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Role of *pfkA* and General Carbohydrate Catabolism in Seed Colonization by *Enterobacter cloacae*

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Enterobacter cloacae **A-11 is a transposon mutant of strain 501R3 that was deficient in cucumber spermosphere colonization and in the utilization of certain carbohydrates (D. P. Roberts, C. J. Sheets, and J. S. Hartung, Can. J. Microbiol. 38:1128–1134, 1992). In vitro growth of strain A-11 was reduced or deficient on most carbohydrates that supported growth of strain 501R3 but was unaffected on fructose, glycerol, and all amino acids and organic acids tested. Colonization by strain A-11 was significantly reduced ($P \leq 0.05$) for cucumber and radish seeds compared to that of strain 501R3, but colonization of pea, soybean, sunflower, and sweet corn seeds was not reduced. Pea seeds released several orders of magnitude more total carbohydrates and amino acids than cucumber and radish seeds and approximately 4,000-fold more fructose. Fructose was the only carbohydrate detected in the seed exudates which supported wild-type levels of in vitro growth of strain A-11. Soybean, sunflower, and sweet corn seeds also released significantly greater amounts of fructose and total carbohydrates and amino acids than cucumber or radish seeds. The exogenous addition of fructose to cucumber and radish seeds at quantities similar to the total quantity of carbohydrates released from pea seeds over 96 h increased the populations of strain A-11 to levels comparable to those of strain 501R3 in sterile sand. Molecular characterization of strain A-11 indicated that the mini-Tn*5* kanamycin transposon was inserted in a region of the genome with significant homology to *pfkA*, which encodes phosphofructo kinase. A comparison of strain A-11 with *Escherichia coli* DF456, a known *pfkA* mutant, indicated that the nutritional loss phenotypes were identical. Furthermore, the *pfkA* homolog cloned from *E. cloacae* 501R3 complemented the nutritional loss phenotypes of both *E. coli* DF456 and *E. cloacae* A-11 and restored colonization by strain A-11 to near wild-type levels. These genetic and biochemical restoration experiments provide strong evidence that the quantities of reduced carbon sources found in seed exudates and the ability of microbes to use these compounds play important roles in the colonization of the spermosphere.**

Colonization of subterranean portions of plants can be an essential process for a number of beneficial microbial activities, including plant growth promotion, plant disease control, and bioremediation (2, 6). Much research has been conducted in attempts to understand the processes by which microbes colonize plants, and several bacterial traits have been correlated with the colonization of seeds and roots in specific systems (e.g., references 3, 4, 7–9, 12, 14, 18, 26, and 41). One trait, microbial growth, has been established as an essential process for colonization, and recent studies have been published concerning the nutritional requirements for microbial growth on subterranean portions of plants (24, 29–32, 35, 36). However, the contributing roles played by the catabolic pathways of beneficial microbes, and the nutrients supplied by the host plant, in growth and colonization are still unclear.

The plant-beneficial bacterium *Enterobacter cloacae* suppresses *Pythium ultimum* damping-off of cucumber and other crops *Pythium ultimum* by (22) and colonizes the spermospheres and rhizospheres of a number of plant species (13, 16, 17, 22, 28–31, 39). It is believed that seeds and roots support growth by bacteria such as *E. cloacae* through the release of complex mixtures of carbohydrates, amino acids, water-soluble and -insoluble organic acids, and other nutrients (5). The growth of strain A-11, a nutritional mutant of *E. cloacae* 501R3 (29), was reduced or deficient on almost all carbohydrates released by seeds and roots that supported the growth of strain 501R3 in vitro (29, 32). The colonization of seeds of a cucumber (but not of a pea) cultivar by strain A-11 was significantly reduced ($P \leq 0.05$) relative to that of strain 501R3 in studies conducted in natural soil and in sterile sand (29, 32). We have characterized the mutation in strain A-11 at the molecular level in an attempt to understand the colonization behavior of this strain. We report here that glycolysis in *E. cloacae* A-11 is blocked by a mutation in *pfkA* and that this gene is most important for colonization of seeds that release limited quantities of reduced carbon sources. Portions of this work have been published previously (32).

