxylem vessels (data not shown). It is therefore not clear why AP activity is confined to *C. xyli* subsp. *cynodontis* inhabiting the protoxylem, at least with strain MDQ1.1688. To date, this strain is the only chromosomal insertion of *phoA* constructed, and the only strain tested in planta. Thus, we cannot rule out the possibility that this particular insertion affects where AP activity is expressed, although that seems unlikely since the population and distribution of this strain are the same as the wild-type. It is known that AP is only active as a dimer. Therefore, it is possible that the reducing environment of the xylem may have prevented disulfide bond formation necessary for proper folding of the enzyme. Development of in planta AP antigen detection assays would help interpret these results. However, histochemical staining of AP antigen in corn stem sections using AP antibodies failed because of a high background due to cross-reactivity of the in planta samples to the antibodies.

We are attempting to develop quantitative assays for expression of proteins secreted from *C. xyli* subsp. *cynodontis* in planta. Results from such experiments are necessary to determine whether the xylem environment is inhibiting AP activity, and therefore to define the limitations of this reporter system for in planta detection. In addition, such quantitation is essential to determine if this endophyte delivery approach can be used successfully to produce useful quantities of a compound in planta.

By using the *phoA* secretion probe vector, we have shown that a number of heterologous signal sequences function to allow secretion in *C. xyli* subsp. *cynodontis*. The vector we have constructed will also be useful for the isolation of native *C. xyli* subsp. *cynodontis* signal sequences by shotgun cloning and for the study of protein export in this organism. We are currently testing *C. xyli* subsp. *cynodontis* recombinant strains that use these signal sequences to direct secretion of proteins with antifungal activity for their crop protection activities.

## MATERIALS AND METHODS

### Bacterial strains, plasmids and growth conditions.

Bacterial strains and plasmids are given in Table 1. *Escherichia coli* AW1061 ( $\Delta phoA phoB phoR$ ), plasmid pH11 (Inouye et al. 1981) and polyclonal antibodies for AP were generous gifts from A. Wright. *Escherichia coli* AW1061 was used as the host for constructions in *E. coli*, and the recombinants were grown in Luria broth (LB) medium containing 100  $\mu$ g ampicillin per ml and 10  $\mu$ g chloramphenicol per ml or 5  $\mu$ g tetracycline per ml. *Clavibacter xyli* subsp. *cynodontis* strains were grown in S27 broth (Anderson et al. 1988), and the recombinants were grown in S27 broth containing 10  $\mu$ g chloramphenicol per ml or 1  $\mu$ g tetracycline per ml. Plasmid pCG188 is a shuttle vector of *E. coli* and *C. xyli* subsp. *cynodontis* (Taylor et al. 1993); while pCG339 is an integrative vector in *C. xyli* subsp. *cynodontis*, a derivative of pCG563 (Lampel et al. 1994).

### Transformations.

Plasmid DNAs were introduced in *C. xyli* subsp. *cynodontis* by electrotransformation as described previously (Lampel et al. 1994). The transformants were selected on BC plates (Anderson et al. 1988) containing 10  $\mu$ g chloramphenicol per ml or 1  $\mu$ g tetracycline per ml.

### PCR primer design and amplifications.

PCR primers were selected on the basis of published DNA sequences of *phoA* (Chang et al. 1986; Inouye et al. 1982), *sti2* (Strickler et al. 1992), *chiC* (Fujii and Miyashita 1993), *melC* (Bernan et al. 1985), *snpA* (Lampel et al. 1992), *aml* (Long et al. 1987), and *dagA* (Buttner et al. 1987). The nucleotide (nt) positions of all the PCR primers are given with respect to the translational start site of the gene. The lower-case letters denote the nucleotide sequences of the non-complementary tails with sites for cloning, and the uppercase letters show the actual DNA sequences of the genes. PCR reagents were purchased from Perkin-Elmer Cetus (Norwalk, CT) and used according to their protocols. Cycling parameters were 25 cycles of denaturation (1 min at 94°C), annealing (30 s at 64°C), and extension (30 s at 72°C). The entire *phoA*

structural gene encoding the mature protein was PCR-amplified. The sequence of the forward PCR primer was 5'-ccgatacCGGACACCAGAAATGCC-3', corresponding to nts 64 to 80 of the *phoA* sequence (Chang et al. 1986). It contains an *EcoRV* site for cloning. The reverse PCR primer was 5'-cctgcagatccGTCTGGTTGCTAACAGC-3' located downstream of the transcriptional terminator of the *phoA* gene, corresponding to nts 1,457 to 1,474. PCR amplifications of pH11 resulted in a 1.4-kb product. Subsequent cloning of *phoA* generated plasmid pCG2001 (Fig. 1A).

