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Increased Fitness of *Pseudomonas fluorescens* Pf0-1 Leucine Auxotrophs in Soil[▽]

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The annotation process of a newly sequenced bacterial genome is largely based on algorithms derived from databases of previously defined RNA and protein-encoding gene structures. This process generally excludes the possibility that the two strands of a given stretch of DNA can each harbor a gene in an overlapping manner. While the presence of such structures in eukaryotic genomes is considered to be relatively common, their counterparts in prokaryotic genomes are just beginning to be recognized. Application of an in vivo expression technology has previously identified 22 discrete genetic loci in *Pseudomonas fluorescens* Pf0-1 that were specifically activated in the soil environment, of which 10 were present in an antisense orientation relative to previously annotated genes. This observation led to the hypothesis that the physiological role of overlapping genetic structures may be relevant to growth conditions outside artificial laboratory media. Here, we examined the role of one of the overlapping gene pairs, *iiv19* and *leuA2*, in soil. Although *iiv19* was previously demonstrated to be preferentially activated in the soil environment, its absence did not alter the ability of *P. fluorescens* to colonize or survive in soil. Surprisingly, the absence of the *leuA2* gene conferred a fitness advantage in the soil environment when leucine was supplied exogenously. This effect was determined to be independent of the *iiv19* gene, and further analyses revealed that amino acid antagonism was the underlying mechanism behind the observed fitness advantage of the bacterium in soil. Our findings provide a potential mechanism for the frequent occurrence of auxotrophic mutants of *Pseudomonas* spp. in the lungs of cystic fibrosis patients.

Sterile liquid media have long served as, and remain, the preferred conduit for studying gene expression in bacteria. Consequently, most current knowledge has come from the perspective of free-living planktonic populations in shaking flasks and test tubes. However, it is widely acknowledged that bacteria in nature rarely exist solely in the planktonic form but rather as constituents of structurally organized communities.

Escherichia coli K-12 has served as the primary model in almost all fundamental aspects of microbiology. One may argue that *E. coli* K-12 has been passaged and studied in shaken broth more often than any other bacterium. Since the initial annotation of the complete genome of K-12 a decade ago, the percentage of identified chromosomal gene products without a function assignment has decreased from approximately 33 percent to 10 percent (28). Although the proportion of genes of unknown function will continue to shrink over time, the functions of some of the remaining genes may have to be elucidated outside the flask. Gene assignment in newly sequenced genomes is an automated process that is largely based on sequence features derived from previously annotated genomes, a process innately biased toward genes identified under laboratory conditions. Understandably, the proportion of genes without a function assignment is markedly higher in other genomes, especially those that are remotely related to *E. coli* K-12.

Pseudomonas fluorescens is a versatile organism that is readily isolated from soil, in bulk and from the rhizosphere. *P. fluorescens* Pf0-1 has long served as a model organism in our ongoing interests in understanding the genetic bases of bacterial proliferation and persistence in soil (7, 9–11, 20, 29). Our experimental systems utilize the same batch of soil from which *P. fluorescens* Pf0-1 was originally isolated (9), presenting an exceptional opportunity to study the organism as close to its natural habitat as possible in the laboratory. Application of in vivo expression technology (IVET) identified a set of 22 discrete loci in the genome of *P. fluorescens* Pf0-1 that were induced specifically in soil (33). This set of *iiv* (*i*nduced *i*n *v*ivo) genes included the so-called "cryptic genes" whose sequences did not share any homology to known genes while overlapping those previously annotated on the opposite DNA strand (34). Overlapping structures as such appear frequently in viral genomes but have been rarely reported in bacteria (31, 34).

One of the obvious challenges in studying the individual function of overlapping genes is that the introduction of a mutation in one also affects the other, especially when one member of the pair is cryptic in nature. We sought to examine the individual roles of one of the previously identified overlapping pairs of genes, *iiv19* and *leuA2*, directly in the soil environment. This gene pair was chosen for study because the function of the *leuA2* gene product had already been described in great detail. Here, we report an unforeseen soil-specific fitness advantage resulting from a mutation in the *leuA2* locus that is independent of its cryptic *iiv19* counterpart.

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MATERIALS AND METHODS

Bacterial strains and culture conditions. *Pseudomonas fluorescens* Pf0-1 was isolated from loam soil (9). For competition experiments, strains were labeled and verified by using the mini-Tn*7* system as previously described (44), with kanamycin resistance via plasmid pHRB2 (24) or streptomycin resistance (33a) via a derivative of the plasmid pUC18T-mini*Tn*7T (8) harboring the cassette from pHRP315 (26). Deletion mutants of Pf0-1 were constructed as already described (33). Splicing by overlap extension-PCR (SOE-PCR) (16) was used to create the deletion constructs with the suicide plasmid pSR47s (22). Pf0-1Δ19 is a construct with a complete deletion of the *leuA2-iiv19* overlapping locus, created by the SOE-PCR primers 19D5f (5′-GCGAAGTGCTGCATTACG) and 19D5r (5′-CTCTCACACCCTCATTCAGGGAACGATCAGGCGCCTC) for the 5′ end and 19D3f (5′-GAGGCGCCTGATCGTTCCCTGAATGAGGGTGTGA GAG) and 19D3r (5′-AGCAACCTGCTGCTGGAC) for the 3′ end. Pf0-1Δ*leuB* is a construct with a complete deletion of the *leuB* locus, created by the SOE-PCR primers leuB5f (5′-TTGAGCAACGTGTCCACG) and leuB5r (5′-GCTT TCCTCGTGTCTTCAGGCTTTCCTCGTGTCTTCAG) for the 5′ end and leuB3f (5′-CTGAAGACACGAGGAAAGCTAATCTCTCGGGCCCGCTG) and leuB3r (5′-ATGGCGCTCATCCACTCG) for the 3′ end. Each deletion construct was cloned into pGEM-T Easy (Promega) then subcloned into the NotI site in pSR47s. Deletions were confirmed by PCR using primers iiv19A (5′-TTGCCAAACGTGAGGAGACG) and iiv19B (5′-GCCGCAACCTCAAT TTCAGT) for the *iiv19-leuA2* locus and primers leuB1 (5′-GTCGCCTCGTTG GTGAAAG) and leuB2 (5′-TTCGGTCGGGTAAGCGTC) for the *leuB* locus. Strain Pf0-1Δ19PE*leuA* is Pf0-1Δ19 complemented with a hybrid *leuA* gene comprising the *leuA2* promoter from Pf0-1 fused to a promoterless *leuA* open reading frame (ORF) from *E. coli* MG1655 by SOE-PCR: primers PleuAF (5′-AGTCCTCGAGTTCTTCAATGGCAACACCG) and PleuAR (5′-GAAAA TAATGACTTGCTGGCTCATCGGAAAATTCCTTG) amplified the Pf0-1 promoter, and primers EleuAF (5′-CAAGGAATTTTCCGATGAGCCAGCA AGTCATTATTTTC) and EleuAR (5′-AGTCGGATCCCGGCAATACGGCA ATATG) amplified the *leuA* ORF from MG1655. The hybrid construct was cloned into pGEM-T Easy, then subcloned into the ApaI and BamHI sites of the mini-Tn*7* plasmid pHRB2 (24), and introduced into Pf0-1Δ19 by conjugation. All routine cloning was done with *E. coli* 10B (Invitrogen), and *E. coli* S17.1λ *pir* (35) was used as the donor strain in conjugations. All *E. coli* strains were grown in LB (Invitrogen) at 37°C.

