

## The Amino Acid Valine Is Secreted in Continuous-Flow Bacterial Biofilms<sup>▽‡</sup>

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**Biofilms are structured communities characterized by distinctive gene expression patterns and profound physiological changes compared to those of planktonic cultures. Here, we show that many gram-negative bacterial biofilms secrete high levels of a small-molecular-weight compound, which inhibits the growth of only *Escherichia coli* K-12 and a rare few other natural isolates. We demonstrate both genetically and biochemically that this molecule is the amino acid valine, and we provide evidence that valine production within biofilms results from metabolic changes occurring within high-density biofilm communities when carbon sources are not limiting. This finding identifies a natural environment in which bacteria can encounter high amounts of valine, and we propose that in-biofilm valine secretion may be the long-sought reason for widespread but unexplained valine resistance found in most enterobacteria. Our results experimentally validate the postulated production of metabolites that is characteristic of the conditions associated with some biofilm environments. The identification of such molecules may lead to new approaches for biofilm monitoring and control.**

Biofilms are matrix-encased bacterial communities that develop on most surfaces and often constitute a reservoir of bacterial pathogens. Detrimental biofilms are difficult to eradicate due to a characteristic tolerance to biocides. They cause serious health and economic problems when they form on medical and industrial devices (11, 19, 24). While biofilm-associated antibiotic tolerance is considered a major physiological trait that distinguishes free cells from surface-attached cells, biofilms are also characterized by gene expression patterns that are distinctive compared to those of planktonic cultures (4, 35, 40, 41, 58). These changes are likely to result from modifications of growth conditions within biofilms, and they have been proposed to correspond to ill-understood responses triggered by the biofilm lifestyle (3, 26).

Recently, studies conducted with simplified, mixed communities composed of two bacterial species revealed that biofilm bacteria express competitive or cooperative behavior that does not take place within classical planktonic cultures (7, 22). These results suggested that biofilm-associated weaponry could contribute to the dynamics of bacterial biofilm populations. However, thus far, the production of molecules involved in antagonistic bacterial relationships within biofilms has been poorly investigated.

Here we show that the continuous-flow biofilms formed by many *Escherichia coli* strains and other gram-negative bacteria

accumulate high levels of a small-molecular-weight compound with inhibitory activity against *E. coli* K-12 strains. We demonstrate both genetically and biochemically that this compound is the amino acid valine. We provide evidence that valine secretion within biofilms could be the cause of the long-known but unexplained widespread valine resistance observed for most enterobacteria. Beyond the biological significance of in-biofilm valine secretion, our results experimentally support the hypothesis that metabolites can be secreted more particularly under biofilm conditions, a finding that opens up novel research alleys for the development of markers with which to monitor biofilm formation.

### MATERIALS AND METHODS

**Bacterial strains, plasmids, and growth conditions.** Bacterial strains and plasmids are listed in Table 3. Bacteria were grown at 37°C in 0.4% glucose-minimal M63B1 medium (M63B1glu) (31), unless specified otherwise, supplemented with the appropriate antibiotics, as follows: kanamycin (Km; 50 µg ml<sup>-1</sup>), chloramphenicol (Cm; 25 µg ml<sup>-1</sup>), ampicillin (Amp; 100 µg/ml), apramycin (Apr; 30 µg ml<sup>-1</sup>), or zeocin (Zeo; 50 µg ml<sup>-1</sup>). Nonessential- and essential-amino-acid solutions were purchased from Invitrogen. The nonessential-amino-acid solution contained L-alanine, L-asparagine, L-aspartic acid, L-glutamic acid, glycine, L-proline, and L-serine at a concentration of 50 µg ml<sup>-1</sup> each. The essential-amino-acid solution contained L-arginine · HCl, L-cysteine, L-histidine · HCl · H<sub>2</sub>O, L-isoleucine, L-leucine, L-lysine · HCl, L-methionine, L-phenylalanine, L-threonine, L-tryptophan, L-tyrosine, and L-valine at a concentration of 50 µg ml<sup>-1</sup> each. Single-amino-acid solutions were prepared in distilled water at 2,500 µg ml<sup>-1</sup> each and used at a final concentration of 50 µg ml<sup>-1</sup> each. For growth curve experiments or for supplementation in agar plates, filter-sterilized supernatant (20% [vol/vol]) was added to the M63B1glu. For complementation experiments, *E. coli* strain CFT073 genes *sspA* and *tufA* were PCR amplified using specific primers (see Table S1 at [www.pasteur.fr/recherche/unites/Ggb/supmat.html](http://www.pasteur.fr/recherche/unites/Ggb/supmat.html)) and cloned into plasmid pZE12 under the control of a synthetic *plac* promoter (29), leading to the plasmids pSpA and pTufA. Plasmids pSM11 and pMM100 (*lacI*<sup>q</sup>) were used for the overexpression of ppGpp. One millimolar isopropyl-β-D-thiogalactopyranoside (IPTG) was used for the activation of the *plac* promoters.

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**DNA manipulations and genetic techniques.** DNA techniques were performed as described by Sambrook et al. (39). Oligonucleotides were purchased from Sigma, France. DNA sequencing was performed using MWG services. Mutants were constructed using a three-step PCR procedure described previously (see reference 28 and <http://www.pasteur.fr/recherche/unites/Ggb/matmet.html>).

To obtain a CFT073 $\Delta$ relA  $\Delta$ spoT mutant growing on minimal medium, a spontaneous prototroph was selected. In a relA spoT double mutant, the absence of (p)ppGpp prevents growth on minimal medium. This auxotrophy can be suppressed by spontaneous mutations in RNA polymerase subunits (9). Such a spontaneous mutant (presumably an rpoB mutant) was selected by plating a relA spoT double mutant previously grown in LB broth on M63B1glu. To construct the MG1655ilvG<sup>+</sup> mutant, a 950-bp PCR fragment amplified from the ilvG gene of CFT073 was recombined with the ilvG gene of MG1655. This enabled the correction of the frameshift mutation present in the ilvG gene of MG1655 mariner and led to the mutant strain MG1655ilvG<sup>+</sup>.

**Transposon mutagenesis.** Mariner transposon mutagenesis of *E. coli* CFT073 and MG1655F' was performed as described previously (12). To select MG1655 mutants resistant to the CFT073 inhibitor, 10,000 transposon mutants were tested for their ability to grow on plates of M63B1glu supplemented with the CFT073 supernatant (10% [vol/vol]). The mutants that were isolated were transduced to MG1655F' to verify that their resistance was due to the transposon insertion only. To screen for CFT073 mutants able to secrete valine in a planktonic culture, 10,000 transposon mutants were grown for 24 h in M63B1glu at 37°C in 96-well microtiter plates. The inhibitory effect was analyzed by spotting 5  $\mu$ l of culture on an overlay of strain MG1655 cells. Transposon insertion sites were determined as described previously (12). Homology searches were performed using BLAST 2.0. Deletion mutants were generated as described elsewhere ([www.pasteur.fr/recherche/unites/Ggb/3SPCRprotocol.html](http://www.pasteur.fr/recherche/unites/Ggb/3SPCRprotocol.html)) using primers described in Table S1 ([www.pasteur.fr/recherche/unites/Ggb/supmat.html](http://www.pasteur.fr/recherche/unites/Ggb/supmat.html)).

**Purification and toxicity test of the supernatants. (i) Planktonic supernatant.** Bacteria cultured to an optical density of 1 at 600 nm (OD<sub>600</sub>) (1 ml at an OD of 1) for 48 h in M63B1glu were centrifuged for 30 min at 5,000 rpm at 4°C. The supernatant was filtered through a 0.2- $\mu$ m filter.