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MATERIALS AND METHODS

Bacterial strains and plasmids. Descriptions of the bacterial strains and plasmids are in Table 1. Unless otherwise indicated, *E. cloacae* and *Escherichia coli* strains were grown to the stationary phase at 35°C and 250 rpm. The following media were used: Luria-Bertani (LB) broth or agar (19), M9 basal salts broth or agar (19), and M56 basal salts broth or agar (23) supplemented as previously described. The antibiotic levels used to maintain or select strains and plasmids were 100 μg/ml for rifampin (RIF) and streptomycin (STR), 50 μg/ml for kanamycin (KAN), and 12.5 μg/ml for tetracycline (TET).

In vitro growth on reduced carbon and nitrogen sources. Growth of bacterial strains on various reduced carbon compounds was compared spectrophotometrically in basal salts broth or on basal salts agar amended with 0.2% carbohydrate or with 0.5% L-amino acid or 0.5% organic acid as previously described (19, 29, 30). The carbohydrates used were arabinose, cellobiose, fructose, galactose, glucose, *N*-acetylglucosamine, lactose, maltose, mannitol, mannose, raffinose, rhamnose, ribose, salicin, stachyose, sucrose, trehalose, and xylose. The L-amino acids and organic acids tested were alanine, asparagine, proline, glutamate, glutamine, serine, pyruvate, and malate. The growth of *E. cloacae* 501R3 and

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Description[a]	Reference or source
Strains		
E. cloacae 501R3	Spontaneous Rif^r mutant of *E. cloacae* Ec CT501	29
E. cloacae A-11	Mini-Tn*5* KAN mutant of 501R3; Rif^r Km^r	29
E. coli DH5α	(ϕ80d*lacZ*ΔM15) Δ(*lacZYA-argF*) *U169 glnV44 deoR gyrA96 recA1 relA91 endA1 thi-1 hsdR17*	27
E. coli DF456	*glnV44 rpsL104 pfkA300*::Mu *metB1 lacY* Sm^r	40
Plasmids		
pLAFR3	Tc^r, pLAFR1 derivative	11
pRK2013	Km^r	10
pRK415	Tc^r	N. Keen
p82	*pfkA*$^+$ Tc^r	This work
pSubB	*pfkA*$^+$ Tc^r	This work
pSubB-3	Tc^r	This work
pBB1415	*pfkA*$^+$ Tc^r	This work
pBS1415	*pfkA*$^+$ Tc^r	This work

[a] Km^r, Rif^r, Sm^r, and Tc^r designate resistance to kanamycin, rifampin, streptomycin, and tetracycline, respectively; *pfkA*$^+$, phosphofructo kinase positive.

A-11 on alanine, asparagine, cysteine, glutamate, glutamine, proline, and serine as sources of nitrogen were measured as previously described (30).

Seed colonization assays. *E. cloacae* strains were grown, washed, resuspended, and applied to single cucumber (*Cucumis sativum* cv. Marketmore 76), radish (*Raphinus sativus* cv. Cherry Bomb), pea (*Pisum sativum* cv. Sugar snap), soybean (*Glycine max* cv. Chesapeake), sunflower (*Helianthus giganteus*), or sweet corn (*Zea mays* cv. Stowells Evergreen) seeds at approximately 10^4 CFU per seed as previously described (29). Seeds were buried in 4 g of a natural Galestown gravelly loamy sand soil (77.8% sand, 12.6% silt, 9.6% clay, and 0.6% organic matter) that had been previously equilibrated to −75 kPa or in 4 g of washed, sterile sand moistened with 4 ml of sterile distilled water (SDW) in 14-ml sterile snap cap tubes and incubated at 22°C. CFU were determined by spiral plating (Spiral Systems, Cincinnati, Ohio) the contents of the tubes onto LB agar containing 100 μg of cycloheximide/ml and the appropriate antibiotics for each bacterial strain.

Experiments were performed three times each with three replicates for each seed-strain combination at each of three sampling times (24, 45, and 96 h) for experiments comparing colonization of seeds by strains A-11 and 501R3. The mean $\log_{10}$ CFU per seed were determined and compared for strains 501R3 and A-11 at each time for each seed type by using Student's *t* test (SAS Institute, Cary, N.C.). Data from all three experiments were combined prior to analysis. There was a significant experiment effect ($P \leq 0.05$); however, there was no significant experiment×treatment effect for experiments conducted in natural soil and in sterile sand.

