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Cocolonization of the Rhizosphere by Pathogenic *Agrobacterium* Strains and Nonpathogenic Strains K84 and K1026, Used for Crown Gall Biocontrol

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The crown gall biocontrol agent strain K84 and three mutants derived from it, K1026 (Tra⁻ deletion mutant of pAgK84), K84 Agr⁻ (lacking pAgK84), and K1143 (lacking pAgK84 and pNoc), significantly reduced gall formation caused by two pathogenic strains resistant to agrocin 84 in peach × almond seedlings planted in infested soil. Cocolonization of roots by pathogenic and nonpathogenic strains was observed in these biocontrol experiments under field conditions. In spite of the efficient biocontrol observed, average populations consisting of 10^2 and 10^6 pathogenic agrobacteria per g of root were found 8 months after planting. The total numbers of pathogenic bacteria on roots were similar for plants treated with the biocontrol strains and for the untreated plants. Strain K84 and the genetically engineered organism K1026 survived at a level of 10^6 agrocin 84-producing bacteria per g of root. The population size of genetically engineered strain K1026 was not significantly different than the population size of wild-type strain K84 8 months after root inoculation. Strains K84 and K1026 controlled two pathogens resistant to agrocin 84 without reducing the total number of pathogenic bacteria in the root system. In addition, this study shows that some biological control activity of strain K84 against agrocin 84-resistant pathogens is independent of plasmids pAgK84 and pNoc.

Pathogenic *Agrobacterium* strains cause crown gall disease in many dicotyledonous plants, including stone fruit trees (17). Biological control of this disease with nonpathogenic strain K84 is the most efficient method of control (3, 7, 13). Strain K84 produces a highly specific antibiotic, agrocin 84, which is effective against many pathogenic *Agrobacterium* strains. Synthesis of agrocin 84 is encoded by plasmid pAgK84 (8). Transfer of pAgK84 from strain K84 to pathogenic strains could reduce the effectiveness of biocontrol (30). To avoid this transfer, a stable Tra⁻ deletion mutant of K84, K1026, has been constructed (12). Strain K1026 is as efficient as K84 in biocontrol of pathogenic strains, both strains susceptible to agrocin 84 and strains resistant to agrocin 84, on different hosts (11, 30). In addition to pAgK84, strain K84 harbors two other indigenous plasmids, pAgK434 encoding agrocin 434 production (5) and pNoc encoding catabolism of nopaline (3). Production of agrocin 84 is required for efficient control of crown gall disease caused by strains that are susceptible to agrocin 84 (4, 14, 17). However, it has been shown that strain K84 controls pathogens that are resistant to agrocin 84 in different countries (2, 16, 17, 30); the mechanisms involved in control of agrocin 84-resistant pathogens have not been determined, but it is known that these mechanisms are not associated with the production of agrocin 84 or with pAgK84 (17). These findings suggest that the biocontrol properties of K84 are complex and that production of agrocin 84 is only one of the components (9). Moreover, nothing is known about the possible role of plasmids pAgK434 and pNoc in biocontrol of crown gall disease caused by strains resistant to agrocin 84.

In addition to agrocin 84, strain K84 produces two other antibiotic substances, agrocin 434 (5) and the antibiotic-like substance ALS84 (24). Production of agrocin 434 is encoded by pAgK434, and this compound is effective only against biovar 2 pathogens (5). Recently, McClure et al. (19) suggested that agrocin 434 may play a role in biocontrol of agrocin 434-susceptible pathogens of biovar 2. Production of ALS84 is encoded in the chromosome, and ALS84 is effective against agrobacteria belonging to all biovars (24). ALS84 inhibitory activity is associated with the production of siderophores by strain K84 only under iron-limiting conditions (25).

According to Farrand and Wang (9), despite the fact that strain K84 is a commercially successful biocontrol agent, a careful analysis of the literature indicated that there is no good evidence concerning what makes this strain so effective against pathogens resistant to agrocin 84. Thus, new information is required to understand this complex process, and any knowledge obtained can be used to improve the process.