**(ii) Biofilm supernatant.** Bacterial biofilms were obtained using a continuous-flow system (60-ml microfermentors) as described previously (see reference 16 and [www.pasteur.fr/recherche/unites/Ggb/biofilmfermenter.html](http://www.pasteur.fr/recherche/unites/Ggb/biofilmfermenter.html)). Briefly, inoculation was performed by dipping the microfermentor's removable spatula into a culture containing 10<sup>8</sup> bacteria/ml for 2 min and subsequently reintroducing it into the microfermentor. The medium was pumped through the microfermentors at a constant rate (0.75 ml/min). After 48 h, the biofilm formed was rapidly resuspended in 10 ml of M63B1 medium (without glucose) by vortexing for 30 s, the equivalent of an OD<sub>600</sub> of 1 of the bacterial resuspension was centrifuged, and the supernatant was filtered through a 0.2- $\mu$ m filter. For supernatants purified from colonies grown overnight on M63B1glu plates at 37°C, a sufficient number of colonies was picked from the plate and resuspended in 1 ml of M63B1 medium to obtain a minimum concentration of an OD<sub>600</sub> of 1. Bacteria were centrifuged, and the supernatant was filtered through a 0.2- $\mu$ m filter. For supernatants purified from static biofilms, the biofilms were grown in M63B1glu in microtiter plates at 37°C. After 48 h of growth, the cultures in the microtiter plates were rinsed briefly with M63B1 medium, and biofilms were resuspended in M63B1 medium to reach an OD of 1 and treated as described above.

**(iii) Growth inhibition test.** The inhibitory effect of filter-sterilized supernatants was evaluated by spotting 8  $\mu$ l of the supernatant on an overlay of MG1655 cells on M63B1glu plates. Plates were incubated overnight at 37°C before pictures were taken. When specified, the filtered supernatant was mixed at a 1:1 ratio with other solutions (LB, amino acids, etc.), and 8  $\mu$ l of the mixed solution was spotted onto an overlay of MG1655 cells on M63B1glu plates.

**Molecular characterization of the inhibitory compound.** The filter-sterilized supernatant was concentrated using a Vivaspin 2 column with a 10,000-Da cutoff (Sartorius). The eluting fraction was further fractionated with a Vivaspin 2 column with a 5,000-Da cutoff (Sartorius). Each concentrated fraction was washed twice with sterilized water before being assayed for inhibition on an overlay of MG1655 cells on M63B1glu plates.

**Role of the stress response in valine production analysis.** CFT073 cells were cultured for 48 h at 37°C in M63B1glu supplemented with 0.05, 0.1, 0.5, and 1 mM paraquat or in M63B1glu whose pH had been decreased to pH 5 by the addition of HCl. Supernatants from these cultures were purified, and their toxicity levels were tested on an overlay of MG1655 cells on M63B1glu plates, as described above.

**Amino acid composition analysis.** The supernatants were prepared as described above. Amino acid composition was determined by total hydrolysis by F. Baleux at the Unité de Chimie Organique of the Institut Pasteur.

**Competitive growth experiment.** For competition experiments, we used strains labeled with an antibiotic marker inserted at the chromosomal  $\lambda$ att site, using a three-step PCR procedure. Cultures were grown overnight in LB medium at 37°C and diluted to an OD of 1 with fresh LB medium. Diluted cultures of MG1655att $\lambda$ -amp and MG1655ilvG<sup>+</sup>/att $\lambda$ -Km or of MG1655att $\lambda$ -Km and MG1655ilvG<sup>+</sup>/att $\lambda$ -amp were mixed at a 1:1 ratio and grown at 37°C for 24 h. A sample of the mixed culture was taken at time zero and at 24 h, serially diluted, and plated on LB, LB plus Km, and LB plus Amp plates, in order to estimate the CFU of the two strains in the mixed culture over time.

**Selection of valine-resistant mutants under valine selection pressure.** Planktonic cultures of MG1655F', CFT073 $\Delta$ mchB  $\Delta$ kpsD, and KS272 were grown overnight in M63B1glu at 37°C. Cultures were diluted to an OD of 1 with M63B1glu and mixed at a 1:1 ratio. The mixed cultures were grown under planktonic conditions at 37°C for 24 h and simultaneously used to inoculate microfermentors, as described above, to allow the formation of mixed biofilms grown for 24 h at 37°C. Serial dilutions of the mixed planktonic and biofilm cultures were plated onto M63B1glu plus Apr with or without 20% CFT073 filtered biofilm supernatant. The frequency of the appearance of valine-resistant mutants was measured by determining the ratio of clones resistant to both Apr and CFT073 in-biofilm supernatant to the total number of Apr<sup>r</sup> clones.

## RESULTS

**Inhibitory molecules are secreted within *E. coli* CFT073 biofilms.** We previously showed that the uropathogenic *E. coli* (UPEC) isolate CFT073 and most extraintestinal pathogenic *E. coli* isolates release capsular polysaccharides that inhibit bacterial adhesion and could contribute to competitive interactions within bacterial communities encountered during the UPEC colonization process (54). We hypothesized that the secretion of bacterial interference molecules could occur within bacterial biofilms. To test this, 48-h-old biofilms formed in a continuous-flow biofilm microfermentor, using CFT073 or the stronger former of biofilm, the capsule-deficient mutant CFT073 $\Delta$ kpsD (54), were resuspended in minimal medium and filter sterilized. The resulting biofilm-derived supernatants (hereafter referred to as in-biofilm supernatants), as well as the supernatants of equivalent 48-h-old planktonic cultures (planktonic supernatants) of these strains, were spotted onto an overlay of the *E. coli* K-12 MG1655 strain on M63B1glu plates. Interestingly, both the CFT073 and the CFT073 $\Delta$ kpsD in-biofilm supernatants produced a large growth inhibition halo that was not observed for the planktonic supernatants (Fig. 1A). MG1655 planktonic growth was also severely impaired in the presence of the CFT073 and the CFT073 $\Delta$ kpsD in-biofilm supernatants (Fig. 1B). Bacteria incubated for 10 h in the presence of a CFT073 in-biofilm supernatant resumed growth after dilution in fresh medium and displayed a wild-type level of sensitivity to in-biofilm supernatants, indicative of bacteriostatic rather than bactericidal activity at the concentration used (data not shown). The supernatants extracted either from resuspended 48-h biofilms formed in microtiter plates or from resuspended colonies formed on agar plates did not inhibit MG1655 growth. These results indicate that growth-inhibiting molecules are produced and accumulate within mature CFT073 biofilms formed under continuous-flow conditions.

**The inhibitor secreted within CFT073 biofilms is not microcin H47.** Attempts to determine the size of the inhibitory molecule using cutoff size-differential filters showed that fractions smaller than 5,000 Da still retained activity (data not shown). This small size suggested that the inhibitory molecule could be a microcin, a small peptide with bacteriostatic or bactericidal activity produced by many enterobacteria (17).

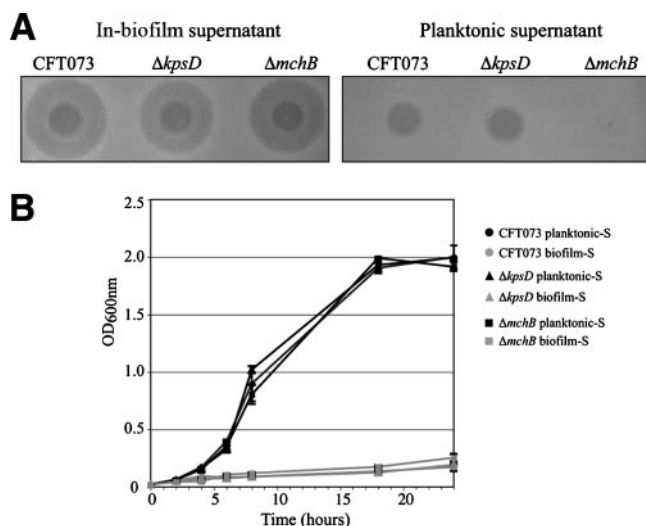


FIG. 1. Bacteriotoxic effect of the CFT073 in-biofilm supernatant. (A) Growth inhibition activity of CFT073, CFT073 $\Delta kpsD$ , and CFT073 $\Delta mchB$  biofilm supernatants on an overlayer of *E. coli* K-12 cells. (B) Growth curves of MG1655 in the presence of 20% CFT073, CFT073 $\Delta kpsD$ , and CFT073 $\Delta mchB$  planktonic supernatant or in-biofilm supernatant. Cultures were performed in M63B1glu. Experiments were performed in duplicate. Error bars represent standard deviations of the means.