The signal sequences and their respective leaders were also PCR amplified. The forward PCR primers were tagged with a *SpeI* site at the 5' end, and the reverse PCR primers were tagged with an *EcoRV* site at the 3' end. The resulting PCR products contain sequences for the leader-signal peptide and the first two amino acids of the mature protein. When cloned into the secretion probe vector pCG2004 (Fig. 1A), the junction of the signal sequence and the mature *phoA* sequence results in the formation of an *EcoRV* site with two extra codons (Asp, Ile) inserted after the processing site. Payne and Jackson (1991) and Nagarajan et al. (1992) have shown that the insertion of an *EcoRV* site preceding the mature *phoA* has no effect on secretion of AP (Fig. 2). The *E. coli* recombinants were blue on LB plates containing XP, indicative of AP activity. The DNA sequences of the PCR inserts were verified by sequencing.

***phoA*.** PCR primers for the *phoA* signal sequence and leader were 5'-ccactagtGCTTTGTTTTATTTTTTAATG-3', corresponding to nts -45 to -24; and 5'-ccgatacGGCTTTGTCACAGGGGTAAAC-3', corresponding to nts 42 to 63. The DNA template was pH11 (Inouye et al. 1981).

***sti2*.** The trypsin inhibitor gene from *Streptomyces longisporus*. PCR primers for the *sti2* signal sequence and leader were 5'-ccactagtGCTCGCCGGTCCGGCACCAAAC-3', corresponding to nts -68 to -47; and 5'-ccgatacCGGGGTGGC-GAGCGCGGCTCCGGTG-3', corresponding to nts 66 to 90. The DNA template was *S. longisporus* ATCC 23931 DNA (Strickler et al. 1992).

***chiC*.** The chitinase gene from *Streptomyces lividans* 66. PCR primers for the *chiC* signal sequence and leader were 5'-ccactagtACTGCGGGCATGAGGCTCAGTC-3', corresponding to nts -58 to -37; and 5'-ccgatacCGTGGCCGCCTGGG-CCGGGCTC-3', corresponding to nts 75 to 96. The DNA template was *S. lividans* DNA (Fujii and Miyashita 1993).

***melC*.** The ORF438 is located 5' to the tyrosinase gene in *Streptomyces antibioticus*. PCR primers for the *melC* signal sequence and leader were 5'-ccactagtAGGTATGCGGGGTC-GTCAACCC-3', corresponding to nts -44 and -23; and 5'-ccgatacGTCCGCGCGGGCGGCGGGAAG-3', corresponding to nts 73 to 93. The DNA template was pIJ702 (Bernan et al. 1985).

***snpA*.** The gene coding for the small neutral proteinase from *Streptomyces lividans* strain C5. PCR primers for the *snpA* signal sequence and leader were 5'-ccactagtCGGACAC-TACGATGTCTC-3' corresponding to nts -51 to -33; and 5'-ccgatacGGCCGCCGTGCTCTTCGGATTG-3', corresponding to nts 228 to 249. The DNA template was pANT25 (Lampel et al. 1992).

***dagA*.** The gene coding for the extracellular agarase from *Streptomyces coelicolor* A3(2). PCR primers for *dagA* were 5'-ccactagtGCCAGGCGGGAATCGAAGAAG-3', corresponding to nts -31 to -11; and 5'-ccgatacGTCTGCGGCATGAGCGGC