For biochemical restoration of seed colonization, *E. cloacae* strains were grown, washed, and resuspended in SDW or SDW containing 6% fructose. Suspensions (40 μl) were applied to individual cucumber or radish seeds in sterile sand, and populations were determined after 18 h as described above. The quantities of fructose added to the cucumber and radish seeds were similar to the quantity of carbohydrates exuded from pea seeds over the initial 96 h after the onset of imbibition. Experiments were performed twice with six replicates for each treatment. For genetic restoration of seed colonization, *E. cloacae* strains were added to cucumber seeds in sterile sand as described above and sampled at 18 h. Experiments were performed three times with three replicates for each treatment. Means were determined and compared by least significant differences (SAS) for both the biochemical and the genetic restoration experiments. Results from both sets of experiments were combined prior to analysis.

Analysis of aqueous seed extracts. Seed extracts were made essentially as previously described (21, 29). Seeds (25 g) in a 250-ml Erlenmeyer flask were surface disinfested in 10% Clorox for 20 min followed by two 20-min rinses in SDW. Surface-disinfested seeds (2.5 g) were added to 10 ml of SDW and incubated at room temperature in the dark. At the sampling times (24, 45, and 96 h) the aqueous contents of the flasks were decanted and checked for microbial contamination by spotting 10-μl aliquots onto nutrient agar. SDW (10 ml) was subsequently added to the flasks, and the flasks were incubated as described above until the next sampling time. All noncontaminated samples from each sampling time for each seed type were pooled, frozen, and lyophilized to dryness.

Total carbohydrates in the samples were estimated by the anthrone assay (20) with glucose as the standard. Individual carbohydrates were identified and quantified as trifluoroacetyl derivatives by using gas chromatography in 24-, 45-, and 96-h samples as previously described (32, 38). Total amino acids in samples were estimated by the ninhydrin assay (37) with L-leucine as the standard.

Molecular techniques and bacterial matings. DNA isolations, transformations, restriction digests, electrophoresis, ligations, and Southern blot hybridizations were performed as previously described (34). Complementation of strain A-11 was performed by mobilizing a total genomic DNA cosmid library of *E. cloacae* 501R3 in the cosmid vector pLAFR3 (constructed by J. Loper, Agricultural Research Service, Corvallis, Oreg.) into strain A-11 by triparental mating (10) and selecting directly for transconjugants which grew on M56 basal salts agar containing 0.2% *N*-acetylglucosamine, RIF, and TET. All other transconjugants were selected by growth on LB agar supplemented with RIF and TET, except matings into *E. coli* DF456, which were selected by growth on LB agar supplemented with STR and TET.

Nucleotide sequence was determined by the dideoxy chain termination method by fluorescence labelling with Amplitaq (Applied Biosystems Inc. [ABI]) run on an ABI model 373 automated sequencer. Overlapping sequences were generated by using a series of nested deletions of fragments subcloned into pGEM7Z(+) generated by digestion with *Exo*III (15) or by the use of selected primers. Sequences were analyzed using the DNA analysis programs of DNAStar (Lasergene, Inc.). BLAST searches of databases (1) were conducted with translated proteins by using the Blastp program available on the National Center for Biotechnology Information web page (20a).

Nucleotide sequence accession number. The nucleotide sequence and the sequences of the translated proteins have been deposited in GenBank under accession no. AF098509.

RESULTS AND DISCUSSION

Seed colonization. *E. cloacae* A-11 increased slightly (17-fold) from 10^4 CFU per seed to approximately 10^5 CFU per seed over the initial 24 h after application to cucumber seeds in natural soil. Populations of strain A-11 remained at this level 45 and 96 h after application (Table 2). Populations of strain 501R3 increased continuously (200-fold) and more rapidly than those of strain A-11 over this 96-h period to approximately 10^6 CFU per seed. Populations of strain 501R3 were significantly greater ($P \leq 0.02$) than those of strain A-11 at 96 h on cucumber seeds in natural soil. Similar results were obtained for experiments performed in radish spermospheres in natural soil. Populations of both strains increased over this 96-h period. However, populations of strain A-11 were significantly lower than those of strain 501R3 at 45 h ($P \leq 0.001$) and at 96 h ($P \leq 0.01$) after application. Similar results were also obtained when seed colonization studies were performed in sterile sand. Populations of strain A-11 grew more slowly and were significantly smaller ($P \leq 0.005$) than those of strain 501R3 at 24 h in both cucumber and radish spermospheres (Table 2).