Under certain environmental conditions, CFT073 was shown to secrete microcin H47, which inhibits the growth of *E. coli* and several phylogenetically related enterobacterial strains (25, 45). Growth inhibition associated with CFT073 in-biofilm supernatants could therefore be due to the overproduction of microcin H47 under biofilm conditions. To test this hypothesis, we constructed a mutation in *mchB*, one of four chromosomal genes involved in microcin synthesis (*mchABCD*) in CFT073 and tested the activity of its in-biofilm supernatant on an MG1655 overlayer. As shown in Fig. 1A, H47 microcin is responsible only for the slight growth inhibition displayed by CFT073 planktonic supernatants, while supernatants from the  $\Delta mchB$  mutant biofilms still produced a large growth inhibition halo and impaired MG1655 liquid growth (Fig. 1B). These results indicate that an inhibitor distinct from microcin H47 is produced within the CFT073 biofilms.

**Genetic and biochemical evidence for high levels of valine in the CFT073 biofilm matrix.** To determine the nature of the inhibitory molecule produced within CFT073 biofilms, we reasoned that *E. coli* MG1655 mutants resistant to the molecule might provide insight into its entry pathway or cellular target. We performed *mariner* transposon mutagenesis in the sensitive strain MG1655 and plated the mutants onto M63B1glu agar plates supplemented with filter-sterilized in-biofilm supernatant. One resistant transposon mutant carried a mutation in the *livH* gene, encoding a membrane component of the high-affinity branched-chain amino acid transport system (for the import of monomeric isoleucine, leucine, and valine). The allelic exchange deletion of *livH* from MG1655 also led to a high level of resistance to CFT073 in-biofilm supernatants, confirming that the inhibitor studied used the LivH transporter to enter the cell (Fig. 2A). Interestingly, the inhibition of *E. coli* K-12 growth by exogenous valine is a well-known phenomenon

(49). It is due to the loss in *E. coli* K-12 of one of the three heterodimeric acetohydroxy acid synthase (AHAS) isozymes. While feedback from AHAS I and III is inhibited by valine, the valine-insensitive isozyme AHAS II (*ilvGM*) is inactivated in *E. coli* K-12 by a frameshift mutation in *ilvG* (14) (Fig. 3B). Valine inhibition of the growth of K-12 $\Delta ilvG$  is due to accumulation of the AHAS substrate  $\alpha$ -ketobutyrate, a toxic inhibitor of the glucose phosphotransferase system (38). This accumulation results both from the valine stimulation of threonine deaminase (*ilvA*) production, which catalyzes  $\alpha$ -ketobutyrate formation, and from the valine inhibition of the remaining valine-sensitive AHAS I and AHAS III isozymes, required for isoleucine synthesis (Fig. 3A). Valine inhibition is reversed by isoleucine, which inhibits IlvA activity, thus blocking the accumulation of  $\alpha$ -ketobutyrate caused by valine (Fig. 3A) (38).

To determine whether *E. coli* K-12 growth inhibition by CFT073 in-biofilm supernatants reflected valine secretion, we first checked the effect of L-isoleucine on CFT073 in-biofilm supernatant activity. The addition of L-isoleucine to a CFT073 in-biofilm supernatant alleviated its inhibition of *E. coli* K-12 MG1655, whereas the addition of other amino acids had no effect (Fig. 2B). Moreover, no growth inhibition was observed for rich LB medium plates (data not shown). We then used the CFT073 *ilvG*<sup>+</sup> sequence to correct the *ilvG* frameshift mutation in MG1655 (MG1655 $\Delta ilvG$ <sup>+</sup>) (see Fig. 3B) and tested the sensitivity of the valine-resistant MG1655 $\Delta ilvG$ <sup>+</sup> to CFT073 in-biofilm supernatant. While MG1655 (*ilvG*) growth is severely inhibited on minimal medium plates by a CFT073 in-biofilm supernatant, strain MG1655 $\Delta ilvG$ <sup>+</sup> displayed a high level of resistance (Fig. 2A). Finally, analysis of the amino acid composition of a CFT073 in-biofilm supernatant showed that it indeed contains much higher levels of L-valine as well as L-alanine than a CFT073 planktonic supernatant (Fig. 2C). The concentration of valine found in the CFT073 biofilm supernatant is about 12  $\mu$ g/ml. Consistently, whereas L-alanine has no inhibitory activity (data not shown and Fig. 2B), the addition of exogenous L-valine at a concentration of 15  $\mu$ g/ml to the planktonic (inactive) supernatant of CFT073 reproduced the inhibitory effect of the CFT073 in-biofilm supernatant on an overlayer of MG1655 cells and not on an overlayer of the  $\Delta livH$  mutant cells (see Fig. S1 in the supplemental material). Taken together, these results demonstrate that the growth inhibition of *E. coli* K-12 by CFT073 in-biofilm supernatants is due to accumulation of monomeric valine.

**Many *E. coli* isolates and other bacteria secrete valine in biofilms.** In order to investigate the occurrence of valine production under biofilm conditions by *E. coli* isolates and other bacteria, the growth inhibition activities of in-biofilm supernatants of 44 *E. coli* isolates of different origins and supernatants of *Klebsiella pneumoniae*, *K. oxytoca*, *Enterobacter cloacae*, *Serratia marcescens*, *Salmonella enterica* serovar Enteritidis, and *Pseudomonas aeruginosa* were tested with an MG1655 overlayer. As shown in Table 1, 24 out of 44 (55%) *E. coli* in-biofilm supernatants, as well as those from *K. pneumoniae*, *E. cloacae*, *Salmonella* serovar Enteritidis, and *P. aeruginosa*, produced a growth inhibition halo. This inhibition was likely to be due to valine secretion, since inhibition was alleviated by isoleu-



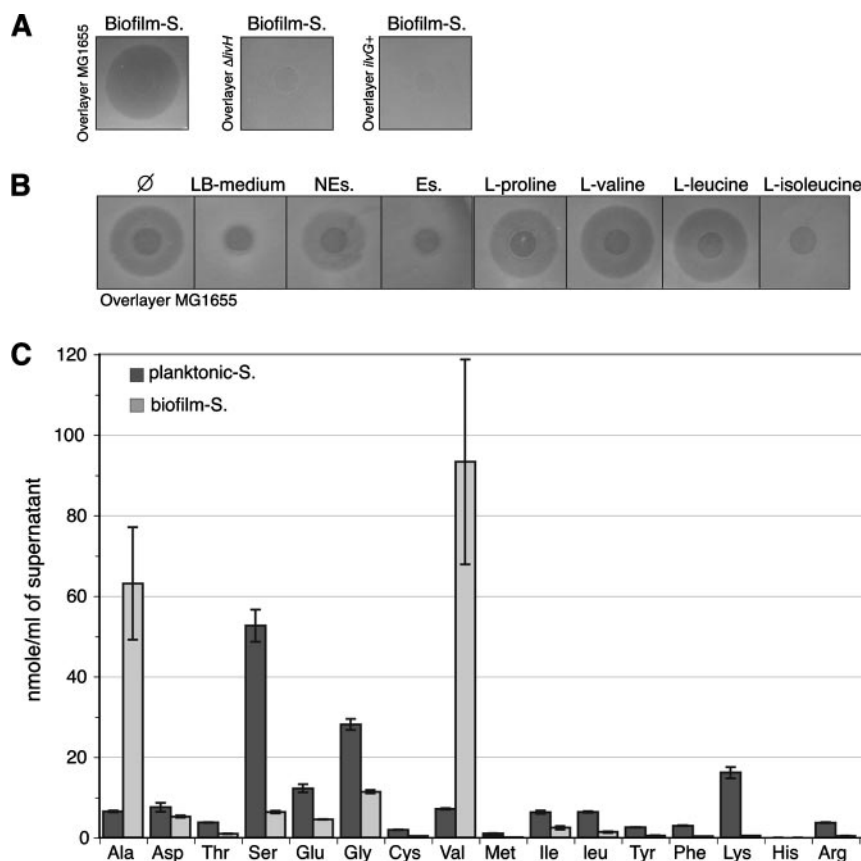


FIG. 2. A high level of valine is present in biofilm supernatant. (A) Inhibitory activity of the CFT073 in-biofilm supernatant (Biofilm-S) on overlays of MG1655, MG1655Δ*livH*, and MG1655*livG*<sup>+</sup> cells. (B) Inhibitory activity of CFT073 in-biofilm supernatant supplemented at a 1:1 ratio with LB medium, nonessential amino acids (NEs; which includes L-alanine; see Materials and Methods), essential amino acids (Es), or the amino acid L-proline, L-valine, L-leucine, or L-isoleucine (all amino acids at a concentration of 50 μg ml<sup>-1</sup> each) on an overlay of MG1655 cells. LB and Es solutions contained L-isoleucine. Ø means that the CFT073 in-biofilm supernatant was not supplemented with exogenous amino acids. (C) The amino acid composition of CFT073 supernatants purified from planktonic culture (Planktonic-S) and biofilm (Biofilm-S). Experiments were performed in triplicate. Error bars represent standard deviations of the means.