P. fluorescens strains were grown in *Pseudomonas* minimal medium (PMM) (18) with shaking at 250 rpm at 30°C. We assessed the growth of strains individually or in competition with another strain in 2.5 ml PMM inoculated with ca. 10^4 CFU. Each culture was serially diluted 16 h later and enumerated on PMM solidified with 1.5% agar. Leucine, isoleucine, valine, and histidine were used as supplements at 200 μM each, and ampicillin, kanamycin, and streptomycin were each added at 50 μg/ml when required. All amino acids and antibiotics were purchased from Sigma.

Reverse transcription-PCR (RT-PCR) and 5′ RACE. RNA was purified from Pf0-1 grown in PMM by using the RNeasy minikit (Qiagen). Each sample was treated with DNase I (Qiagen) on-column and later with RQ1 DNase I (Promega) off-column. Each sample was then repurified using the RNeasy minikit. cDNA was synthesized from 0.5 μg RNA by using the Superscript III kit (Invitrogen), primer 19RT-F (5′-ACCTGGCTGAATTCGATCTG) for *leuA2*, and primer 19RT-R2 (5′-AACGTCGACCTCGTGACTG) for *iiv19*. PCR was performed using the cDNA as templates and 19RT-F and 19RT-R2 as primers. cDNA generated by use of 19RT-R2 was used as the template for the 5′ rapid amplification of cDNA ends (RACE) system (Invitrogen) with primers 19GSP2 (5′-CGAAGAGTGCAACCAGATC) and 19GSP3 (5′-GGTGCACACAGCGT TCTC). 5′ RACE products from three discrete cDNA preparations were cloned into pGEM-T Easy (Promega) and sequenced using the T7 and SP6 primers at the Tufts University Core Facility (Boston, MA).

Soil assays. Gamma-irradiated loam soil (11) was inoculated and sampled as previously described (33) with slight modifications. Strains were grown in PMM overnight and serially diluted in distilled water to ca. 10^4 CFU/ml, and 1 ml was mixed with 5 g of soil. This resulted in ca. 50% water-holding capacity. The initial population density (CFU/g soil) was determined by sampling 0.5 g of soil at 30 minutes following inoculation. Each soil sample was vortexed for 30 seconds in 1 ml distilled water, briefly rested to settle large particles, and then serially diluted and enumerated on PMM solidified with 1.5% agar. Soil was supplemented with leucine, isoleucine, valine, or histidine by the addition of 20 μl of 1 mM stock solution during bacterial inoculation or as noted otherwise. Competition experiments were carried out in the same manner except that both strains at 0.5 ml each were mixed together prior to being mixed with soil and enumerated using PMM supplemented with the appropriate antibiotic. Competitive index values were calculated as the output ratio of the mutant population density to the wild-type population density divided by the input ratio of the mutant population density to the wild-type population density as previously described (41).

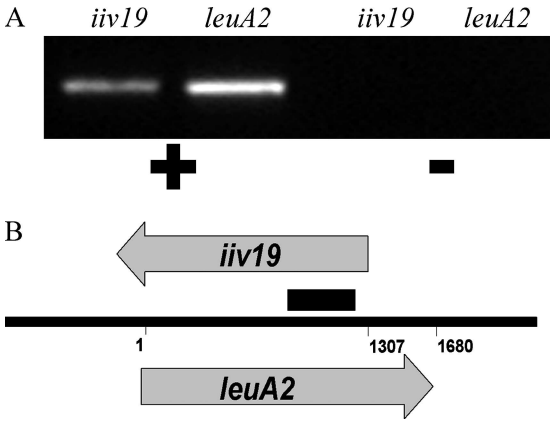

FIG. 1. Transcription and organization of *iiv19* and *leuA2* genes. (A) Results of RT-PCR using gene-specific primers; "+" and "−" indicate the presence and absence of reverse transcriptase in the reaction, respectively. (B) Schematic of the overlapping genetic organization of *iiv19* and *leuA2*. The numbers indicate the nucleotide positions of the *leuA2* ORF (bp 1 to 1680). The transcript start site for *iiv19* (bp 1307 relative to the start site of the *leuA2* ORF) was mapped by 5′ RACE. The 3′ end of *iiv19* is stretched out to the first translational stop site, but the precise location of the 3′ terminus of the *iiv19* transcript is unknown. The horizontal bar (bp 843 to 1232) represents the RT-PCR product indicated in panel A.

RESULTS AND DISCUSSION

Molecular analyses of the *iiv19-leuA2* locus. The *leuA2* gene specifies the 2-isopropylmalate synthase, the first specific enzyme in the leucine biosynthesis pathway (38). In *P. fluorescens* Pf0-1, antisense to *leuA2* resides a cryptic promoter termed *iiv19*, whose activity was sufficient for detection by IVET in soil but not in minimal medium (PMM) (33). To determine whether *iiv19* is indeed capable of transcribing a message, RT-PCR was carried out using strand-specific oligonucleotides. Products from both strands were detected only when reverse transcriptase was supplied (Fig. 1A), confirming that each of the two genes—*iiv19* and *leuA2*— is actively transcribed. Furthermore, the transcription initiation site was mapped to be 373 bp downstream from the 3′ end of the *leuA2* gene on the opposite strand by 5′ RACE (Fig. 1B). BPROM (http://www.softberry.com/berry.phtml?topic=bprom&group=programs&subgroup=gfindb) and NNPP (http://www.fruitfly.org/seq_tools/promoter.html) analyses of the immediate upstream region did not reveal recognizable σ^{70}-dependent promoter sequences, and the termination site of the transcript remains elusive, as 3′ RACE attempts did not yield conclusive results.