Early studies suggested that the incidence of crown gall disease was strongly correlated with the proportion of pathogenic and nonpathogenic agrobacteria in the rhizosphere of plants (21). Strain K84 has proven to be a good colonizer of the root systems of different hosts (18, 27, 30), and it has been shown that large populations of a mutant of K84 that is resistant to rifampin and streptomycin are maintained in the rhizosphere of cherry plants for up to 2 years (28). Additional studies have suggested that the ability of strain K84 to colonize and persist on roots is important in the biocontrol process (6, 27). However, there are no data available on differential colonization of the rhizosphere by pathogenic bacteria and the biocontrol agent obtained in situ in biocontrol experiments, in which the pathogen is introduced into the soil, like under natural conditions of infection. Likewise, there is no information about the environmental fitness or persistence in the rhizosphere of genetically engineered strain K1026 compared to the environmental fitness or persistence of parent strain K84.

The present study was undertaken to (i) study the populations of pathogenic and nonpathogenic (biocontrol agent) bac-

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TABLE 1. Characteristics of nonpathogenic and pathogenic *Agrobacterium* strains used in crown gall biocontrol experiments

Strain	Biovar	Antibiotic production[a]			Susceptibility to[a]:			Plasmid(s)[b]	Description	References
		Agrocin 84	Agrocin 434	ALS84	Agrocin 84	Agrocin 434	ALS84			
Nonpathogenic strains										
K84	2	+	+	+	NA	NA	NA	pAgK434, pNoc, pAgK84	Utilized for biological control of crown gall	14, 24
K1026[c]	2	+	+	+	NA	NA	NA	pAgK434, pNoc, pAgK1026	Mutant strain derived from K84 with Tra deletion in pAgK84	12, 24
K84 Agr[−d]	2	−	+	+	NA	NA	NA	pAgK434, pNoc	Strain K84 cured of pAgK84	4, 24
K434[e]	2	−	+	+	NA	NA	NA	pAgK434, pNoc	Spontaneous mutant of K84 lacking pAgK84	5, 24
K1143[e]	2	−	+	+	NA	NA	NA	pAgK434	Strain K84 lacking pNoc and pAgK84	5, 24
Pathogenic strains										
B6	1	NA	NA	NA	R	R	S	ND		
66R	2	NA	NA	NA	R	R	R	ND	Resistant to rifampin and streptomycin	

[a] Production of and susceptibility to agrocin 84, agrocin 434, and ALS84 were determined as described in Materials and Methods. +, producer; −, non producer; NA, not applicable; R, resistant; S, susceptible.
[b] ND, not determined.
[c] Strain K1026 was kindly supplied by G. C. Bullard (Bio-Care Technology, Woy-Woy, Australia).
[d] Strain K84 Agr− was kindly supplied by L. M. Moore (Oregon State University, Corvallis).
[e] Strains K434 and K1143 were kindly supplied by B. Clare (Waite Agricultural Institute, Glen Osmond, Australia).

teria on roots in a field biocontrol experiment under conditions similar to those of natural infection, (ii) study the ability of genetically engineered strain K1026 to persist in the rhizosphere compared to the ability of parent strain K84 to persist, and (iii) evaluate the effectiveness of derivatives of strain K84 lacking either pAgK84 (mutant K84 Agr−) or both pNoc and pAgK84 (mutant K1143) in controlling two pathogenic *Agrobacterium* strains resistant to agrocin 84. The results obtained may help elucidate the complex mechanisms that strain K84 uses to control crown gall disease caused by strains resistant to agrocin 84.