cine and the tested in-biofilm supernatants had no effect on the valine-resistant mutant MG1655Δ*livG*<sup>+</sup> (Table 1). These results therefore indicate that the secretion of valine within the biofilm matrix is not restricted to CFT073 but is a widespread phenotype among natural *E. coli* isolates, as well as other gram-negative bacteria.

***E. coli* stress responses do not contribute to valine production within biofilm.** Mature biofilms constitute a heterogeneous stationary phase-like environment in which the induction of several stress responses has been demonstrated (4, 58). To investigate the potential link between bacterial stress responses and valine secretion, we first analyzed the level of valine secretion by CFT073 grown planktonically when bacteria were exposed to acid or oxidative stresses. Neither the use of different levels of superoxide radicals generated by a range of paraquat concentrations (up to 1 mM) nor acidic pH conditions (pH 5) induced valine secretion by CFT073 planktonic cells (data not shown). We then studied the role of several major regulators of *E. coli* stress or signaling responses, including H-NS, RpoS (stationary phase), OxyR, SoxR (oxidative stress), CpxR (envelope stress), and LuxS (quorum sensing). None of the mutants lacking one of these regulators displayed altered valine production in

filter-sterilized planktonic or in-biofilm supernatants (data not shown). These results showed that none of the tested stresses or stress response regulators is involved in valine secretion within biofilms.

**Valine secretion within biofilms is not due to the stringent response.** The stringent response, a complex cellular adaptation to starvation for amino acids and sources of carbon, nitrogen, and phosphate, has been shown to be important for *E. coli* biofilm formation under slow-growth or nutrient-limited conditions (2, 9). The bacterial stringent response is essentially mediated by two proteins, RelA and SpoT, which control the intracellular level of ppGpp. This nucleotide acts as a general starvation signal leading simultaneously to down-regulation of nucleic acid and protein synthesis and to up-regulation of protein degradation and amino acid synthesis. Whereas both RelA and SpoT convert GTP to pppGpp (which is further converted to ppGpp), only SpoT mediates pppGpp and ppGpp hydrolysis (9). To explore the potential relationship between in-biofilm valine secretion and the stringent response, we first tested the phenotypic consequences of the absence of ppGpp and introduced either a single *relA* or a *relA spoT* double mutation into CFT073. In their planktonic supernatants, neither the Δ*relA*

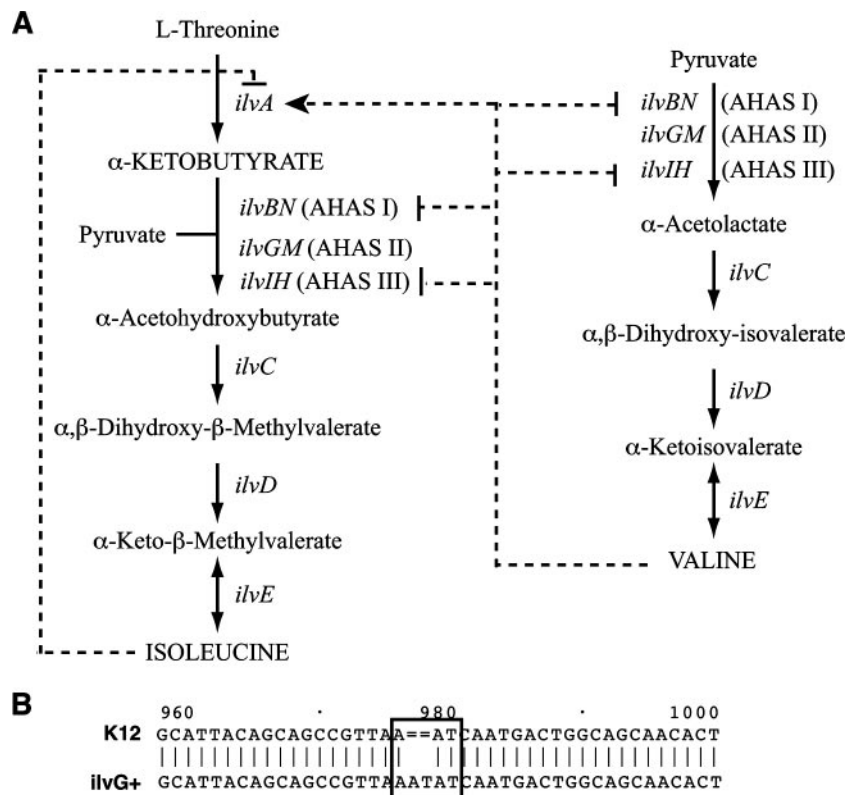


FIG. 3. Isoleucine and valine biosynthesis pathways. (A) *ilvBN*, *ilvGM* and *ilvIH* code for the heterodimeric acetohydroxy acid synthase isozymes AHAS I, AHAS II, and AHAS III, respectively. AHAS I and AHAS III are inhibited in the presence of high amounts of valine, whereas isozyme AHAS II is valine insensitive. Regulations by valine and isoleucine are shown by dashed lines terminated by an arrow for activation or by a vertical line for inhibition. (B) In *E. coli* K-12, AHAS II is inactivated by a frameshift mutation in *ilvG*. *ilvG* sequences of *E. coli* K-12 and MG1655 *ilvG*<sup>+</sup> are aligned. The numbers above indicate base numbers from the ATG translation start according to the *ilvG*<sup>+</sup> sequence.

mutant nor the  $\Delta relA \Delta spoT$  mutant produced valine, and their in-biofilm supernatants still displayed valine-associated inhibition (Fig. 4A).

We then tested the consequence of an increased level of ppGpp by introducing into CFT073 a plasmid-borne *plac*-con-

trolled allele of *relA* (pRelA\*), which has been shown to lead to starvation-independent ppGpp production (9). We did not observe any valine-related inhibition by the planktonic supernatant of the CFT073(pRelA\*) strain, and the presence of the

TABLE 1. Occurrence of growth inhibition activity of in-biofilm supernatant of different gram-negative bacteria on *E. coli* MG1655

Organism	No. of strains tested	Biofilm-S strains inducing inhibition <sup>a</sup>	
		No. of strains	% of strains tested
<i>E. coli</i> <sup>b</sup>	44	24	55
<i>K. oxytoca</i>	3	0	0
<i>K. pneumoniae</i>	4	3	75
<i>E. cloacae</i>	2	1	50
<i>S. marcescens</i>	1	0	0
<i>Salmonella</i> serovar Enteritidis	2	2	100
<i>P. aeruginosa</i>	4	4	100

<sup>a</sup> Number and percentage of the strains tested whose in-biofilm supernatants (Biofilm-S) induced the appearance of a growth inhibition halo on an overlayer of MG1655 cells on M63B1glu plates and for which the inhibition was alleviated by the addition of isoleucine.

<sup>b</sup> The *E. coli* strains tested for growth inhibition activities of their in-biofilm supernatants are specified in Table 3.