Since *iiv19* and *leuA2* directly overlap one another (Fig. 1B), transcription of the *leuA2* gene should have a direct influence on *iiv19* expression, and vice versa, through DNA template competition. The *leuA* gene of *E. coli* is strongly repressed in the presence of exogenous leucine by a regulatory mechanism known as transcription attenuation (23). Although the *iiv19* transcript can be detected by RT-PCR from Pf0-1 grown in PMM (Fig. 1A), its expression is below the threshold level of

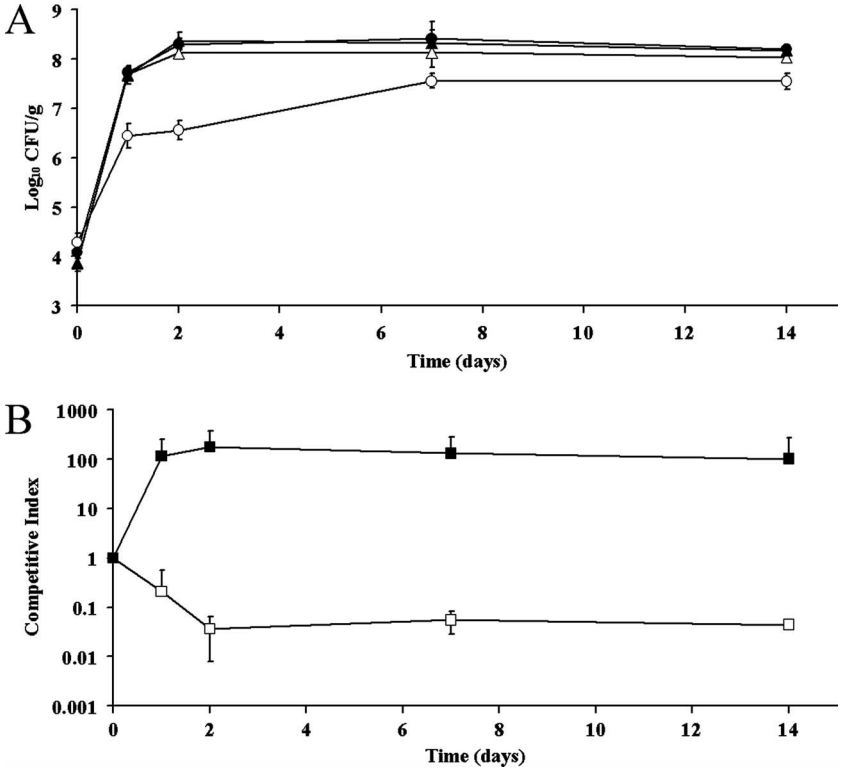

FIG. 2. Comparisons of growth of the Pf0-1Δ19 mutant and the Pf0-1 parent strains in individual and competitive soil assays with (filled symbols) or without (open symbols) leucine. (A) Pf0-1 (triangles) and Pf0-1Δ19 (circles) were introduced into individual soil systems and the data are presented as $\log_{10}$ CFU/g soil. (B) Equal amounts of Pf0-1 and Pf0-1Δ19 were mixed prior to being introduced into the same soil systems, and the data are presented as competitive index values. The error bars indicate the standard deviations of the means from at least three independent experiments.

the IVET screen (33). We reasoned that the addition of leucine to PMM would reduce *leuA2* transcription, which in turn may allow *iiv19* to be transcribed at a higher rate. Northern analyses were carried out using RNA isolated from Pf0-1 grown in PMM and the rich King's B medium supplemented with leucine at various concentrations ranging between 0 to 1 mM. We did not observe any notable differences in the *leuA2* transcript levels up to the highest level of leucine supplied, and *iiv19* transcripts were not detected in any of the samples (data not shown). These results indicated that expression of the *leuA2* gene in Pf0-1 was not regulated by leucine. In agreement with these observations, a recent bioinformatics study reported that *leuA2* genes in *Pseudomonas* spp. do not contain the upstream attenuator elements that are present in *E. coli* (43).

Assessment of the ability of the *iiv19 leuA2* double mutant to colonize soil. Understanding the role of *iiv19* in survival in soil represented a rather complicated challenge, given the overlapping orientation of the two genes and the unknown precise structure of the *iiv19* gene. Since the function of the *leuA2* gene is well established, we reasoned that the role of *iiv19* could potentially be deduced by complementing the *leuA2* mutation biochemically in a double mutant. We therefore constructed the strain Pf0-1Δ19 from *P. fluorescens* Pf0-1; this mutant has a deletion in the genomic region spanning both *leuA2* and the predicted *iiv19* gene. As anticipated, Pf0-1Δ19 behaved as an auxotroph, requiring exogenous supplementation of leucine for growth in PMM.

The impact of the mutation on soil colonization was assessed by comparing Pf0-1Δ19 to the parent strain Pf0-1 in our sterile soil system (33). Growth of the two strains was monitored when they were inoculated alone in soil (i.e., each strain assessed individually) and in competitive soil (i.e., the two strains mixed together). To differentiate the strains, neutral kanamycin and streptomycin resistance markers were introduced into each chromosome (see Materials and Methods) and verified not to influence colonization of individual and competitive soil (data not shown). Pf0-1 introduced alone rapidly colonized soil, reaching the maximum population density afforded by the soil system at ca. 10^8 CFU/g by the second day (Fig. 2A). Pf0-1 remained at this density even after three months following inoculation, provided that moisture loss was kept to a minimum (data not shown). In contrast, Pf0-1Δ19 exhibited a markedly reduced rate of colonization, gradually reaching a maximum of ca. 10^7 CFU/g, about 10-fold less than Pf0-1 (Fig. 2A). Thus, the soil system appeared to contain sufficient amounts of leucine and/or leucine-containing peptides (LCP) to support the growth of the auxotrophic Pf0-1Δ19 but became limiting eventually. An alternative explanation is that leucine was not the limiting factor, but the absence of the *iiv19* gene resulted in reduced fitness. However, supplementation of leucine to the soil system fully restored the colonization ability of Pf0-1Δ19 (Fig. 2A), indicating that the absence of the *leuA2* gene was likely the main reason behind the observed defect. A concentration of 0.4 μmol/g was the minimum amount of

leucine required for Pf0-1Δ19 to consistently reach the same population density as Pf0-1. This concentration is far below the level of hydrolysable leucine detected in a variety of natural soils (12, 21), and supplementation of soil with leucine neither increased nor decreased the population density of Pf0-1 (Fig. 2A).