MATERIALS AND METHODS

Bacterial strains. It has been proposed that the two biovars of the genus *Agrobacterium* corresponded to distinct species, but in this study *Agrobacterium* strains were classified as members of biovar 1 or biovar 2. Nonpathogenic *Agrobacterium* strains K84, K1026, K84 Agr−, and K1143 were used as biocontrol agents. Strains B6 and 66R were used as challenge pathogens. The characteristics of the strains are described in Table 1. Strain 66R is resistant to rifampin (100 μg/ml) and streptomycin (500 μg/ml). Resistance of the pathogens to agrocin 84 and susceptibility to ALS84 were determined as described by Peñalver et al. (24). Susceptibility to agrocin 434 was determined as described by Donner et al. (5) by using strain K434 as the producer strain.

Biocontrol assays. The abilities of strains K84, K1026, K84 Agr−, and K1143 to control strains B6 and 66R were assessed under field conditions in nine open-air containers (5 by 2 by 0.5 m) filled with a natural soil typical of the area (a loamy, calcareous, sandy clay with a pH of 7.6). Pathogenic agrobacteria were not isolated from this soil before inoculation. The experiments were performed as previously described by Vicedo et al. (30). Briefly, just before planting, the soil was inoculated with pathogens by pouring onto the soil enough water suspension containing strain B6 or 66R to obtain a final concentration of about 10^6 CFU/g (dry weight) of soil. One hundred rooted 1-year-old plants of peach × almond (*Prunus persica* × *P. dulcis*) hybrid GF677 were used in each treatment. One untreated control and four treatments (strains K84, K1026, K84 Agr−, and K1143) were evaluated in soil inoculated with strain B6. One untreated control and three treatments (strains K84, K1026, and K1143) were used for assays performed with strain 66R. The biocontrol strains were grown in a fermentor for 60 to 72 h and were mixed with Padul peat moss at a ratio of 1:1. The mixtures were packed in polyethylene bags and allowed to mature for 10 days at 4°C. The peat inoculum used to treat the plants with strain K84 and strains derived from strain K84 was prepared as described by López et al. (16). The final concentration of strain K84 and the strains derived from K84 in the aqueous suspension of the peat preparation was 1.0×10^9 CFU/ml. The plants were grown for 8 months and then dug up and examined to determine whether galls were formed. The results of the crown gall biocontrol experiment were expressed as percentages of disease reduction in each treatment compared to the corresponding control, calculated as follows: Index of control gall biocontrol = 100% − [(% of disease incidence in treatment × 100)/(% of disease incidence in corresponding control)].

The proportions of plants with galls for each treatment and the corresponding control were compared by using the approximate Z test ($P = 0.05$) as described by Vicedo et al. (29). Bacteria were isolated from 10 galls from each treatment by plating tumor tissue macerates onto two selective media (20, 26). Up to five typical *Agrobacterium* type colonies were purified from each isolation plate and characterized.

Root colonization and survival in the rhizosphere. Root colonization by soil-inoculated strains B6 and 66R and survival in the rhizosphere of root-inoculated strain K84 and Tra− mutant K1026 were studied as described by Vicedo et al. (30). The sizes of populations of pathogenic and nonpathogenic agrobacteria in the rhizospheres of at least five symptomless plants per treatment were determined after 8 months. Each plant was analyzed as a separate sample. Strain B6 was recovered on biovar 1 selective medium (26), and the identity of the colonies was confirmed by an indirect enzyme-linked immunosorbent assay. Twenty-five *Agrobacterium*-like colonies from each sample were analyzed with OH2437 antiserum specific for strain B6 as described by Alarcón et al. (1). Suspensions of strains B6 and K84 were used as positive and negative controls for the enzyme-linked immunosorbent assay, respectively. In previous experiments, the OH2437 antiserum did not react with nonpathogenic strains introduced onto the roots of treated plants (1). A colony was identified as a strain B6 colony when the serological relationship with the positive control was greater than 60%. The serological relationship was determined as follows: $[(x - y)/(z - y)] \times 100$, where x is the absorbance at 405 nm of a suspension of a colony, z is the absorbance of the standard positive control, and y is the absorbance of the standard negative control (1). Antibiotic-resistant mutant 66R was recovered on PGYA medium (30) supplemented with cycloheximide (250 μg/ml), rifampin (100 μg/ml), and streptomycin (500 μg/ml). The biocontrol strains did not grow on this selective medium. Strains K84 and K1026 were isolated on the selective medium of New and Kerr (20). Production of agrocin 84 by a proportion of the colonies recovered was used to confirm the identity of K84 or K1026 as described by Peñalver et al. (24). The colonization data were expressed as $\log_{10}$ CFU per gram (fresh weight) of root (22) and were analyzed by performing an analysis of variance ($P = 0.05$) with the computer program Statgraphics (Statistical Graphics Corporation, Inc.).