There was evidence of strain A-11 achieving slightly lower populations than strain 501R3 on pea, soybean, sunflower, and sweet corn seeds at 45 and 96 h after application. However, populations of strains A-11 and 501R3 increased significantly and were statistically similar ($P \leq 0.05$) in pea, soybean, sunflower, and sweet corn spermospheres at 24, 45, and 96 h after application in both natural soil and sterile sand (Table 2). This suggests that compounds other than reduced carbon compounds are limiting to colonization in the spermospheres of these pea, soybean, sunflower, and sweet corn seed cultivars.

TABLE 2. Growth of *E. cloacae* 501R3 and A-11 on various seeds in natural soil and in sterile sand[a]

Seed	Time (h)	Growth in natural soil (log_{10} CFU/seed)		*P*	Growth in sterile sand (log_{10} CFU/seed)		*P*
		501R3	A-11		501R3	A-11	
Cucumber	24	5.17	5.22	0.92	7.21	4.49	0.0001*
	45	5.75	4.99	0.11	7.40	7.01	0.11
	96	6.35	5.25	0.02*	7.73	7.90	0.46
Radish	24	4.95	4.67	0.56	7.50	6.79	0.005*
	45	5.89	4.36	0.001*	7.69	7.69	0.98
	96	6.54	5.36	0.01*	8.17	7.94	0.35
Pea	24	7.31	7.24	0.88	8.51	8.28	0.36
	45	8.10	7.39	0.13	8.89	8.99	0.68
	96	8.22	7.35	0.07	9.30	9.08	0.37
Soybean	24	7.01	6.89	0.59	8.11	8.17	0.81
	45	7.52	7.13	0.09	8.38	8.40	0.92
	96	8.06	8.06	0.97	9.07	8.61	0.06
Sunflower	24	6.68	6.16	0.27	8.32	8.37	0.83
	45	6.03	5.80	0.63	8.60	8.62	0.92
	96	6.69	5.90	0.10	8.78	8.90	0.64
Sweet corn	24	6.81	6.73	0.72	8.28	8.23	0.86
	45	7.20	7.08	0.60	8.55	8.57	0.91
	96	7.46	7.29	0.54	9.39	8.95	0.08

[a] Similar populations (approximately 10^4 CFU per seed) of strains 501R3 and A-11 were applied to all seed cultivars. Populations of these strains were determined by spiral plating 24, 45, and 96 h after application. Asterisks indicate statistically significant ($P \leq 0.05$) differences between the strains.

Analysis of seed exudate. The cultivars of pea, soybean, and sweet corn seeds tested released approximately 1,000-fold more glucose equivalents, as determined by the anthrone assay, than radish or cucumber seeds, while sunflower seeds released approximately 20-fold more glucose equivalents (Table 3). This trend was confirmed by gas chromatography (Fig. 1). Fructose comprised a major portion of the carbohydrates released by each seed for all six seed cultivars over 96 h. However, there were approximately 2 to 3 orders of magnitude more fructose released by the cultivars of the sunflower, pea, soybean, and sweet corn seeds tested than by those of the cucumber and radish seeds. Other prevalent carbohydrates detected were glucose, galactose, sucrose, and stachyose. Significantly more total amino acids, as determined by the ninhydrin assay, were also released by pea, soybean, sunflower, and sweet corn seeds than by the cultivars of cucumber or radish seeds tested over this 96-h period (Table 3).

Characterization of *E. cloacae* A-11. Strain 501R3 grew on 13 of 16 carbohydrates detected by gas chromatography in the seed exudates (Fig. 1) when supplied as sole sources of reduced carbon. Strain 501R3 did not grow on D-lactose, L-rhamnose, or stachyose. In contrast, strain A-11 showed significant growth on only fructose. Growth on fructose by strain A-11 was similar to that by strain 501R3 (data not shown). Strains 501R3 and A-11 had similar growth on the L-amino acids alanine, asparagine, glutamine, glutamate, proline, and serine and on pyruvate and malate when supplied as sole sources of reduced carbon. Strains 501R3 and A-11 also had similar growth on glycerol supplied as a reduced carbon source and on the L-amino acids alanine, asparagine, cysteine, glutamate, glutamine, proline, and serine when supplied as sole sources of nitrogen (data not shown). This nutritional utilization profile is consistent with that of *pfkA* mutants of the closely related bacterium *E. coli* (33).