Given the reduced colonization efficiency exhibited by the mutant in soil, we predicted that Pf0-1 would be dominant over Pf0-1Δ19 in competitive soil. Indeed, Pf0-1Δ19 was outcompeted by Pf0-1 by at least an order of magnitude (Fig. 2B). However, we observed an unexpected reversal in trend when leucine was added to the soil: Pf0-1Δ19 outcompeted Pf0-1 by two orders of magnitude (Fig. 2B). One explanation for this result may be that leucine had a toxic effect on Pf0-1, but this is unlikely since leucine supplementation did not adversely affect its ability to colonize soil alone (Fig. 2A). Alternatively, since *iiv19* is known to be preferentially expressed in soil (33), the consequence of its activity under an elevated level of exogenous leucine may have somehow been manifested as a competitive defect. In order to address this hypothesis, it was necessary to study the effect of the mutation in each of these genes independently.

The absence of the *iiv19* gene does not affect soil colonization. There are two distinct classes of homologous genes, *leuA* and *leuA2*, that encode the 2-isopropylmalate synthase (43). Unlike most members of the class *Gammaproteobacteria*, *Pseudomonas* spp. possess the *leuA2* homolog. Given that the two classes share only 30% sequence identity (43), it is unlikely that the *iiv19* gene is also present antisense to the *leuA* homologs. We designed a hybrid construct by fusing the *leuA2* promoter from Pf0-1 to the *leuA* gene from *E. coli* MG1655. We then introduced the hybrid construct into a noncoding region in the genome of Pf0-1Δ19 by using the Tn*7* system. The resulting strain, Pf0-1Δ19PE*leuA*, was capable of growing in PMM without leucine, indicating that the *E. coli leuA* gene was correctly being transcribed by the native promoter from Pf0-1. Since Pf0-1Δ19PE*leuA* is capable of expressing a functional 2-isopropylmalate synthase while lacking the *iiv19* gene, we had the opportunity to assess specifically the impact of the *iiv19* mutation in the soil environment. In competition experiments, Pf0-1Δ19PE*leuA* was capable of colonizing the soil equally as well as Pf0-1, both in the presence and absence of leucine supplementation (Fig. 3A). These results indicate that *iiv19* does not play a significant role in soil colonization and its presence did not contribute to the apparent reduced fitness of Pf0-1 in competition with Pf0-1Δ19 (Fig. 2B).

Although the *iiv19* gene may not be present antisense to the *leuA* gene in *E. coli*, there exists the possibility that an unrelated *iiv19*-like element is present antisense to the *E. coli leuA* gene in Pf0-1Δ19PE*leuA*. If such an element indeed exists and is expressed in Pf0-1Δ19PE*leuA*, the lack of competition observed against Pf0-1 (Fig. 3A) does not necessarily refute a role of *iiv19* in soil colonization. To address this issue, a *leuB* deletion mutant was constructed. The *leuB* gene encodes the 3-isopropylmalate dehydrogenase complex, which functions two steps after the *leuA* gene product in the leucine biosynthesis pathway (15). Pf0-1Δ*leuB* is incapable of producing leucine but harbors the wild-type *leuA2-iiv19* locus. Pf0-1Δ*leuB* exhibited characteristics similar to those of Pf0-1Δ19: it had a reduced rate of colonization without leucine (Fig. 3B), but it outcompeted Pf0-1 when leucine was supplied exogenously (Fig. 3C). These results confirm that *iiv19* is not involved in soil colonization, nor does its presence affect the decreased fitness of Pf0-1 in competition with Pf0-1Δ19.

Effect of leucine supplementation on isoleucine-valine pseudoauxotrophy in PMM. Leucine, like other amino acids, has a transient existence in soil, be it free or as a component in peptides and proteins. Radiolabeled leucine is often utilized in assays to estimate bacterial growth rates in environmental samples, such as soil samples (1). It is not readily apparent why the addition of leucine, which is present at a limiting concentration in our soil system (Fig. 2A), negatively affects Pf0-1 only in competition against strains that cannot synthesize leucine (Fig. 2B and 3C).

Given the chemical and physical complexities of the soil environment, we explored the underlying mechanism behind this phenomenon under a more-defined setting. For growth in PMM, Pf0-1Δ19 requires 100 to 200 μM leucine, which is considered to be the general range needed to support the growth of most *P. fluorescens* auxotrophs (36). However, despite leucine supplementation, Pf0-1Δ19 consistently grew to a density less than that of Pf0-1 (Fig. 4A), and providing more leucine did not increase its density. In contrast, when cocultured with Pf0-1 in leucine-supplemented PMM, Pf0-1Δ19 reached a density much higher than that obtained in pure culture (Fig. 4A). A similar finding was observed when cells were grown on the surface of PMM solidified with agar (data not shown). Taken together, these results suggest that some factor other than leucine affected the relative fitness of Pf0-1Δ19, and it is somehow related to cohabitation in medium with Pf0-1. Cell-free supernatant of Pf0-1 grown in leucine-supplemented PMM did not have any noticeable effect on Pf0-1Δ19 and vice versa (data not shown), indicating that a secreted factor was presumably not involved. However, we cannot exclude the possibility that such a factor may have been unstable and/or below the effective concentration in our assays.

Amino acids can inhibit growth as a consequence of antagonism between related amino acids (13). In the case of leucine, an increased intracellular concentration inhibits the activity of acetohydroxy acid synthase (14). Since this enzyme performs the first common step in the biosynthesis of all branched-chain amino acids, inhibition of its activity halts growth, as the cells are no longer able to synthesize leucine, isoleucine, and valine. Growth resumes when these amino acids are exogenously supplied (30) or the acetohydroxy acid synthase is actively derepressed (19). To test the antagonism theory, Pf0-1Δ19 and Pf0-1 were individually grown in PMM supplemented with leucine alone or a cocktail of leucine, isoleucine, and valine. The addition of isoleucine and valine increased the density of Pf0-1Δ19 by almost an order of magnitude and also increased the density of Pf0-1 by a smaller margin (Fig. 4B). The addition of isoleucine and valine also closed the remaining gap between cocultured Pf0-1Δ19 and Pf0-1 values (Fig. 4C). These results collectively support the notion that leucine supplementation was indeed exerting an antagonistic influence on isoleucine and valine biosynthesis.