RESULTS

Biological control of agrocin 84-resistant pathogens. The abilities of strain K84 and mutants K1026, K84 Agr−, and K1143 to reduce gall formation caused by strains B6 and 66R are shown in Table 2. There were significant differences in the

TABLE 2. Control of strains B6 and 66R on peach × almond hybrid plants by nonpathogenic strain K84 and strains derived from K84[a]

Pathogen	Treatment	No. of plants analyzed	% of plants with galls	Index of crown gall biocontrol (%)[b]
B6	Control	76	9.2	
	K84	56	0.0[c]	100
	K1026	70	0.0[c]	100
	K84 Agr$^-$	70	2.8[c]	69
	K1143	67	0.0[c]	100
66R	Control	66	18.2	
	K84	80	0.0[c]	100
	K1026	61	1.6[c]	91
	K1143	74	8.1[c]	56

[a] The numbers of plants with galls were determined 8 months after inoculation.

[b] The index of crown gall biocontrol was calculated as described in the text.

[c] Value that is significantly different from the corresponding control value. The data were analyzed by using the approximate Z test for the different treatment values and the corresponding control values as described in the text.

TABLE 3. Root colonization by soil-inoculated pathogens B6 and 66R and survival in the rhizosphere of root-inoculated biocontrol agent K84 and genetically engineered derivative strain K1026 in biocontrol assays

Pathogen	Treatment	Log_{10} no. of pathogenic cells per g (fresh wt) of root[a]	Log_{10} no. of nonpathogenic K84 or K1026 per g (fresh wt) of root[b]
B6	Control	4.1 ± 0.2	
	K84	4.8 ± 0.4	5.9 ± 0.3
	K1026	4.9 ± 0.6	6.2 ± 0.4
	K84 Agr$^-$	5.7 ± 1.1	
	K1143	4.7 ± 0.5	
66R	Control	3.6 ± 0.3	
	K84	3.5 ± 0.3	6.2 ± 0.3
	K1026	2.8 ± 0.3	5.8 ± 0.8
	K1143	4.0 ± 0.7	

[a] Mean $\log_{10}$ ± standard deviation. Data were analyzed by performing an analysis of variance as described in the text. The population sizes (CFU per gram [fresh weight] of root) of the treated samples did not differ significantly from the population sizes of the corresponding controls.

[b] Mean $\log_{10}$ ± standard deviation. Data were analyzed by performing an analysis of variance as described in the text. The population size (CFU per gram [fresh weight] of root) of genetically engineered strain K1026 did not differ significantly from the population size of wild-type strain K84.

number of plants with galls when the untreated control plants were compared with plants treated with any of the biocontrol agents in assays performed with the challenge pathogen B6. All of the derivatives of strain K84 tested were as able to control the formation of crown galls induced by the agrocin 84-resistant pathogen B6 as wild-type strain K84 was. The biocontrol index for mutant K84 Agr$^-$ was 69%, and the biocontrol index for strains K84, K1026, and K1143 was 100%. Similarly, strain K84 and mutants K1026 and K1143 significantly reduced the incidence of the disease on seedlings planted in soil infested with the other agrocin 84-resistant pathogen, strain 66R. The index of biocontrol ranged from 56% for K1143 to 91 and 100% for strains K1026 and K84, respectively. In all of the treatments in which plants with galls were obtained, bacteria isolated from the galls were identical to the introduced pathogens.

