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Fitness, Stress Resistance, and Extraintestinal Virulence in *Escherichia coli*

Alexandre Bleibtreu,[a,b] Pierre-Alexis Gros,[a,b] Cédric Laouénan,[c,d] Olivier Clermont,[a,b] Hervé Le Nagard,[c,d] Bertrand Picard,[a,e] Olivier Tenaillon,[a,b] Erick Denamur[a,b]

UMR-S 722, INSERM, Paris, France[a]; UMR-S 722, Université Paris Diderot, Sorbonne Paris Cité, Paris, France[b]; UMR-S 738, INSERM, Paris, France[c]; UMR-S 738, Université Paris Diderot, Sorbonne Paris Cité, Paris, France[d]; UMR-S 722, Université Paris Nord, Bobigny, France[e]

The extraintestinal virulence of *Escherichia coli* is dependent on numerous virulence genes. However, there is growing evidence for a role of the metabolic properties and stress responses of strains in pathogenesis. We assessed the respective roles of these factors in strain virulence by developing phenotypic assays for measuring *in vitro* individual and competitive fitness and the general stress response, which we applied to 82 commensal and extraintestinal pathogenic *E. coli* strains previously tested in a mouse model of sepsis. Individual fitness properties, in terms of maximum growth rates in various media (Luria-Bertani broth with and without iron chelator, minimal medium supplemented with gluconate, and human urine) and competitive fitness properties, estimated as the mean relative growth rate per generation in mixed cultures with a reference fluorescent *E. coli* strain, were highly diverse between strains. The activity of the main general stress response regulator, RpoS, as determined by iodine staining of the colonies, H_2O_2 resistance, and *rpoS* sequencing, was also highly variable. No correlation between strain fitness and stress resistance and virulence in the mouse model was found, except that the maximum growth rate in urine was higher for virulent strains. Multivariate analysis showed that the number of virulence factors was the only independent factor explaining the virulence in mice. At the species level, growth capacity and stress resistance are heterogeneous properties that do not contribute significantly to the intrinsic virulence of the strains.

Human extraintestinal infections with *Escherichia coli* represent a major public health problem that is often underestimated, being largely overshadowed by the rare but highly sensationalized outbreaks of intraintestinal *E. coli* infection (1). Nevertheless, *E. coli* extraintestinal infections have a high incidence, with extraintestinal *E. coli* causing 130 million to 175 million cases of uncomplicated cystitis per year worldwide and 127,500 cases of sepsis per year in the United States (2). This high incidence is associated with high morbidity, mortality, and costs. In France, the mortality rate for *E. coli* sepsis was 13% in 2005 (3), and the annual costs of sepsis have been estimated to be $1.1 million to $2.8 million in the United States (2). However, not all *E. coli* strains are virulent; some are gut commensal organisms. *E. coli* is the major facultative anaerobic bacterium in the gut, with an abundance of 10^8 to 10^9 CFU per gram of feces in humans (4). An understanding of the bacterial determinants underlying the development of virulence is essential to the control of extraintestinal infections.

Population genetic studies have shown that the *E. coli* species is mostly clonal (5), with the clear delineation of at least four phylogenetic groups (A, B1, B2, D) (4). Some degree of specialization has been observed in the strains of the various phylogroups. For example, extraintestinal pathogenic strains often belong to phylogroup B2 and, to a lesser extent, phylogroup D (6, 7), whereas the commensal strains present in the human intestine mostly belong to phylogroup A (8). Many gene inactivation (9–11) and character association (12) studies have demonstrated the role of specific genes, known as "virulence genes," in the emergence of extraintestinal virulence. These genes, often colocalized on genomic islands (13), mostly encode adhesins, toxins, iron capture systems, and protectins (14).

Convincing data concerning the role of metabolism in *E. coli* extraintestinal pathogenesis have recently been obtained. Amino acids are a key carbon source in urine, and genes encoding proteins involved in arginine (15) and serine (16) metabolism and in gluconeogenesis (17) are positive fitness traits in strains causing urinary tract infection. A genomic island involved in carbohydrate uptake has also been shown to be important for sepsis (18). Commensal *E. coli* strains growing in the intestine mostly use sugars, gluconate, in particular, as the principal carbon source (19). In addition, commensal *E. coli* strains have to compete for resources with other bacteria, including other *E. coli* strains, and they must protect themselves against bacteriocins, phages, and protozoa, all of which are very common in the digestive tract. Stress tolerance is also a key element in the successful adaptation of bacteria to their various environments. One of the most important regulators of the stress response in *E. coli* is RpoS, an alternative sigma factor of RNA polymerase that regulates more than 10% of *E. coli* genes (20). It plays a critical role in survival under conditions of exposure to several types of stress, including acid, heat, and oxidative stress (21). However, its precise role in extraintestinal virulence remains a matter of debate (21). RpoS seems to be important in some strains for the invasion of brain microvascular epithelial cells (22) and for biofilm formation (23), but its role in urinary