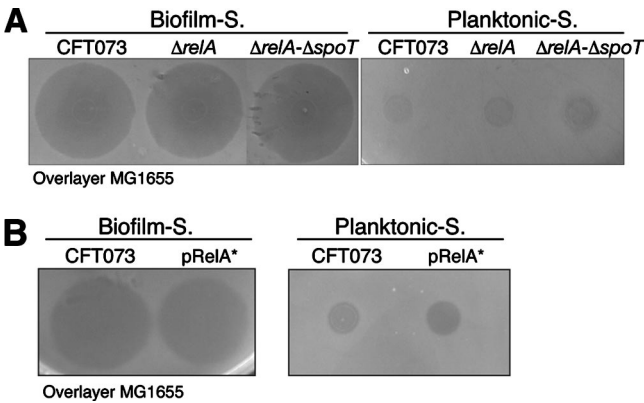


FIG. 4. Effect of the stringent response on valine-related inhibition. Valine-related inhibition on an MG1655 overlayer of (A) the in-biofilm (Biofilm-S) and planktonic (Planktonic-S) supernatants of the CFT073 $\Delta relA$  and CFT073 $\Delta relA \Delta spoT$  mutants and (B) the supernatants of CFT073 cells overproducing ppGpp (pRelA\*) and non-overproducing cells, purified from biofilm (Biofilm-S) or planktonic culture (Planktonic-S).

TABLE 2. *E. coli* CFT073 transposon mutants producing valine under planktonic conditions

COG classification <sup>a</sup>	Protein	CFT073 gene no.	BLAST description
Amino acid biosynthesis genes	IlvA	C4694	Threonine dehydratase biosynthesis
	IlvY	C4695	Transcriptional activator protein
Growth-rate-dependent regulatory genes	SspA	C3981	Stringent starvation protein
	TufA	C4111	Elongation factor Tu
Other	NadB	C3098	L-Aspartate oxidase
	NadC	C0128	Nicotine-nucleotide pyrophosphorylase
	H protein	C0746	Hypothetical protein
	AceE	C0142	Pyruvate dehydrogenase

<sup>a</sup> COG, cluster of orthologous groups.

plasmid did not prevent valine secretion in biofilm (Fig. 4B). These results suggest that an increased ppGpp level does not lead to biofilm-independent valine secretion nor does it inhibit in-biofilm valine secretion. Hence, no correlation between in-biofilm valine secretion and the stringent response could be demonstrated.

**Screening for CFT073 mutants secreting valine in planktonic cultures.** We sought to investigate further the genetic basis for valine secretion within CFT073 biofilms and performed *mariner* transposon mutagenesis with CFT073. However, due to the practical difficulty of screening for the loss of growth inhibition activity of in-biofilm supernatants extracted from a large number of mutants grown in microfermentors, we instead screened approximately 10,000 *mariner* transposon mutants for biofilm-independent inhibitory activity when grown as colonies on an overlayer of *E. coli* MG1655. We identified eight mutants that produced a larger growth inhibition halo than the wild-type CFT073. These mutants corresponded to a transposon insertion at eight different loci (Table 2). Two of the mutations were in genes involved in the branched-chain amino acid biosynthesis pathway (*ilvA* and *ilvY*). Most of the mutants (*ilvA*, *ilvY*, *nadB*, *nadC*, C0746, and *aceE* mutants) had a reduced growth rate on minimal glucose plates compared to that of wild-type CFT073 (data not shown), although two mutants, the *tufA* and *sspA* mutants, displayed wild-type levels of growth. The stringent starvation protein A (SspA) was previously described as an RNA polymerase-associated activator for the lytic development of phage P1, which also has global regulatory functions and is essential for stationary phase-induced acid tolerance in *E. coli* (20, 21). *tufA* is one of two unlinked genes (with *tufB*) coding for the translation elongation factor Tu, which promotes GTP-dependent binding of aminoacyl-tRNA to the A site of ribosomes during protein synthesis. Like *sspA*, *tufA* may play an important regulatory role in the bacterial response to nutrient deprivation (57, 59).

In order to confirm the role of these mutations in MG1655 growth inhibition, we performed nonpolar allelic replacement of the *sspA* and *tufA* genes in *E. coli* CFT073. The planktonic supernatant of the two mutants displayed the same inhibitory phenotype as those of the corresponding transposon mutants (Fig. 5A). Furthermore, the MG1655 growth inhibition activity of the CFT073Δ*sspA* and CFT073Δ*tufA* planktonic superna-

tants was independent of *mchB*; it was abolished by the addition of isoleucine and was inefficient against MG1655*ilvG*<sup>+</sup> (Fig. 5B to D). Finally, the two mutants' planktonic supernatants contained high levels of L-valine (Fig. 5E). We then cloned *sspA* and *tufA* under the control of the *plac* promoter, leading to plasmids pSspA and pTufA, respectively. As expected, no valine-associated inhibition was observed in the planktonic supernatants of the mutants CFT073Δ*sspA* and CFT073Δ*tufA* complemented by pSspA and pTufA, respectively (Fig. 5F). These results therefore demonstrated that we had identified mutations in strain CFT073 leading to valine secretion under planktonic growth conditions.

**Although an *ilvG* strain outcompetes an *ilvG*<sup>+</sup> strain under planktonic conditions, valine-sensitive *E. coli* isolates are rare in natural populations.** Valine-sensitive strains occur at very low frequencies among natural *E. coli* isolates, according to an early study (30). In agreement with that study, we identified only two valine-sensitive strains (*E. coli* 195 and *E. coli* 057) among 552 commensal *E. coli* isolates of extremely varied origins (Table 3). The sequences of their *ilvG* alleles indicated that at least one of these strains (*E. coli* 057) did indeed carry *ilvG* (see Fig. S2 at <http://www.pasteur.fr/recherche/unites/Ggb/supmat.html>). Hence, although it is sometimes considered a laboratory allele, an inactive *ilvG* gene may give bacteria a selective advantage under certain environmental conditions. To test whether the presence of a wild-type *ilvG*<sup>+</sup> allele is associated with a detectable fitness loss, we used the corrected valine-resistant MG1655*ilvG*<sup>+</sup> strain to compare the growth of isogenic MG1655*ilvG* with that of the *ilvG*<sup>+</sup> strain under planktonic conditions. Growth curves indicated a clear yield defect of MG1655*ilvG*<sup>+</sup> strains in LB medium but not in M63B1glu (Fig. 6A and data not shown). These results suggest that, under some growth conditions, the expression of *ilvG* may constitute a genetic burden that is alleviated in *ilvG* strains. To further investigate the potential consequences of this fitness loss, we performed competition experiments in which a 1:1 ratio of tagged isogenic MG1655*ilvG*<sup>+</sup> and MG1655*ilvG* strains were mixed under planktonic culture conditions in LB medium. After 24 h of growth, plating assays revealed a competitive advantage for the *ilvG* strain, with a 1.5:1 ratio of *ilvG* to *ilvG*<sup>+</sup> cells (Fig. 6B).

This suggests that, although a valine-sensitive *ilvG* *E. coli* strain may have a selective advantage over an *ilvG*<sup>+</sup> strain in LB broth, valine-sensitive *ilvG* frameshift mutants are not favored over valine-resistant *E. coli* in most natural ecological niches.