Root colonization by the introduced pathogens in biocontrol assays. In the same biocontrol assays in which strain K84 and mutants derived from K84 efficiently controlled two agrocin 84-resistant pathogens, we measured the numbers of pathogenic and nonpathogenic bacteria on the root systems. The levels of root colonization by soil-introduced pathogenic strains B6 and 66R after 8 months are shown in Table 3. In soil inoculated with strain B6, the average sizes of populations of pathogenic bacteria were 10^4 to 10^6 CFU/g (fresh weight) of root (Table 3). No significant differences were found in the mean population sizes for strain B6 when control plants were compared to plants treated with the four nonpathogenic strains. In soil inoculated with strain 66R, the average population sizes of pathogenic bacteria ranged from 10^2 to 10^4 CFU/g (fresh weight) of root. No significant differences were found between the sizes of the populations of strain 66R on control plants and plants treated with nonpathogenic strains, although biocontrol was efficient.

Survival in the rhizosphere of strain K84 and genetically engineered derivative strain K1026 as determined in biocontrol assays. In the same biological control assays we measured the numbers of nonpathogenic bacteria producing agrocin 84 on roots in the treatments with the biocontrol agents K84 and K1026 8 months after root inoculation. The mean size of the population of strain K84 or K1026 was 10^6 CFU/g (fresh weight) of root for K84- or K1026-treated plants in the biocontrol assays performed with B6 or 66R (Table 3). The differences in the sizes of the populations of genetically engineered strain K1026 and wild-type strain K84 were not significant.

DISCUSSION

Pathogenic strains B6 and 66R are not inhibited in vitro by agrocin 84, but gall formation caused by these strains was reduced by strains K84 and K1026 in vivo. These data, together with previous results (11, 30) confirmed the efficacy of Tra$^-$ mutant strain K1026 for crown gall biocontrol. Moreover, it has been shown that strain K84 is able to control agrocin 84-resistant pathogens under conditions similar to natural infection, suggesting that in addition to agrocin 84 production other traits of strain K84 are involved in its ability to control crown gall disease.

As far as we know, no comparative data concerning root colonization by the pathogen and the biocontrol agent have been obtained in situ in crown gall biocontrol experiments. We measured the amounts of pathogenic and nonpathogenic bacteria in biocontrol assays in which two agrocin 84-resistant pathogens were controlled efficiently. We confirmed that *Agrobacterium* strains were able to colonize the rhizosphere (22, 31). Average population sizes of 10^2 to 10^6 pathogenic bacteria per g of root were recovered from plants 8 months after they were planted in infested soil. The mean population sizes recovered from the rhizosphere were similar to the mean population sizes reported in other studies (22, 27), which supported the assertion that our soil inoculation procedure resulted in colonization of roots by the pathogens. Surprisingly, the population sizes of strains B6 and 66R on roots were similar for plants treated with any of the biocontrol agents and for untreated plants. The presence of strain K84 on roots did not affect root colonization by pathogens resistant to agrocin 84, independent of whether K84 produced agrocin 84. Strain B6 has been shown to be slightly susceptible in vitro to the ALS84 produced by strain K84 and mutants derived from it; however, any of these treatments with these biocontrol agents resulted in a reduction in the total number of B6 cells on roots compared to the number of B6 cells on roots of the untreated plants. These data suggest that antagonism by ALS84 did not

affect global root colonization by pathogenic strain B6 in these experiments. In vitro, strains K84 and K1026 and mutant K1143 produced agrocin 434, but in vivo any of these treatments resulted in a reduction in the total number of 66R bacteria on roots compared to the number of 66R bacteria on roots of untreated plants. Thus, these data suggest that antagonism by agrocin 434 does not affect general root colonization by pathogenic strain 66R (a biovar 2 strain).

Strain K84 and genetically engineered mutant K1026 survived at levels of 10^6 agrocin 84-producing bacteria per g of root 8 months after they were inoculated onto the roots. The population size of genetically engineered strain K1026 did not differ significantly from the population size of wild-type strain K84 in roots of plants growing in soil inoculated with agrocin 84-resistant *Agrobacterium* strains. Deletion of *tra* on pAgK84 in strain K84 did not affect the ability of this strain to colonize and survive in the rhizosphere.