Extension of the amino acid antagonism theory into the soil environment. Although the antagonism theory can account for most of the experimental data derived from the use of PMM, it is not readily applicable for an understanding of the outcome of the soil experiments. Unlike growth in PMM, leucine sup-

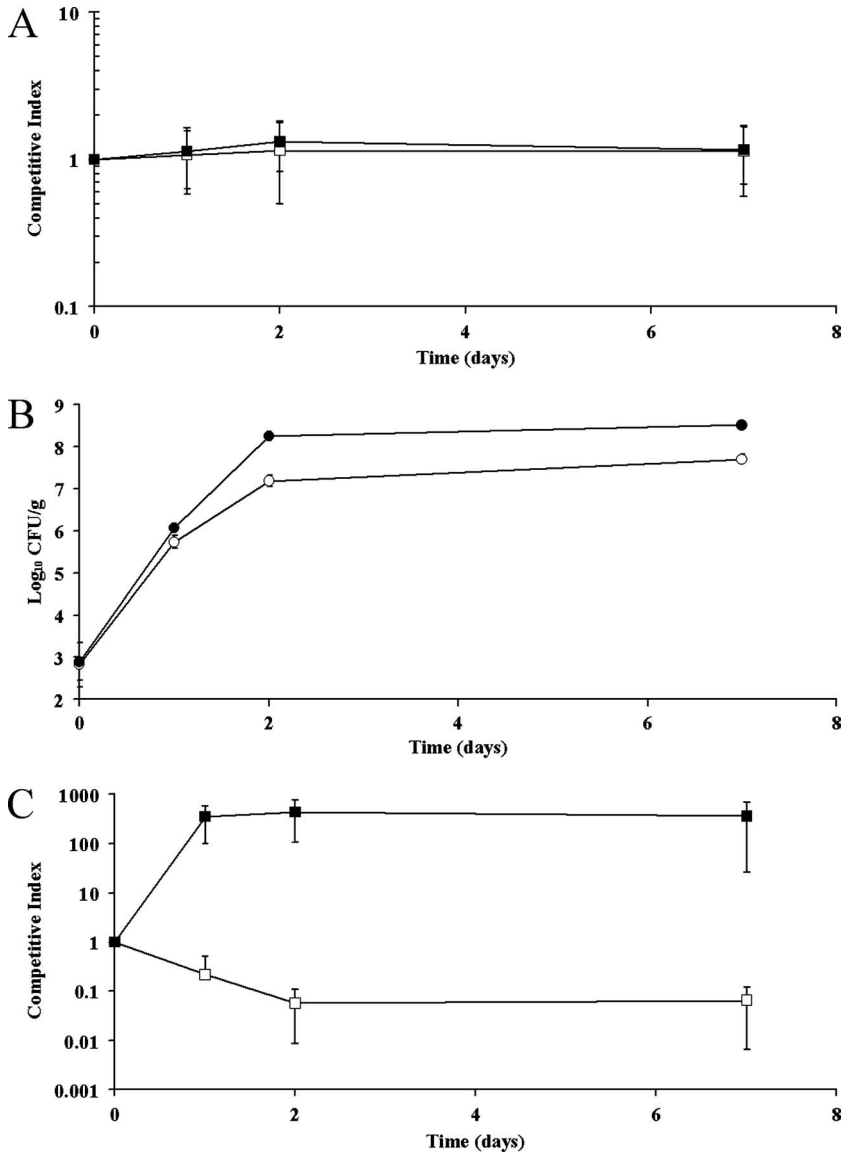

FIG. 3. Growth of Pf0-1Δ19PE*leuA* and Pf0-1Δ*leuB* compared to growth of Pf0-1 in soil with (filled symbols) or without (open symbols) leucine. (A) Competitive index values for soil inoculated with a mixture of the partially complemented mutant strain Pf0-1Δ19PE*leuA* and the parent strain Pf0-1. (B) Growth of the Pf0-1Δ*leuB* mutant in soil inoculated with the individual strain. (C) Competitive index values for soil inoculated with a mixture of the Pf0-1Δ*leuB* mutant and the parental Pf0-1 strain. The error bars indicate the standard deviations of the means from at least three independent experiments.

plementation supported Pf0-1Δ19 colonization of soil as effectively as it supported Pf0-1 soil colonization, and it also supported dominance over Pf0-1 when the strains were placed in competition together. The antagonism theory was directly tested in soil by setting up groups of competitions between Pf0-1Δ19 and Pf0-1 in leucine-supplemented soil. To a given group, either histidine or a cocktail of isoleucine and valine was further added. The relative changes in the dominance of Pf0-1Δ19 over Pf0-1 following exposure to either isoleucine and valine or histidine are shown in Fig. 5. Relative fitness of 100% indicates that a given treatment did not influence Pf0-1Δ19's advantage over Pf0-1. The addition of isoleucine and valine significantly reduced the relative fitness, indicating that their presence made Pf0-1 more competitive. An alternative interpretation is that leucine supplementation in a competitive soil assay has a negative impact on Pf0-1 greater than its negative impact on Pf0-1Δ19. Histidine, which was included as a non-branched-chain amino acid control, did not influence the relative fitness, strengthening the relevance of the antagonism theory in the soil competitions.

So what is the underlying basis for Pf0-1's elevated sensitivity to antagonism by leucine? Leucine is taken up by the cell through two major routes: (i) as a free amino acid via the LIV transport systems, which are repressed by leucine or other amino acid substrates (17, 27), and (ii) as a component of peptides via peptide transport systems that are constitutively active (2). The rate of leucine metabolism is considered to be lower than the combined rates of transporting in LCP and