Cosmid p82, which was identified from a genomic library of strain 501R3, restored the ability of strain A-11 to grow on M56 basal salts agar amended with either 2% *N*-acetylglucosamine or 2% salicin. Plasmid pSubB, containing an 8.5-kb subcloned fragment from p82 (Fig. 2), restored the growth of strain A-11 on M56 minimal salts medium amended individu-

TABLE 3. Analysis of aqueous seed extracts[a]

Seed	Total carbohydrates (μg of glucose equivalents/seed) at:			Total amino acids (μg of leucine equivalents/seed) at:		
	0–24 h	24–45 h	45–96 h	0–24 h	24–45 h	45–96 h
Cucumber	1.0 ± 0.4	0.7 ± 0.1	1.6 ± 0.1	UD[b]	1.0 ± 1.0	UD
Radish	5.9 ± 0.8	1.8 ± 0.2	0.9 ± 0.1	2.4 ± 0.3	0.9 ± 0.1	0.9 ± 0.9
Pea	2,981.8 ± 184.0	409.2 ± 16.9	95.7 ± 6.2	336.8 ± 30.7	131.2 ± 0	83.1 ± 4.4
Soybean	987.7 ± 42.8	105.6 ± 7.5	54.5 ± 2.3	190.0 ± 44.3	12.9 ± 12.9	17.2 ± 17.2
Sunflower	52.1 ± 0.2	21.3 ± 1.5	43.8 ± 0.6	31.2 ± 31.2	9.8 ± 9.8	15.6 ± 15.6
Sweet corn	1,762.2 ± 193.3	1,664.7 ± 92.1	1,649.9 ± 86.8	92.9 ± 16.4	88.8 ± 20.5	120 ± 11.0

[a] Extracts were collected at the indicated time intervals after the start of imbibition. Total carbohydrates were determined by the anthrone assay, and total amino acids were determined by the ninhydrin assay.

[b] UD, undetectable.