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Address correspondence to Erick Denamur, erick.denamur@inserm.fr.
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tract infection assessed in a mouse model remains unclear (24, 25). Under commensal conditions in the mouse digestive tract, *rpoS* mutants outcompete wild-type strains (26). Extensive, rapid diversification of isolates has been reported to occur during extraintestinal infection in humans, with RpoS levels varying as a result of the trade-off between protection against stress and the preservation of metabolic properties (27). Such a balance between carbon source use and oxidative stress has also recently been reported to be critical in urinary tract infection (28).

In the present work, we developed phenotypic assays for the estimation of individual and competitive fitness *in vitro*. We investigated the determinants involved in extraintestinal virulence in *E. coli* by using these assays and *rpoS* sequencing to determine the growth rate and behavior in competition in various media and to assess the general stress response in a collection of 82 commensal and extraintestinal pathogenic *E. coli* strains representative of the phylogenetic diversity of the species. These data were then analyzed as a function of the intrinsic extraintestinal virulence of the strains previously determined in a mouse model of sepsis (7).

MATERIALS AND METHODS

Strains. Strains from the IAI collection, encompassing 15 commensal *E. coli* strains and 67 extraintestinal pathogenic *E. coli* strains (from blood [$n = 6$], urine [$n = 53$], and miscellaneous samples [$n = 8$]), were studied (7). The strains, isolated in the 1980s in various parts of France, were sent to our laboratory in stab cultures and then stored in glycerol at −80°C. Another short passage in stab culture was made, because the lab moved in the 1990s, followed again by −80°C storage. We used the new quadruplex PCR assay (29) and an *Escherichia* clade detection assay (30) to identify the phylogroup to which each strain belonged, and data for the presence/absence of 18 extraintestinal virulence factors (VFs; i.e., genes for adhesins, toxins, iron capture systems, protectins, uropathogenic-specific protein) and lethality in a mouse septicemia model were already available (12) (see Table S1 in the supplemental material). Strains were described to be nonkiller strains if no more than 2 of the 10 mice inoculated died and killer strains if more than 2 of the 10 mice inoculated died. This categorization was used, as only 5 strains killed between 3 and 7 mice of the 10 inoculated (see Table S1 in the supplemental material). However, excluding these 5 strains or using a third category of intermediate killer did not change the results (data not shown).

For the competition assay, the *E. coli* K-12 BW25113 Δ*lacZ*::*tsr-venus kan* strain (known as the Venus strain) was used as the reference. This strain was derived from *E. coli* strain SX4 (31) by the incorporation into the *E. coli* chromosome of a single copy of the chimeric gene *tsr-venus* as a replacement for the native *lacZ* gene.

Media. Strains were grown in four different media: Luria-Bertani (LB) broth, LB broth supplemented with 100 μM 2-2′dipyridyl (DPY), an iron chelator (LB-DPY), minimal medium supplemented with 20 mM gluconate (MMA), and urine. Urine was collected from healthy male volunteers who had not taken any antibiotic medication for the last 3 months. Samples were filtered, pooled, and stored at −20°C. Competition assays were performed in LB medium supplemented with 0.1 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) (see below) and in LB-DPY-IPTG.

Individual fitness assays. Strains were grown from a glycerol stock for 18 h in propylene plates with 96 deep wells (2 ml) in 500 μl of the various media with shaking at 250 rpm. The plates were covered with a sterile gas-permeable film, and relative humidity was maintained at 70% in the incubator, to prevent evaporation. Each well contained a glass bead, to prevent cell aggregation and improve oxygenation. The plates were incubated overnight, and 1 μl of the culture was then diluted in 200 μl of sterile phosphate-buffered saline (PBS). We used 2 μl of the resulting suspension to inoculate 200 μl of the various media. Growth curves were then generated with an automatic spectrophotometer (Tecan Infinite M200). The optical density at 600 nm (OD_{600}) was measured at 5-min intervals over a period of 24 h. The experiment was repeated three times.