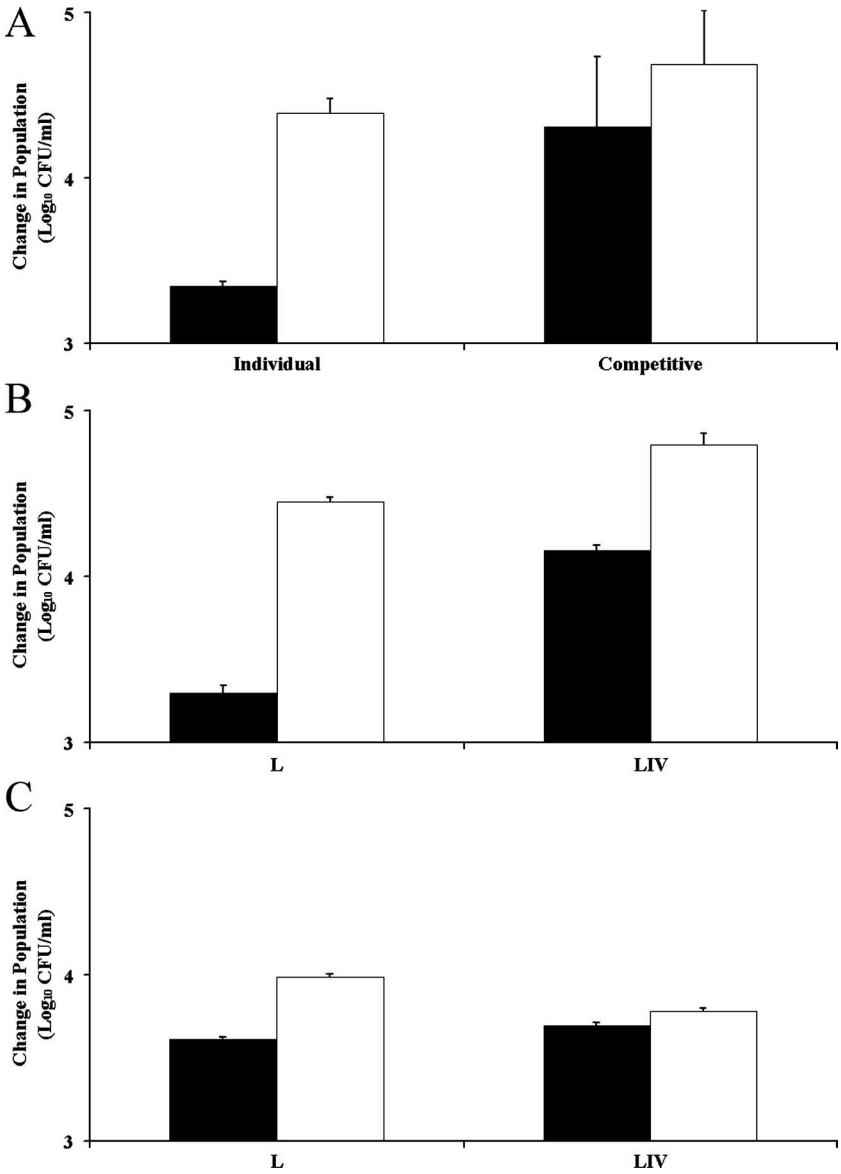

FIG. 4. Effect of various branched-chain amino acids on the growth of Pf0-1Δ19 and Pf0-1 in PMM. Pf0-1Δ19 (black bars) and Pf0-1 (white bars) were grown overnight in PMM supplemented with one or more branched-chain amino acids. The data are presented as relative increases in cell density ($\log_{10}$ CFU/ml) compared to the initial density of ca. 10^4 CFU/ml. (A) Strains were inoculated in PMM supplemented with leucine individually or together (competitive) in the same tubes. In separate experiments, strains were inoculated in individual tubes (B) or the same tubes (C) containing PMM supplemented with leucine (L) or a combination of leucine, isoleucine, and valine (LIV). Each amino acid was added at 200 μM. The error bars indicate the standard deviations of the means from at least three independent experiments.

cleaving them into free amino acids (39). Thus, in the presence of increasing levels of LCP, the intracellular accumulation rate of leucine would increase, eventually leading to isoleucine-valine pseudoauxotrophy. One of the obvious differences between Pf0-1Δ19 and Pf0-1 is that the former does not synthesize leucine de novo and the only source for intracellular accumulation would be through uptake. It is estimated that more than 99% of amino acids in soil exist in a polymeric state, such as in peptides and proteins (37). With these data taken together, the initial uptake rates of exogenous leucine and LCP would be the same for Pf0-1Δ19 and Pf0-1 in leucine-supplemented soil. However, the intracellular accumulation rate would be higher for Pf0-1, since it is capable of synthesizing leucine de novo and especially since none of the leucine-specific biosynthesis genes are predicted to contain the transcription attenuation motif (43). The net effect would be that inhibition of acetohydroxy acid synthase activity would occur more rapidly among the Pf0-1 cells and quicken the onset of pseudoauxotrophy. Such a relative effect would not be predicted to manifest as a colonization defect in soils inoculated with the strain alone (Fig. 2A).

Among the obvious physiological conditions that are difficult to represent in constantly shaken broth are spatial chemical gradations (e.g., branched-chain amino acids). One of the main

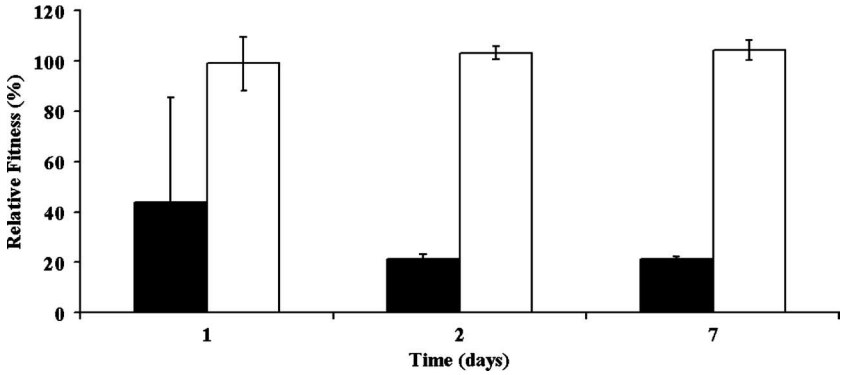

FIG. 5. Effect of adding branched-chain amino acids or histidine to leucine-supplemented soil. Equal amounts of Pf0-1Δ19 and Pf0-1 cells were mixed and introduced into leucine-supplemented soil. Each soil system was further supplemented with a mixture of isoleucine and valine (black bars) or histidine (white bars). The competitive index values derived from each treatment were compared to the competitive index values derived from soil supplemented with leucine alone, and the data are presented as percent relative fitness. One hundred percent relative fitness indicates that a given treatment generated the same competitive index values as leucine alone. The error bars indicate the standard deviations of the means from at least three independent experiments.

physical differences between soil and PMM is that leucine was present only in the free form in the latter. Leucine uptake via the LIV transport systems is quickly repressed when the intracellular level reaches a certain point (27). Thus, Pf0-1 is not expected to be dominated by Pf0-1Δ19 in PMM as it was in soil. It is, however, less apparent why leucine-supplemented Pf0-1Δ19 grows to a higher density in the presence of Pf0-1 than on its own in PMM, especially when Pf0-1 does not appear to secrete any factors that benefit Pf0-1Δ19.

Relevance of findings. Leucine has long been considered a major regulatory signal in *E. coli*, exerting both positive and negative effects, largely through the global transcriptional regulator Lrp (6). Fluctuating intracellular levels of leucine may have stimulated significant physiological changes that were not captured in our assays, or perhaps they were somehow reflected in Pf0-1Δ19's ability to reach a higher density in the presence of Pf0-1 in PMM. Although *iiv19* was demonstrated to be a nonfactor in this study, the relevance of its existence antisense to *leuA2* remains to be characterized. Because of technical difficulties associated with extracting mRNA from soil (32), we could not establish whether a transcript of *iiv19* can be detected directly from soil. This finding is necessary to confirm that *iiv19* is indeed expressed in soil, since IVET can detect only promoters or promoter-like elements. Moreover, the precise nature of each of the cryptic genes remains to be elucidated. There is at least one convincing piece of evidence that each member of an overlapping pair of genes, Pf1-0939 and *iiv14*, encodes a protein and that the *iiv14* gene product plays a role in soil colonization (33a). Perhaps when a larger set is characterized in greater detail, a common pattern that may reveal the path toward function will emerge.