**In-biofilm valine secretion selects valine resistance in mixed *E. coli* biofilms.** Valine-resistant mutants with mutations that correct the translational reading frame of the *ilvG* gene can be readily isolated among mutants growing when a moderate concentration of valine (1 μg/ml or more) is present in isoleucine-free growth medium. This genetic switch from *ilvG* (valine-sensitive) to *ilvG*<sup>+</sup> (valine-resistant) *E. coli* K-12 strains is one of the best-documented examples of activation of an otherwise inactive ("cryptic") pseudogene (18). The high frequency of valine-resistant strains among natural *E. coli* isolates found in early studies led to the hypothesis that in unidentified valine-rich environments, valine resistance might be an advantageous phenotype. Our finding that many *E. coli* and other bacterial isolates naturally secrete high amounts of valine within bio-

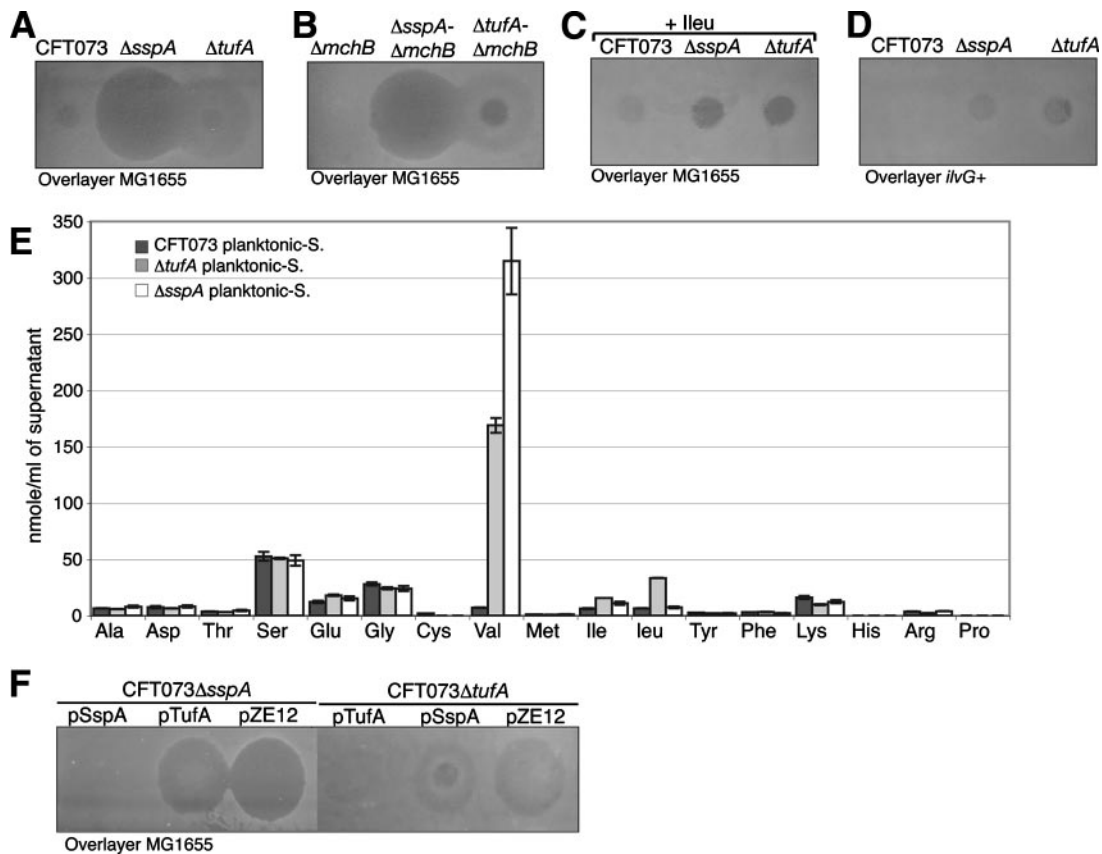


FIG. 5. Phenotypic analysis of the planktonic valine-secreting mutants. Valine-related inhibition by supernatants purified from planktonic cultures of CFT073, CFT073 $\Delta sspA$ , and CFT073 $\Delta tufA$  (A) and CFT073 $\Delta mchB$ , CFT073 $\Delta sspA \Delta mchB$ , and CFT073 $\Delta tufA \Delta mchB$  (B) on an overlayer of MG1655 cells. (C) Effect of L-isoleucine addition to the CFT073 $\Delta sspA$  and the CFT073 $\Delta tufA$  planktonic supernatant activity on an overlayer of MG1655 cells. (D) Inhibitory activity of the CFT073 $\Delta sspA$  and the CFT073 $\Delta tufA$  planktonic supernatants on an overlayer of MG1655 *ilvG*<sup>+</sup>. (E) Analysis of the amino acid composition of the CFT073 $\Delta sspA$  and the CFT073 $\Delta tufA$  planktonic supernatants. Experiments were performed in triplicate. Error bars represent standard deviations of the means. (F) Complementation of the CFT073 $\Delta sspA$  and CFT073 $\Delta tufA$  mutants with pSspA, pTufA, and the empty plasmid pZE12 as the control.

films led us to hypothesize that this widespread valine-rich environment may be the selection pressure favoring genetic maintenance of the *ilvG*<sup>+</sup> genotype.

To test this hypothesis, we compared the frequency of valine-resistant derivatives produced in mixed biofilm or planktonic cultures in which valine-sensitive *E. coli* MG1655 F', which does not produce in-biofilm valine, was mixed 1:1 with the valine-producing and valine-resistant strain CFT073 $\Delta mchB \Delta kpsD$ . As shown in Fig. 7, after 24 h, 15-fold more valine-resistant derivatives were isolated from mixed biofilm cultures than from mixed planktonic cultures. In contrast, the frequency of valine-resistant derivatives in a mixed biofilm formed with two *E. coli* K-12 strains that do not secrete valine (KS272 and MG1655 F') was similar to that obtained under planktonic conditions with *E. coli* MG1655 F' and CFT073 $\Delta mchB \Delta kpsD$  (Fig. 7). These results thus indicate that the secretion of valine within bacterial biofilms increases the frequency of valine-resistant derivatives in the valine-sensitive MG1655 F' population. In-biofilm valine secretion is therefore a plausible selective force leading to the persistence of valine-resistant strains in natural *E. coli* populations living mostly in mixed biofilm-like environments where valine can be produced by many different bacteria.

## DISCUSSION

Over 60 years ago, Tatum noted that in the absence of isoleucine, valine inhibits the growth of *E. coli* K-12 (49). The cause of this toxicity is now well understood (see Fig. 3), and amino acid antagonisms have been instrumental in the study of the branched-chain amino acid biosynthetic pathways and have led to the elucidation of several important biological concepts such as feedback inhibition and allosterity (10, 14, 38, 52). However, we still know little about the actual biological or ecological relevance of these antagonisms.

In this study, we observed that many *E. coli* strains, irrespective of their specific phylogenetic groups, have the ability to produce valine when grown as continuous-flow biofilms in glucose-rich minimal medium but not after incubation in batch cultures of the same medium where carbon sources are rapidly used and depleted. Although valine is a bacteriostatic inhibitor of *E. coli* K-12 derivatives and a few natural isolates, it does not inhibit most of the 552 *E. coli* strains of extremely varied origin tested in this study. This narrow range of target strains therefore strongly suggests that valine is unlikely to be a biofilm bacteriotoxin. Nevertheless, valine could play several other