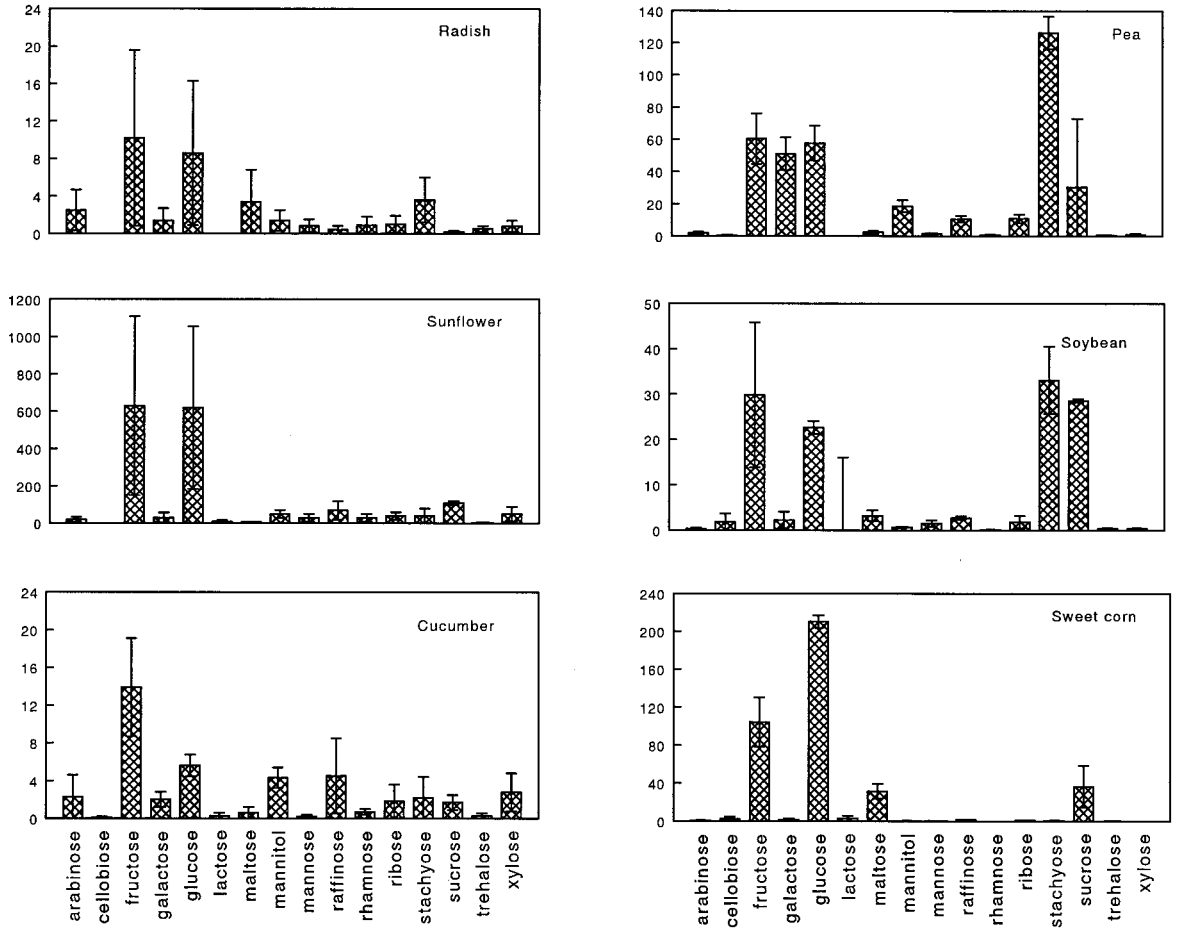

FIG. 1. Individual carbohydrates released by cucumber, radish, pea, soybean, sunflower, and sweet corn seeds. Quantities represented are summed from 0 to 24 h, 24 to 45, and 45 to 96 h samples. Error bars represent one standard deviation from the mean. Note that quantities are micrograms/seed for pea, soybean, and sweet corn seeds and nanograms/seed for cucumber, radish, and sunflower seeds.

ally with salicin, *N*-acetylglucosamine and all 12 other carbohydrates tested. The 8.5-kb DNA fragment from pSubB was used as a probe in Southern hybridizations to genomic DNA from strains A-11 and 501R3 digested with *Eco*RI. Analysis of the blot (data not shown) indicated that transposon mini-Tn*5* KAN was inserted within a 1.5-kb *Eco*RI fragment located within pSubB (Fig. 2). A 2.5-kb *Bam*HI fragment which contained this *Eco*RI fragment was subsequently subcloned from pSubB in both orientations into pRK415, resulting in plasmids pBB1415a and pBB1415b. Both plasmids restored the growth of strain A-11 on salicin, *N*-acetylglucosamine, and all 12 other carbohydrates tested, indicating that this 2.5-kb *Bam*HI fragment contained the gene of interest in its entirety. In contrast, pSubB-3, which consisted of a 2-kb *Bgl*II deletion of pSubB, did not restore the growth of strain A-11 on either salicin or *N*-acetylglucosamine (Fig. 2).

Nucleotide sequence analysis of the 2.5-kb *Bam*HI fragment from pBB1415 indicated the presence of two complete open reading frames (ORFs) and part of a third ORF, all with the same transcriptional orientation (Fig. 2). Each of these ORFs contained a predicted ATG start codon that was preceded within 6 to 10 bases by sequences resembling ribosome binding sites. The predicted start codon of ORF1 was located 212 bases from the *Bam*HI site and encoded a predicted protein product of 320 amino acids. Two DNA inverted repeat sequences, representing potential transcriptional termination sites, were identified immediately downstream of the stop codon. The first repeat was located 12 bases from the stop codon and consisted of the 7-base inverted repeat sequence of GCCCGGT-N_{12}-ACCGGGC. The second repeat was located 20 bases downstream from the first repeat and consisted of the sequence GCCGGGT-N_{12}-ACCCGGC. Database searches revealed that the predicted protein for this ORF had an amino acid sequence identity of 88% to the *pfkA* gene product of *E. coli* (GenBank accession no. P06998). *pfkA* encodes phosphofructo kinase, a key enzyme in glycolysis catalyzing the conversion of fructose-6-phosphate to fructose-1,6-phosphate (33). To verify that the mutation in strain A-11 was located within *pfkA*, a 1.5-kb *Bam*HI-*Sal*I fragment containing the entire *pfkA* ORF and only the 5′ end of the *sbp* ORF was subcloned into pRK415. The resultant plasmid, pBS1415 (Fig. 2), restored the growth of strain A-11 on all 14 carbohydrates tested. In addition, pBS1415 was mobilized into *E. coli* DF456, a *pfkA*-deficient strain, restoring growth on *N*-acetylglucosamine.