**Table 1.** Bacterial strains and plasmids

Strain or Plasmid	Description <sup>a</sup>	Source or Reference
<i>Clavibacter xyli</i> subsp. <i>cynodontis</i>		
MDM1.6	White mutant of <i>C. xyli</i> subsp. <i>cynodontis</i> strain MDE1	This study
MDQ1.1688	MDM1.6 with <i>sti2-phoA</i> as a chromosomal insertion	This study
MDQ1.1696	MDM1.6 with <i>aml-phoA</i> fusion	This study
MDQ1.1698	MDM1.6 with <i>snpA-phoA</i> fusion	This study
MDQ1.1704	MDM1.6 with <i>phoA</i>	This study
MDQ1.1705	MDM1.6 with <i>sti2-phoA</i> fusion	This study
MDQ1.1756	MDM1.6 with pCG2004	This study
MDQ1.1759	MDM1.6 with <i>chiC-phoA</i> fusion	This study
MDQ1.1778	MDM1.6 with <i>melC-phoA</i> fusion	This study
MDQ1.1781	MDM1.6 with <i>dagA-phoA</i> fusion	This study
<i>Escherichia coli</i>		
AW1061	$\Delta phoA phoB phoR$	Hoffman and Wright 1985
<i>Streptomyces</i>		
<i>S. lividans</i> 66	Strain carrying the <i>chiC</i> gene	Fujii and Miyashita 1993
<i>S. longisporus</i>	Strain carrying the <i>sti2</i> gene	Strickler et al. 1992
Plasmids		
pH11	<i>phoA</i> Tet <sup>r</sup>	Inouye et al. 1981
pCG188	Chimeric plasmid of <i>C. xyli</i> subsp. <i>cynodontis</i> and <i>E. coli</i> . Autonomous in <i>C. xyli</i> subsp. <i>cynodontis</i> , cam <sup>r</sup>	Taylor et al. 1993
pCG2001	pGEM5Z carrying <i>phoA</i> devoid of signal sequence	This study
pCG2004	pCG188 carrying <i>phoA</i> devoid of signal sequence	This study
pCG339	<i>C. xyli</i> subsp. <i>cynodontis</i> integration vector, Tet <sup>r</sup>	J. Kelly; Lampel et al. 1994
pCG2017	pGEM5Z carrying <i>sti2-phoA</i> fusion	This study
pCG2021	pCG339 carrying <i>sti2-phoA</i> fusion	This study
pIJ702	Streptomyces plasmid carrying <i>melC</i>	Bernan et al. 1985
pANT25	pUC19 carrying <i>snpA</i>	Lampel et al. 1992
pIJ2027	pUC18 carrying <i>dagA</i>	Buttner et al. 1987
pCetI	pUC18 carrying <i>aml</i>	Long et al. 1987

<sup>a</sup> cam = chloramphenicol; Tet = tetracycline; r = resistant.



GGG-3', corresponding to nts 76 to 96. The DNA template was pIJ2027 (Buttner et al. 1987).

**aml.** The  $\alpha$ -amylase gene of *Streptomyces limosus* ATCC 19778. PCR primers for *aml* were 5'-ccactagtTTTGCTGCA-AGAGAAATTCAGCCCTC-3', corresponding to nts -51 to -27; and 5'-ccgatatcCGGGGCGGCAGCGGCGGGTGTG-3', corresponding to nts 69 to 90. The DNA template was pCetI (Long et al. 1987).

### Immunodetection of AP.

The presence of AP in the culture supernatants of *C. xyli* subsp. *cynodontis* recombinants was detected by Western blotting (Towbin et al. 1979). Forty-milliliter cultures of *C. xyli* subsp. *cynodontis* were harvested during log phase, and the culture supernatants were precipitated by addition of solid  $(\text{NH}_4)_2\text{SO}_4$  to 85% of saturation. The  $(\text{NH}_4)_2\text{SO}_4$  precipitates were dissolved in 200  $\mu\text{l}$  of 10 mM Tris, 1 mM EDTA buffer, and dialyzed extensively in the same buffer. Aliquots of  $(\text{NH}_4)_2\text{SO}_4$  concentrates were boiled with sample buffer and separated by electrophoresis on 4 to 20% gradient sodium dodecyl sulfate polyacrylamide gels (Enprotech, Natick, MA). Gels were electrophoretically blotted onto nitrocellulose membranes (type BA-S83, Schleicher and Schuell, Keene, NH). The membranes were blocked with 1% skim milk, 0.5% Tween 20 in phosphate-saline buffer, treated with rabbit anti-AP antibody, and visualized using an AP conjugated goat anti-rabbit antibody (Pierce, Rockford, IL) and the NBT-BCIP color reagent kit (KPL Laboratories, Gaithersburg, MD). Sizes of immunoreactive bands were determined with pre-stained molecular weight markers (Enprotech).

### Assay of *phoA* activity.

*phoA* activity was measured by production of p-nitrophenol from p-nitrophenyl phosphate as described by Nagarajan et al. (1992).

### Histochemical staining for *phoA* activity in planta.

*Clavibacter xyli* subsp. *cynodontis* strain MDM1.6 and recombinant MDQ1.1688 were stab-inoculated into corn plants as described previously (Lampel et al. 1994). The stem portion closest to the soil was harvested after 6 weeks. The green sheaths on the stem were removed. Thin cross-sections were made with a razor blade and soaked for 5 min in 10 ml of NBT-BCIP color reagent in a petri dish, and the reaction stopped by addition of deionized water. The stem sections were examined under the microscope. The development of brown color is indicative of *phoA* activity.

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