TABLE 3. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Reference or source
<b>Strains</b>		
<i>E. coli</i>		
MG1655	Commensal <i>E. coli</i> K-12 containing a frameshift mutation in <i>ilvG</i>	Laboratory collection
MG1655 $\Delta$ <i>livH</i>	MG1655 $\Delta$ <i>livH::km</i> Km <sup>r</sup>	This study
MG1655F'	MG1655 F' <i>tet-<math>\Delta</math>traD::apra</i> plasmid, Apr <sup>r</sup> , Tet <sup>r</sup>	54
KS272	Commensal <i>E. coli</i> K-12	Laboratory collection
BL21	Commensal <i>E. coli</i> K-12	Laboratory collection
MG1655 $\lambda$ <i>attgfp-amp</i>	MG1655 with a <i>gfp</i> gene inserted at the $\lambda$ <i>att</i> site; Amp <sup>r</sup>	13
MG1655 $\lambda$ <i>attgfp-Km</i>	MG1655 with a <i>gfp</i> gene inserted at the $\lambda$ <i>att</i> site; Km <sup>r</sup>	13
MG1655 <i>ilvG</i> <sup>+</sup>	MG1655 with a hybrid active <i>ilvG</i> gene of K-12 and CFT073	This study
CFT073	Uropathogenic isolate	Laboratory collection
CFT073 $\Delta$ <i>kpsD</i>	CFT073 $\Delta$ <i>kpsD::kan</i> ; Km <sup>r</sup>	54
CFT073 $\Delta$ <i>mchB</i>	CFT073 $\Delta$ <i>mchB::kan</i> ; Km <sup>r</sup>	This study
CFT073 $\Delta$ <i>soxR</i>	CFT073 $\Delta$ <i>soxR::kan</i> ; Km <sup>r</sup>	This study
CFT073 $\Delta$ <i>oxyR</i>	CFT073 $\Delta$ <i>oxyR::kan</i> ; Km <sup>r</sup>	This study
CFT073 $\Delta$ <i>cpxR</i>	CFT073 $\Delta$ <i>cpxR::kan</i> ; Km <sup>r</sup>	This study
CFT073 $\Delta$ <i>spoS</i>	CFT073 $\Delta$ <i>spoS::kan</i> ; Km <sup>r</sup>	This study
CFT073 $\Delta$ <i>hns</i>	CFT073 $\Delta$ <i>hns::kan</i> ; Km <sup>r</sup>	This study
CFT073 $\Delta$ <i>relA</i>	CFT073 $\Delta$ <i>relA::cm</i> ; Cm <sup>r</sup>	This study
CFT073 $\Delta$ <i>relA-<math>\Delta</math>spoT</i>	CFT073 $\Delta$ <i>relA::cm <math>\Delta</math>spoT::zeo</i> ; Cm <sup>r</sup> Zeo <sup>r</sup>	This study
CFT073 p <i>RelA</i> <sup>*</sup>	Strain with plasmids pMM100 and pSM11	This study
<i>E. coli</i> 195	Commensal isolate belonging to phylogenetic group B1, obtained from a Pyrenees farm sheep	44; also see Table S2 <sup>c</sup>
<i>E. coli</i> 057	Commensal isolate belonging to phylogenetic group B1.341 from a Pyrenean chamois	44; also see Table S2
1092 <sup>a</sup>	Commensal isolate from a healthy patient	C. Le Bouguenec
1091 <sup>a</sup>	Commensal isolate from a healthy patient	C. Le Bouguenec
1102 <sup>a</sup>	Commensal isolate from a healthy patient	C. Le Bouguenec
1103 <sup>a</sup>	Commensal isolate from a healthy patient	C. Le Bouguenec
1110 <sup>a</sup>	Commensal isolate from a healthy patient	C. Le Bouguenec
1125 <sup>a</sup>	Commensal isolate from a healthy patient	C. Le Bouguenec
536 <sup>a</sup>	Uropathogenic isolate (O6:K15:H31)	J. Hacker
J96 <sup>a</sup>	Uropathogenic isolate (O4:K6)	J. Hacker
<i>E. coli</i> collection		33; also see Table S2
ECOR <sup>b</sup>		
<i>E. coli</i> collection	341 commensal isolates constituted from several animal populations	44; also see Table S2
<i>E. coli</i> collection	Commensal isolates constituted from human populations	15; also see Table S2
UPEC collection	Uropathogenic clinical isolates U1–3, U6–7, U9–11, U13–20	54; also see Table S2
<b>Klebsiella spp.</b>		
<i>K. oxytoca</i> U12	Clinical isolate	C. Forestier
<i>K. oxytoca</i> U10	Clinical isolate	C. Forestier
<i>K. oxytoca</i> U33	Clinical isolate	C. Forestier
<i>K. pneumoniae</i> U112	Clinical isolate	C. Forestier
<i>K. pneumoniae</i> U22	Clinical isolate	C. Forestier
<i>K. pneumoniae</i> U12	Clinical isolate	C. Forestier
<i>K. pneumoniae</i> U21	Clinical isolate	C. Forestier
<b>Enterobacter</b>		
<i>E. cloacae</i> U28	Clinical isolate	C. Forestier
<i>E. cloacae</i> U4	Clinical isolate	C. Forestier
<i>Serratia marcescens</i> U11	Clinical isolate	C. Forestier
<b>Salmonella enterica</b>		
serovar Enteritidis		
3934	Clinical isolate	46
921	Clinical isolate	46
<b>Pseudomonas aeruginosa</b>		
PAK	Laboratory strain	8
KK02	Clinical isolate	S. de Bentmann
KK14	Clinical isolate	S. de Bentmann
KK11	Clinical isolate	S. de Bentmann
<b>Plasmids</b>		
pMM100	Derivative of pACYC184 that expresses the LacI repressor	34
pSM11	pSM11 contains 455 codons from the <i>E. coli relA</i> gene placed under the control of the <i>lac</i> promoter; promoter induction leads to increased ppGpp production in the cell	42
pZE12	Used as a cloning vector; Amp <sup>r</sup> ColE1 <i>ori</i>	29
pSpA	CFT073 <i>sfpA</i> placed under the control of the synthetic <i>lacP</i> promoter on the pZE12 vector; Amp <sup>r</sup> ColE1 <i>ori</i>	This study
pTufA	CFT073 <i>tufA</i> placed under the control of the synthetic <i>lacP</i> promoter on the pZE12 vector; Amp <sup>r</sup> ColE1 <i>ori</i>	This study

<sup>a</sup> Tested for growth inhibition activity of in-biofilm supernatant on an MG1655 overlayer (Table 1).<sup>b</sup> ECOR, *Escherichia coli* Reference Collection (33). Isolates 1 to 4, 7 to 12, and 14 to 19 were used; see Table S2 in the supplemental material.<sup>c</sup> In the supplemental material.



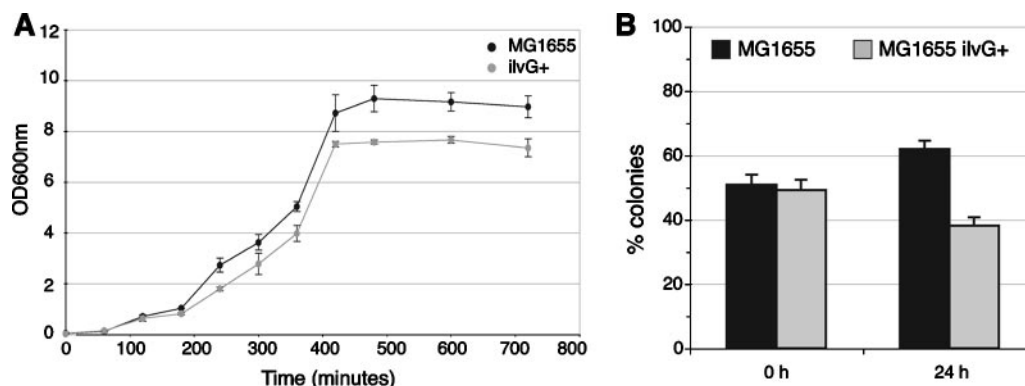


FIG. 6. Inactivation of *ilvG* confers an advantageous phenotype on *E. coli* K-12. (A) Growth curves of MG1655 and MG1655*ilvG*<sup>+</sup> in LB medium at 37°C. (B) Competition experiments: MG1655Δ*attgfp-Km* (MG1655) and MG1655Δ*attgfp-amp* (MG1655*ilvG*<sup>+</sup>) cells grown overnight and diluted to an OD of 1 with fresh LB medium were mixed at a 1:1 ratio and grown in LB at 37°C. The population composition was estimated by serial dilutions and plating in order to estimate the CFU of the two strains in the mixed culture. Results are shown as the percentages of colonies of MG1655 and MG1655*ilvG*<sup>+</sup> in the mixed culture at 24 h after mixing. Experiments were performed in triplicate. Error bars represent standard deviations of the means.

specific roles, such as that of protective osmolyte or a role signaling (32, 37, 43, 48). However, none of the tested stress or adaptive response regulators affected in-biofilm valine production, and mutants overexpressing valine (*sspA* or *tufA*) or impaired in valine acquisition (*livH*) displayed a wild-type biofilm phenotype (data not shown). Moreover, the lack of effect of exogenously added in-biofilm supernatant on biofilm growth of several valine-resistant strains (see Table S2 at [www.pasteur.fr/recherche/unites/Ggb/supmat.html](http://www.pasteur.fr/recherche/unites/Ggb/supmat.html)) led us to favor the hypothesis that in-biofilm valine secretion is rather a metabolic adaptation taking place within the mature biofilm formed under our test conditions.