The second ORF (ORF2) within the 2.5-kb *Bam*HI fragment was positioned 1,384 bp from the *Bam*HI site, beginning 202 bases from the termination codon of the *pfkA* homolog, and consisted of a predicted protein product of 329 amino acids. Database searches indicated 83% sequence identity to the sulfur binding protein of *E. coli* encoded by *sbp* (GenBank

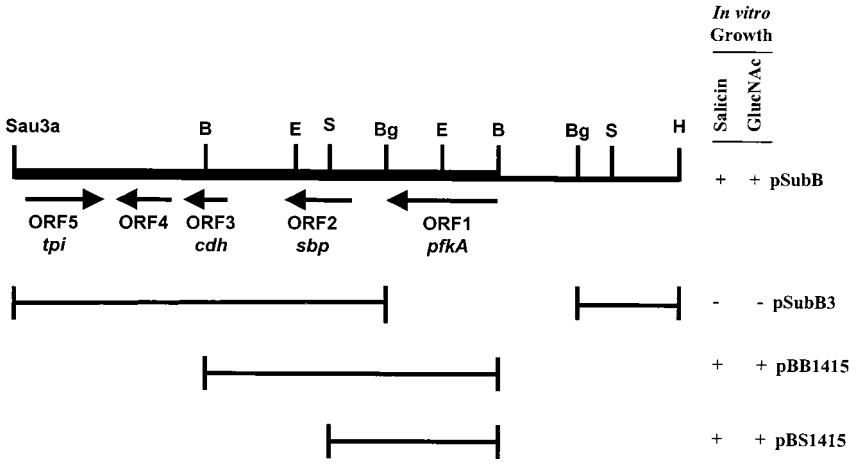

FIG. 2. Physical maps of plasmid pSubB and pSubB-derived subclones. Abbreviations: B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; S, *Sal*I. Arrows indicate the direction of transcription. The thick line indicates the sequenced portion of pSubB. *E. cloacae* A-11 harboring the indicated plasmid was capable (+) or incapable (−) of in vitro growth on M56 minimal medium plus 0.2% salicin or *N*-acetylglucosamine.

accession no. S40860). Analysis of the nucleotide sequence extending an additional 3.25 kb downstream from the 2.5-kb *Bam*HI fragment indicated that *pfkA* and *sbp* are physically linked to a gene with 76% sequence identity to *cdh* of *E. coli*, which encodes CDP-diglyceride hydrolase (GenBank accession no. P06282) and an ORF (ORF4) of unknown function. Database searches for this ORF did not identify any matches of significance at the nucleotide or predicted amino acid sequence levels. A fifth ORF (ORF5) encodes a protein with 91% identity to triose phosphate isomerase of *E. coli* (GenBank accession no. P04790) (Fig. 2).

Genetic and biochemical restoration of seed colonization by strain A-11. Populations of strain 501R3 increased from approximately 10^4 CFU per seed to greater than 10^7 CFU per seed in 18-h assays on surface-disinfested cucumber seeds in sterile sand (Table 4). Populations of strains A-11 and A-11(pRK415) showed no substantial increases after 18 h when approximately 10^4 CFU per seed were added to the cucumber seeds. Populations of strains A-11 and A-11(pRK415) were substantially smaller than those of strain 501R3 at this time. Plasmids pBB1415 and pBS1415 (Fig. 2) restored the ability of A-11 to grow on cucumber seeds to near the levels associated with the wild-type strain, strain 501R3 (Table 4).

Addition of fructose, a carbohydrate capable of supporting wild-type growth of strain A-11, to treatments containing strain A-11, also restored the ability of strain A-11 to grow on both cucumber and radish seeds to levels similar to those of strain 501R3 (Table 5). The quantity of fructose added to cucumber and radish seeds in these treatments was similar to the quantity of total carbohydrates exuded from pea seeds over the initial 96 h after the onset of imbibition.