We have demonstrated that an auxotroph can outcompete its prototrophic parent in the soil environment, provided that the lacking amino acid is exogenously provided. Since soil bacteria are generally described to lead a lifestyle of feast and famine, selecting against genes that ensure survival for fleeting moments of advantage over your neighbors would be considered a poor evolutionary strategy. However, as shown via the unlikely conditions presented in this study, the potential to dominate as an auxotroph is clearly present in the genome of *P. fluorescens* Pf0-1. In addition, it appears that amino acid antagonism may represent a mechanism for competition in nature, as it was recently demonstrated that valine is actively secreted by various bacterial biofilms, including *E. coli* and *P. aeruginosa* (42). The practical significance of this study, however, may lie across the species barrier. There have been several reports of the isolation of significant numbers of *P. aeruginosa* auxotrophs, including leucine auxotrophs, from the lungs of cystic fibrosis patients and healthy individuals (3–5, 40). Although there has not yet been a strong claim regarding the mechanism driving auxotrophy selection, this mechanism has largely been explained as one of increased fitness over the prototrophs as a consequence of not expending energy for de novo biosynthesis. Unlike bulk soil, sputum represents an ideal environment where auxotrophy may be selected as a competitive trait, beyond the classic explanation described here. A recent microarray analysis indicated that, in sputum, amino acid biosynthesis genes are down-regulated and transport genes are up-regulated (25). From the outside looking in, this surely looks like an ideal place to invoke the amino acid antagonism theory.

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REFERENCES

1. **Bååth, E.** 1998. Growth rates of bacterial communities in soils at varying pH: a comparison of the thymidine and leucine incorporation techniques. Microb. Ecol. **36:**316–327.
2. **Barak, Z., and C. Gilvarg.** 1975. Peptide transport. Biomembranes **7:**167–218.
3. **Barth, A. L., and T. L. Pitt.** 1995. Auxotrophic variants of *Pseudomonas aeruginosa* are selected from prototrophic wild-type strains in respiratory infections in patients with cystic fibrosis. J. Clin. Microbiol. **33:**37–40.
4. **Barth, A. L., and T. L. Pitt.** 1995. Auxotrophy of *Burkholderia* (*Pseudomonas*) *cepacia* from cystic fibrosis patients. J. Clin. Microbiol. **33:**2192–2194.
5. **Barth, A. L., and T. L. Pitt.** 1996. The high amino-acid content of sputum from cystic fibrosis patients promotes growth of auxotrophic *Pseudomonas aeruginosa*. J. Med. Microbiol. **45:**110–119.
6. **Calvo, J. M., and R. G. Matthews.** 1994. The leucine-responsive regulatory protein, a global regulator of metabolism in *Escherichia coli*. Microbiol. Rev. **58:**466–490.