Increased valine concentrations due to an imbalance in amino acid biosynthetic pathways have been reported to result from acid stress or from the addition of exogenous homocysteine to the medium (50, 51, 53). Valine secretion under several unusual growth conditions, such as high-cell-density

planktonic *E. coli* cultures (with ODs of up to 50) used in recombinant protein production biotechnology, has also been described previously (1). These conditions are reminiscent of those within biofilms, raising the possibility that in-biofilm valine production may be a consequence of complex alterations of metabolic pools within biofilms. Webb reported previously that the growth inhibition of *Klebsiella pneumoniae* by aminopterin led to the production of valine and L-alanine as a metabolic consequence of pyruvate accumulation (55, 56). Aminopterin is an inhibitor of dihydrofolate reductase and thus of tetrahydrofolate, a precursor of all one-carbon units. Under aerobic conditions, the central metabolite pyruvate leads to acetyl-coenzyme A (acetyl-CoA) and the citric acid cycle, while under anaerobic conditions, in addition to forming acetyl-CoA, it is the precursor of most fermentation products. Because pyruvate is a precursor of valine (the condensation of two pyruvates leads to one valine) and L-alanine (by transamination), Webb's studies suggest that in the absence of a limited source of carbon, reduced growth or oxygen rarefaction may slow down the citric acid (Kreb's) cycle and consequently lead to an increased drain of pyruvate toward biosynthesis of L-alanine and L-valine, both of which are increased in the CFT073 in-biofilm supernatant (55, 56).

In an attempt to investigate the genetic basis for in-biofilm valine production, we screened for mutants of CFT073 that were able to secrete valine under nonbiofilm conditions. We found eight mutants that secreted valine in their planktonic supernatant (Table 2). Two of the mutations affect the valine biosynthetic pathway genes *ilvA* and *ilvY*, which could cause an increase in the pyruvate intracellular pool by reducing its incorporation into α-acetoxybutyrate by the lack of α-keto-butyrate (the *ilvA* mutation) or by the reduction of conversion of α-acetoxybutyrate into α-β-keto-β-methylvalerate (IlvY is a transcriptional activator of *ilvC*) (Fig. 3). One mutation (*aceE*) affected pyruvate dehydrogenase, the inactivation of which has been recently shown to cause pyruvate accumulation and valine secretion (5, 6). The *sspA* and *tufA* mutations affect the protein synthesis rate, and the amino acid pool utilization may be also associated with a reduced pyruvate

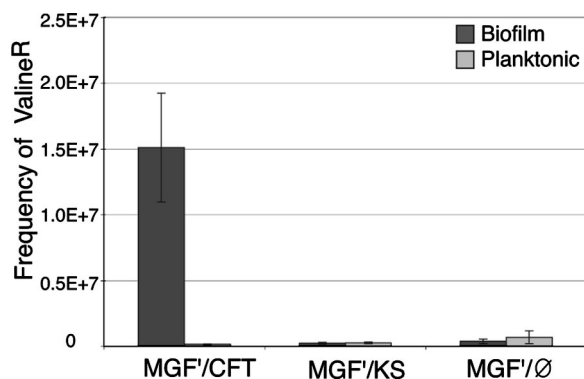


FIG. 7. Frequency of MG1655 valine-resistant derivative mutants. MG1655F' valine-resistant derivatives were isolated from mixed cultures of MG1655F' and the valine-producing strain CFT073Δ*mchB*Δ*kpsD* (MGF'/CFT), MG1655F' and the non-valine-producing strain KS272 (MGF'/KS) or MG1655F' alone (MGF'/Ø) grown in biofilm or in planktonic culture. Results are expressed as the frequency of valine-resistant mutants of MG1655 isolated after 24 h of growth of the mixed culture. Experiments were performed in triplicate. Error bars represent standard deviations of the means.

utilization. Mutations leading to valine secretion in planktonic cultures do not cause alanine secretion and, *sensu stricto*, may not be fully correlated with events leading to in-biofilm valine secretion. However, a transcriptome analysis performed under similar experimental conditions with *E. coli* K-12 showed consistent biofilm-induced modification of the expression of genes involved in carbohydrate and energy metabolism, including enzymes of the tricarboxylic acid cycle such as fumarate deshydrogenase, malate deshydrogenase, or succinyl-CoA synthetase (4). Our data therefore strongly suggest that in-biofilm valine production reflects physiological adjustments of bacteria to continuous-flow biofilm conditions such as low oxygen and reduced growth rate when carbon sources are not limiting. These physiological conditions would lead to an excess of pyruvate, dissimilated through relaxed synthesis and secretion of valine and alanine. Consistent with this hypothesis, biofilm supernatants from cultures grown in minimal medium containing glycerol or low glucose (0.05%), where pyruvate is not expected to accumulate, did not display valine-related inhibitory activity (data not shown). Although the determination of the metabolic bases of in-biofilm valine production in the UPEC strain CFT073 will require further study, we have demonstrated that this property is widespread among gram-negative bacteria. These bacteria are commonly found in environments that share some of the structural characteristics of multispecific biofilms (e.g., intestinal flora and lung), and we therefore sought to investigate the consequence of in-biofilm valine secretion, in particular with respect to the occurrence of valine resistance. Early studies showed that valine-resistant *E. coli* K-12 mutants were readily isolated on minimal medium in the presence of valine. The activation of the *ilvG* pseudogene of *E. coli* K-12 now even constitutes a classical example of cryptic gene activation. The relative fitness of bacteria with the cryptic or the active gene will depend on the environmental conditions. In the case of the *ilvG* mutant, it has been pointed out that a functional AHAS II isozyme (*ilvG*<sup>+</sup> strains) is not required under most growth conditions, since isozymes AHAS I and III have adequate biosynthetic capacity (see Fig. 3). Previous studies suggested that the *ilvG*<sup>+</sup> allele could constitute a genetic burden, possibly due to the wasteful overexpression of downstream genes (*ilvEDA*) when wild-type *ilvG* is expressed (27, 30). We have shown here that the characteristics of the *ilvG*<sup>+</sup> mutant indeed negatively affect the *E. coli* MG1655 growth yield and fitness compared to that of MG1655 (*ilvG*), although we confirmed that the occurrence of valine-sensitive strains and *ilvG* isolates is rare. Hence, while one of the potential beneficial consequences of naturally occurring frame shifts in the *ilvG* gene may be to alleviate the detrimental effect of *ilvG* expression under certain conditions, selection pressure has been hypothesized as the explanation for the prevalence of *ilvG*<sup>+</sup> valine-resistant strains in nature.

While it was possibly acquired under laboratory conditions, the *E. coli* K-12 frameshift mutation in *ilvG* raised the question of the long-sought rationale for valine resistance within natural *E. coli* isolates. We demonstrate that mixing valine-resistant and valine-sensitive strains led to the accumulation of valine-resistant mutants when the valine-sensitive cells were grown in a mixed biofilm with a valine secretor. These results suggest that in-biofilm valine secretion may be the most relevant environmental condition, leading to the persistence of a valine-

resistant phenotype over that of valine sensitivity in *E. coli* strains, thus providing a plausible explanation for the widespread occurrence of valine-resistant bacterial strains.

Global transcriptome analyses comparing surface-attached planktonic culture conditions revealed that the biofilm lifestyle triggers extensive modifications in gene expression that are proposed to correspond to profound physiological changes (3, 26, 40). However, with the exceptions of increased antibiotic tolerance and cyclic di-GMP-mediated regulation of motility and cell surface adhesins, very few bacterial functions have yet been characterized as restricted to the biofilm lifestyle (23, 24). Here, we identified valine secretion as a metabolic response associated with mature biofilms formed under conditions in which carbon sources are not severely limited. Valine secretion is not strictly restricted to biofilm environments, as it is occasionally produced in some industrial planktonic fermentation bioprocesses; however, these conditions are highly artificial (1). By contrast, high-cell-density biofilms formed under conditions where carbon sources are renewed by environmental flow correspond to a number of natural settings. Valine can then accumulate, since it is locally trapped within this reduced-diffusion environment composed of a high-cell-density biomass and the biofilm matrix (1, 36, 47).

In conclusion, this study validates the postulated existence of molecules whose secretion is associated with the biofilm environment. Further characterization of such metabolic signatures may allow the development of new markers with which to monitor or control the biofilm lifestyle.

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