Genetic and biochemical data presented here strongly suggest that the strain A-11 colonization phenotype is solely due to inactivation of *pfkA* rather than to the loss of other physically linked genes that appear to be involved in general carbohydrate catabolism (Fig. 2). A DNA fragment containing only *pfkA* restored the strain 501R3 phenotype to strain A-11 with regard to in vitro growth and seed colonization. Also, the presence of an inverted repeat indicative of a transcriptional termination signal downstream from *pfkA* suggests this gene is expressed independently of other genes. Finally, the exogenous addition of fructose in colonization experiments circumvented the impact of the mutation in *pfkA* on colonization by supplying strain A-11 with a reduced carbon source utilizable for growth. Fructose is not expected to biochemically complement any of the genes physically linked to *pfkA*. These observations link the loss of *pfkA* function, the loss of carbohydrate catabolic capabilities, and decreased colonization of seeds that release relatively small quantities of reduced carbon such as the cucumber and radish cultivars tested here.

TABLE 4. Genetic restoration of colonization of cucumber spermosphere by *E. cloacae* A-11

Strain[a]	Mean colonization ($\log_{10}$ CFU/seed)[b]
501R3	7.56 A
A-11(pBB1415)	7.07 B
A-11(pBS1415)	6.98 B
A-11	4.64 C
A-11(pRK415)	2.75 D
LSD[c]	0.29

[a] Similar populations (approximately 10^4 CFU per seed) of all strains were applied to cucumber seeds in sterile sand.
[b] Populations at 18 h after application of bacteria to the seed. Numbers followed by the same letters are not significantly different ($P \leq 0.05$).
[c] LSD, least significant difference ($P \leq 0.05$).

TABLE 5. Biochemical restoration of colonization of cucumber and radish spermospheres by *E. cloacae* A-11

Strain and treatment[a]	Mean colonization ($\log_{10}$ CFU/seed)[b] on:	
	Cucumber seeds	Radish seeds
501R3	7.16 A	7.10 A
A-11 + fructose	7.45 A	6.81 A
A-11	6.46 B	6.37 B
LSD[c]	0.33	0.31

[a] Similar populations (approximately 10^4 CFU per seed) were added to cucumber and radish seeds in sterile sand for all treatments.
[b] Populations at 18 h after application to cucumber seed. Numbers followed by the same letters in each column are not significantly different ($P \leq 0.05$).
[c] LSD, least significant difference ($P \leq 0.05$).

Conclusion. Nutrient-rich plant exudates are expected to support an abundance of microbial growth while nutrient-poor exudates are not. Since it has been established that growth is an essential component of microbial colonization processes (24, 29–31, 35), it can be assumed that the catabolic capabilities of a bacterium, with regard to specific reduced carbon compounds found in exudates, contribute directly to its ability to colonize a given host plant. Under nutrient-poor conditions a greater ability to catabolize compounds in exudates is expected to improve colonization. Although these assumptions are obvious and accepted by many, there is little evidence to support them. Our studies with *E. cloacae* 501R3 and the near-isogenic strain A-11 directly support these assumptions for microbial colonization of the spermosphere. Strain A-11 had decreased catabolic capabilities with regard to specific carbohydrates in seed exudates relative to those of strain 501R3. The colonization of cucumber and radish seeds by strain A-11 was also reduced relative to that by strain 501R3, but there were no differences between the two strains relative to the colonization of pea, soybean, sunflower, and sweet corn seeds. The exudates from these cultivars of pea, soybean, sunflower, and sweet corn seeds contained, on average, several orders of magnitude more carbohydrates and amino acids than the exudate from cucumber or radish seeds.

Work with additional carbohydrate utilization mutants of *E. cloacae* and other plant-beneficial bacteria needs to be performed to substantiate the findings presented here. In addition, mutants affected in the utilization of other nutrients such as nitrogen need to be analyzed. The acquisition of reduced carbon compounds and other nutrients is fundamentally important for growth and other desired microbial activities such as biocontrol in the spermosphere and rhizosphere (25). An understanding of the nutritional requirements of beneficial bacteria colonizing subterranean portions of plants and the impact of available nutrients on beneficial activities such as colonization and biocontrol is required if the behavior of beneficial bacteria in specific environments is to be accurately predicted.

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