Our data demonstrated that both pathogenic and nonpathogenic agrobacteria cocolonized roots during the biocontrol experiments. Strains K84 and K1026 and pathogenic bacteria were isolated from roots in another biocontrol experiment (11), but the numbers of pathogenic and nonpathogenic bacteria colonizing roots were not determined. The biocontrol agents on roots were counted by directly quantifying the agrocin 84-producing bacteria instead of using antibiotic-resistant mutants or any other indirect method because the mutants may have differed in fitness compared to the wild-type strain. As far as we know, this is the first time that cocolonization of roots by K84 or K1026 and pathogenic bacteria has been directly quantified in a biocontrol experiment performed under conditions similar to the conditions found in natural infections. Inhibition of tumorigenesis despite root colonization by pathogenic agrobacteria has been observed in plants treated with K84 or K1026. It is known that an *Agrobacterium* strain needs a fresh wound to produce a tumor. The fresh wounds of the plants treated with K84 or K1026 could be colonized by the biocontrol agent, which would have impeded tumor induction by the pathogen, as suggested long ago (15). Quantification of root colonization by the pathogen and biocontrol agent K84 or K1026 revealed that in the rhizospheres of plants treated with K84 or K1026, both pathogenic and nonpathogenic bacteria coexisted during the experiment, but the population sizes of the nonpathogenic bacteria were greater than the population sizes of the pathogenic bacteria (there was a 1- to 3-log difference). In this context, the possibility of producing a K84- or K1026-treated plant with galls could be much less than the possibility of producing an untreated plant with galls when only pathogenic bacteria were present. In summary, control of the two pathogens by K84 treatment was complete, and there was no reduction in the total population of pathogenic bacteria in the root systems.

In this study, the biocontrol assays showed that strain K84 and plasmid-deficient mutants K84 Agr$^-$ (lacking pAgK84) and K1143 (lacking pNoc and pAgK84) efficiently controlled two pathogenic strains resistant to agrocin 84 under field conditions. When the possible involvement of pAgK84 in controlling agrocin 84-resistant pathogens was considered, this report and our previous studies showed that control of such organisms was similar whether K84 or K84 lacking pAgK84 (K84 Agr$^-$) was used (17). Under conditions under which strain K84 efficiently controls agrocin 84-resistant pathogens, pAgK84 is not necessary for maximum control, at least when pNoc and/or pAgK434 is present. This indicates that agrocin 84 production is the unique important trait encoded by pAgK84 that is involved in crown gall biocontrol by strain K84, but this trait plays an important role mainly against agrocin 84-susceptible pathogens (4, 17). When the possible involvement of pNoc in controlling agrocin 84-resistant pathogens was considered in biocontrol assays, mutant K1143 (lacking pNoc and pAgK84) efficiently controlled two pathogens resistant to agrocin 84 under field conditions. This mutant was as efficient as wild-type strain K84 in controlling crown galls induced by strain B6. On the other hand, K1143 still efficiently controlled the disease induced by 66R; however, mutant K1143 was less efficient against this pathogen than wild-type strain K84 was. This difference in the ability of mutant K1143 to control crown gall produced by two agrocin 84-resistant pathogens may be related to the different strains of challenge pathogens. As far as we know, strains B6 and 66R differ in a number of ways, including their nutritional requirements (biovar) and susceptibility to ALS84. Recently, McClure et al. (19) showed that mutant K1143 was as efficient as strain K84 in inhibiting the formation of tumors on almond trees in a biocontrol assay performed with a pathogenic strain susceptible to agrocin 84. Thus, pNoc does not seem to be necessary for efficient control of biovar 1 pathogens (such as strain B6), but according to the data of McClure et al. (19) pNoc may play a role in biocontrol of biovar 2 pathogens (such as strain 66R). Overall, these results show that some biological control activity of strain K84 against agrocin 84-resistant pathogens is independent of pAgK84 and pNoc. Thus, a third plasmid, pAgK434, and chromosomally encoded traits could be involved in the ability of strain K84 to control crown gall disease caused by strains resistant to agrocin 84.