7. **Casaz, P., A. Happel, J. Keithan, D. L. Read, S. R. Strain, and S. B. Levy.** 2001. The *Pseudomonas fluorescens* transcription activator AdnA is required for adhesion and motility. Microbiology **147:**355–361.
8. **Choi, K. H., J. B. Gaynor, K. G. White, C. Lopez, C. M. Bosio, R. R. Karkhoff-Schweizer, and H. P. Schweizer.** 2005. A Tn7-based broad-range bacterial cloning and expression system. Nat. Methods **2:**443–448.
9. **Compeau, G., B. J. Al-Achi, E. Platsouka, and S. B. Levy.** 1988. Survival of rifampin-resistant mutants of *Pseudomonas fluorescens* and *Pseudomonas putida* in soil systems. Appl. Environ Microbiol. **54:**2432–2438.
10. **DeFlaun, M. F., B. M. Marshall, E.-P. Kulle, and S. B. Levy.** 1994. Tn*5* insertion mutants of *Pseudomonas fluorescens* defective in adhesion to soil and seeds. Appl. Environ. Microbiol. **60:**2637–2642.
11. **DeFlaun, M. F., A. S. Tanzer, A. L. McAteer, B. Marshall, and S. B. Levy.** 1990. Development of an adhesion assay and characterization of an adhesion-deficient mutant of *Pseudomonas fluorescens*. Appl. Environ. Microbiol. **56:**112–119.
12. **Friedel, J. K., and E. Scheller.** 2002. Composition of hydrolysable amino acids in soil organic matter and soil microbial biomass. Soil Biol. Biochem. **34:**315–325.
13. **Gladstone, G. P.** 1939. Interrelationship between amino acids in the nutrition of *Bacillus anthracis*. Br. J. Exp. Pathol. **20:**189–200.
14. **Gollop, N., D. M. Chipman, and Z. Barak.** 1983. Inhibition of acetohydroxy acid synthase by leucine. Biochim. Biophys. Acta **748:**34–39.
15. **Hoang, T. T., and H. P. Schweizer.** 1997. Identification and genetic characterization of the *Pseudomonas aeruginosa leuB* gene encoding 3-isopropylmalate dehydrogenase. Mol. Gen. Genet. **254:**166–170.
16. **Horton, R. M., H. D. Hunt, S. N. Ho, J. K. Pullen, and L. R. Pease.** 1989. Engineering hybrid genes without the use of restriction enzymes: gene splicing by overlap extension. Gene **77:**61–68.
17. **Hoshino, T.** 1979. Transport systems for branched-chain amino acids in *Pseudomonas aeruginosa*. J. Bacteriol. **139:**705–712.
18. **Kirner, S., S. Krauss, G. Sury, S. T. Lam, J. M. Ligon, and K. H. van Pee.** 1996. The non-haem chloroperoxidase from *Pseudomonas fluorescens* and its relationship to pyrrolnitrin biosynthesis. Microbiology **142:**2129–2135.
19. **Lee, C. R., B. M. Koo, S. H. Cho, Y. J. Kim, M. J. Yoon, A. Peterkofsky, and Y. J. Seok.** 2005. Requirement of the dephospho-form of enzyme IIANtr for derepression of *Escherichia coli* K-12 *ilvBN* expression. Mol. Microbiol. **58:**334–344.
20. **Marshall, B., E. A. Robleto, R. Wetzler, P. Kulle, P. Casaz, and S. B. Levy.** 2001. The *adnA* transcriptional factor affects persistence and spread of *Pseudomonas fluorescens* under natural field conditions. Appl. Environ. Microbiol. **67:**852–857.
21. **Martens, D. A., and K. L. Loeffelmann.** 2003. Soil amino acid composition quantified by acid hydrolysis and anion chromatography-pulsed amperometry. J. Agric. Food Chem. **51:**6521–6529.
22. **Matthews, M., and C. R. Roy.** 2000. Identification and subcellular localization of the *Legionella pneumophila* IcmX protein: a factor essential for establishment of a replicative organelle in eukaryotic host cells. Infect. Immun. **68:**3971–3982.
23. **Merino, E., and C. Yanofsky.** 2005. Transcription attenuation: a highly conserved regulatory strategy used by bacteria. Trends Genet. **21:**260–264.
24. **Monds, R. D., P. D. Newell, R. H. Gross, and G. A. O'Toole.** 2007. Phosphate-dependent modulation of c-di-GMP levels regulates *Pseudomonas fluorescens* Pf0-1 biofilm formation by controlling secretion of the adhesin LapA. Mol. Microbiol. **63:**656–679.
25. **Palmer, K. L., L. M. Mashburn, P. K. Singh, and M. Whiteley.** 2005. Cystic fibrosis sputum supports growth and cues key aspects of *Pseudomonas aeruginosa* physiology. J. Bacteriol. **187:**5267–5277.
26. **Parales, R. E., and C. S. Harwood.** 1993. Construction and use of a new broad-host-range *lacZ* transcriptional fusion vector, pHRP309, for Gram$^-$ bacteria. Gene **133:**23–30.
27. **Quay, S. C., T. E. Dick, and D. L. Oxender.** 1977. Role of transport systems in amino acid metabolism: leucine toxicity and the branched-chain amino acid transport systems. J. Bacteriol. **129:**1257–1265.
28. **Riley, M., T. Abe, M. B. Arnaud, M. K. Berlyn, F. R. Blattner, R. R. Chaudhuri, J. D. Glasner, T. Horiuchi, I. M. Keseler, T. Kosuge, H. Mori, N. T. Perna, G. Plunkett III, K. E. Rudd, M. H. Serres, G. H. Thomas, N. R. Thomson, D. Wishart, and B. L. Wanner.** 2006. *Escherichia coli* K-12: a cooperatively developed annotation snapshot—2005. Nucleic Acids Res. **34:**1–9.
29. **Robleto, E. A., I. Lopez-Hernandez, M. W. Silby, and S. B. Levy.** 2003. Genetic analysis of the AdnA regulon in *Pseudomonas fluorescens*: nonessential role of flagella in adhesion to sand and biofilm formation. J. Bacteriol. **185:**453–460.
30. **Rogerson, A. C., and M. Freundlich.** 1970. Control of isoleucine, valine and leucine biosynthesis. 8. Mechanism of growth inhibition by leucine in relaxed and stringent strains of *Escherichia coli* K-12. Biochim. Biophys. Acta **208:**87–98.
31. **Rogozin, I. B., A. N. Spiridonov, A. V. Sorokin, Y. I. Wolf, I. K. Jordan, R. L. Tatusov, and E. V. Koonin.** 2002. Purifying and directional selection in overlapping prokaryotic genes. Trends Genet. **18:**228–232.
32. **Saleh-Lakha, S., M. Miller, R. G. Campbell, K. Schneider, P. Elahimanesh, M. M. Hart, and J. T. Trevors.** 2005. Microbial gene expression in soil: methods, applications and challenges. J. Microbiol. Methods **63:**1–19.
33. **Silby, M. W., and S. B. Levy.** 2004. Use of in vivo expression technology to identify genes important in growth and survival of *Pseudomonas fluorescens* Pf0-1 in soil: discovery of expressed sequences with novel genetic organization. J. Bacteriol. **186:**7411–7419.

33a. **Silby, M. W., and S. B. Levy.** Overlapping protein-encoding genes in *Pseudomonas fluorescens* Pf0-1. PLoS Genet., in press.

34. **Silby, M. W., P. B. Rainey, and S. B. Levy.** 2004. IVET experiments in *Pseudomonas fluorescens* reveal cryptic promoters at loci associated with recognizable overlapping genes. Microbiology **150:**518–520.
35. **Simon, R., U. Priefer, and A. Puhler.** 1983. A broad host range mobilization system for in vivo genetic engineering: transposon mutagenesis in Gram-negative bacteria. Bio/Technology **1:**784–791.
36. **Simons, M., H. P. Permentier, L. A. de Weger, C. A. Wijffelman, and B. J. Lugtenberg.** 1997. Amino acid synthesis is necessary for tomato root colonization by *Pseudomonas fluorescens* strain WCS365. Mol. Plant-Microbe Interact. **10:**102–106.
37. **Stevenson, F. J.** 1982. Nitrogen in agricultural soils. Soil Science Society of America, Madison, WI.
38. **Stieglitz, B. I., and J. M. Calvo.** 1974. Distribution of the isopropylmalate pathway to leucine among diverse bacteria. J. Bacteriol. **118:**935–941.
39. **Tavori, H., Y. Kimmel, and Z. Barak.** 1981. Toxicity of leucine-containing peptides in *Escherichia coli* caused by circumvention of leucine transport regulation. J. Bacteriol. **146:**676–683.
40. **Thomas, S. R., A. Ray, M. E. Hodson, and T. L. Pitt.** 2000. Increased sputum amino acid concentrations and auxotrophy of *Pseudomonas aeruginosa* in severe cystic fibrosis lung disease. Thorax **55:**795–797.
41. **Unsworth, K. E., and D. W. Holden.** 2000. Identification and analysis of bacterial virulence genes in vivo. Philos. Trans. R. Soc. Lond. B **355:**613–622.
42. **Valle, J., S. Da Re, S. Schmid, D. Skurnik, R. D'Ari, and J. M. Ghigo.** 2008. The amino acid valine is secreted in continuous-flow bacterial biofilms. J. Bacteriol. **190:**264–274.
43. **Vitreschak, A. G., E. V. Lyubetskaya, M. A. Shirshin, M. S. Gelfand, and V. A. Lyubetsky.** 2004. Attenuation regulation of amino acid biosynthetic operons in proteobacteria: comparative genomics analysis. FEMS Microbiol. Lett. **234:**357–370.
44. **Zuber, S., F. Carruthers, C. Keel, A. Mattart, C. Blumer, G. Pessi, C. Gigot-Bonnefoy, U. Schnider-Keel, S. Heeb, C. Reimmann, and D. Haas.** 2003. GacS sensor domains pertinent to the regulation of exoproduct formation and to the biocontrol potential of *Pseudomonas fluorescens* CHA0. Mol. Plant-Microbe Interact. **16:**634–644.