Agrocin 434, whose production is encoded by pAgK434, inhibits the growth of only agrobacteria belonging to biovar 2 (5). Challenge pathogen B6 (a biovar 1 strain) is itself resistant to agrocin 434, and strain 66R (a biovar 2 strain) has been shown to be resistant when tested in bioassays in which K434 is the producing strain. For these reasons, it is unlikely that agrocin 434 played an important role in the biocontrol observed in our experiments. However, the results obtained with strains that do not produce agrocin 434 suggest that agrocin 434 may play a role in biocontrol of agrocin 434-susceptible pathogens (19). The third antibiotic produced in vitro by strain K84 is the antibiotic-like substance ALS84 (24). ALS84 is a hydroxamate type of siderophore that is produced by strain K84 only under low-iron conditions, and it is encoded by the chromosome (25). ALS84 inhibits the growth of many pathogenic strains (24). However, when the control of strains B6 and 66R was examined in this study, ALS84 did not seem to play an important role because strain B6 exhibited only slight sensitivity in vitro to ALS84 (24) and strain 66R was resistant to ALS84. However, we cannot eliminate the possibility that ALS84 plays a role in the control of ALS84-susceptible pathogens if it is produced in planta.

According to the hypothesis of Farrand and Wang (9), it is not clear whether general colonization is the most important parameter in crown gall biocontrol by strain K84. Strain K84 could act in a specific microhabitat and could inhibit the formation of tumors by different mechanisms at the primary sites of infection. It has been suggested that this complex phenomenon includes physical blocking of the sites of infection (15) and/or antibiosis (5, 14, 24) or a combination of these or other mechanisms. One of the possible mechanisms could be that antibiotics act in some way other than killing the target pathogen. The possible roles of the different antibiotics produced by strain K84 during transformation of plant cells by the pathogen are being investigated. Recently, we observed that strain K84 was not able to block the attachment of strain B6 to tomato root tips cultured in vitro (23). In this work we also found that strain K84 efficiently controlled two pathogens resistant to

agrocin 84 without a reduction in the total number of pathogenic bacteria in the root systems. Both experimental observations fit the hypothesis of Farrand and Wang (9).

Overall, biocontrol is often attributed to antibiosis (10). Even though strain K84 produces three different antiagrobacterial substances, antibiosis is not a unique mechanism that can completely explain the ability of strain K84 to control crown gall disease in the field.

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Interactions between Carbon and Nitrogen Metabolism in *Fibrobacter succinogenes* S85: a ^{1}H and ^{13}C Nuclear Magnetic Resonance and Enzymatic Study

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The effect of the presence of ammonia on [1-^{13}C]glucose metabolism in the rumen fibrolytic bacterium *Fibrobacter succinogenes* S85 was studied by ^{13}C and ^{1}H nuclear magnetic resonance (NMR). Ammonia halved the level of glycogen storage and increased the rate of glucose conversion into acetate and succinate 2.2-fold and 1.4-fold, respectively, reducing the succinate-to-acetate ratio. The ^{13}C enrichment of succinate and acetate was precisely quantified by ^{13}C-filtered spin-echo difference ^{1}H-NMR spectroscopy. The presence of ammonia did not modify the ^{13}C enrichment of succinate C-2 (without ammonia, 20.8%, and with ammonia, 21.6%), indicating that the isotopic dilution of metabolites due to utilization of endogenous glycogen was not affected. In contrast, the presence of ammonia markedly decreased the ^{13}C enrichment of acetate C-2 (from 40 to 31%), reflecting enhanced reversal of the succinate synthesis pathway. The reversal of glycolysis was unaffected by the presence of ammonia as shown by ^{13}C-NMR analysis. Study of cell extracts showed that the main pathways of ammonia assimilation in *F. succinogenes* were glutamate dehydrogenase and alanine dehydrogenase. Glutamine synthetase activity was not detected. Glutamate dehydrogenase was active with both NAD and NADP as cofactors and was not repressed under ammonia limitation in the culture. Glutamate-pyruvate and glutamate-oxaloacetate transaminase activities were evidenced by spectrophotometry and ^{1}H NMR. When cells were incubated in vivo with [1-^{13}C]glucose, only ^{13}C-labeled aspartate, glutamate, alanine, and valine were detected. Their labelings were consistent with the proposed amino acid synthesis pathway and with the reversal of the succinate synthesis pathway.

Fibrobacter succinogenes is a major rumen fibrolytic bacterium. It uses cellulose, glucose, and cellobiose as carbon and energy sources and ammonia as its sole nitrogen source (21). Ammonia is the prime source for protein synthesis in the rumen, accounting for the synthesis of 50 to 70% of bacterial nitrogen (13). Most of the studies of ammonia assimilation in the rumen have dealt with total ruminal content, and only a few papers have given detailed information on individual species (18, 23). In particular, the pathways of ammonia assimilation and amino acid synthesis in *F. succinogenes* are still unknown, although its carbon metabolism has been extensively studied (14, 15, 17). Glucose is metabolized through glycolysis into succinate, acetate, and a little formate. A part of the carbohydrates metabolized is stored as glycogen. Analysis by ^{13}C and ^{1}H nuclear magnetic resonance (NMR) was previously used to monitor in vivo the storage and degradation of glycogen in resting cells of different strains of *F. succinogenes* (8, 16), and a futile cycling of glycogen was shown: glycogen was simultaneously stored and degraded when bacteria were supplied with an exogenous carbon source. Furthermore, *F. succinogenes* was shown to accumulate glycogen throughout the exponential growth phase even when ammonia was not limiting (8, 16), whereas bacteria usually accumulate glycogen when ammonia (or another factor) limits growth (19). In addition to the futile glycogen cycle, a reversal of glycolysis and of the succinate synthesis pathway was found in *F. succinogenes* (16). These unusual features prompted us to investigate the relationship between ammonia assimilation and glycogen storage and also the effect of the presence of ammonia on the futile cycles. The enzymes responsible for ammonia assimilation and amino acid synthesis from intermediates of glucose metabolism were also sought.

The main pathways of ammonia assimilation were glutamate dehydrogenase (GLDH) and alanine dehydrogenase (ADH). The results obtained by ^{13}C-NMR and ^{13}C-filtered spin-echo difference (^{13}C-FSED) ^{1}H-NMR spectroscopy suggest a modulation of carbon metabolism by ammonia in *F. succinogenes* S85.

MATERIALS AND METHODS

Culture conditions. *F. succinogenes* S85 (ATCC 19169) was grown for 15 h on a chemically defined medium (8) with 3 g of cellobiose per liter.

Preparation and incubation of cells. For in vivo NMR experiments, cells were prepared as described by Matheron et al. (16). The cells harvested in the late log phase were spun (6,000 × *g*, 10 min, 4°C) and resuspended in a reduced 50 mM potassium phosphate–0.4% Na_2CO_3–0.05% cysteine buffer (pH 7.1). The cells at a final concentration of 5 mg of protein · ml^{-1} were incubated with 32 mM [1-^{13}C]glucose, with or without 13 mM $(NH_4)_2SO_4$, depending on the experiment. The cells were incubated at 37°C either in the spectrometer (in vivo NMR) or in a water bath and then sampled.

Each experiment was carried out at least three times with three different cultures.

Preparation of cell extracts. For ^{13}C-FSED ^{1}H-NMR and ^{13}C-NMR experiments, samples taken at the end of the incubation were frozen in liquid nitrogen and thawed three times. After being spun (15,000 × *g*, 10 min, 4°C) to remove the cell debris, the supernatants were analyzed by NMR.

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