The growth curves were then analyzed with R software (32). For each curve, the minimal optical density (absorbance of the medium) was determined and subtracted from each of the values obtained. A small positive constant (0.001) was then added to each value, to ensure that they were all strictly positive (we found that the exact value of this constant had a negligible impact on the analysis, provided that it was much smaller than the optical density at the maximal growth rate). We then smoothed the time series with the smooth.spline function and calculated the first (growth rate) and second derivatives, with respect to time, of the data expressed logarithmically. All time points at which the second derivatives changed sign (i.e., time points at which the growth rate was at a local maximum or minimum) were identified. We considered those to correspond to the maximum growth rate (MGR). Smoothing was required to decrease significantly the contribution of measurement noise to the maximum growth rate. Using isogenic strain cultures, we found that well position had no effect on the estimated maximum growth rate and that its coefficient of variation was less than 5% (data not shown). MGR is expressed in h^{-1}, and the doubling time is expressed in minutes, being determined as 60/MGR.

Bacterial competition assays against the Venus strain. Strains were grown from a glycerol stock for 24 h in propylene plates with 96 deep wells (2 ml) in 500 μl of LB medium supplemented with 0.1 mM IPTG or in LB medium with DPY and IPTG with shaking at 250 rpm, as described above. IPTG, which binds to the *lac* repressor and inactivates it, was used to induce expression of the gene encoding the fluorescent fusion protein Tsr-Venus (with the *tsr-venus* gene replacing the native *lacZ* gene). The strains were diluted 1/500 with fresh medium for a second acclimation cycle. The same procedure was applied to the fluorescent Venus strain. After 24 h of culture, each strain was mixed with the fluorescent reference strain in a 1:1 ratio, and the mixture was diluted 1/500 with fresh medium and incubated for 24 h with shaking. The same mixture was also diluted 1/200 with sterile PBS twice and analyzed by cytometry. After a first cycle of competition for 24 h, overnight cultures were again diluted 1/500 with fresh medium and incubated for another cycle of 24 h. After each competition step, the content of each well was diluted with PBS for further analysis.

For each competition step, the fluorescence of ~10,000 cells was acquired in a Guava Easy Cyte Plus flow cytometer (Millipore) with the forward scatter (FSC) and green fluorescence channels and logarithmic filters. Event detection was based on a minimal FSC value adjusted to minimize the debris acquisition (which might otherwise be interpreted as nonfluorescent cells). Data were analyzed with the Bioconductor and flowCore packages of R software (32). For each competition step, we fitted a mixture of two inverse gamma distributions to the distribution of the logarithm of fluorescence, using the mix function of the R package mixdist (32). This made it possible to estimate the relative proportion of each cell type (fluorescent or nonfluorescent). We checked that this method gave the same result as estimations based on plating and colony counts (data not shown).

We estimated the mean growth rate of each strain relative to that of the fluorescent reference strain by dividing the number of cells of the focal strain by the number of cells of the fluorescent strain at each measurement step (step 0, initial ratio; step 1, after the first competition cycle; step 2, after the second competition cycle). We then analyzed the linear regression of the logarithm of this ratio against the step number. The estimated slope was equal to the difference in the mean growth rate between the focal and reference strains over 24 h. The mean relative growth rate per generation was obtained by dividing by the number of generations in one cycle [$\approx \log_2(500) \approx 9$)]. Strains with a positive or a negative mean relative growth rate per generation were described to be winner and loser strains, respectively. The experiments were repeated three times.

Inhibitory interactions due to bacteriocin and/or phage production. The production of bacteriocin and/or phage by IAI collection strains was detected by plaque lysis assays, with *E. coli* Venus used as the sensitive strain. Briefly, 10 μl of an overnight (O/N) culture in LB medium of each IAI strain was spotted onto LB agar medium containing mitomycin (25 μM) with or without DPY onto which an O/N culture of the Venus strain diluted to 0.5 McFarland unit had already been plated. After O/N culture at 37°C, the result of the assay was considered to be positive for strains surrounded by a halo, corresponding to inhibition of the growth of the sensitive strain. The inverse test was performed to assess the sensitivity of the IAI strains to lysis by the Venus strain, with each IAI strain used as the plated strain and Venus used as the spotted strain. When the Venus strain was lysed by IAI collection strains, PCR was carried out to check for the presence of the principal colicin genes (*colIa-colIb*, *colB*, *colE*) (33). Additional PCRs were also carried out to detect the principal microcin genes, *micV* and *micH47*, in *iroN*-positive strains (33).

Iodine staining. We initially evaluated RpoS activity by staining patches of bacteria on LB agar plates with iodine. Plates were incubated for 24 h at 37°C and then left at 4°C for 20 h before being flooded with Lugol's solution (I_2 concentration, 10 g · liter^{-1}). The iodine stained glycogen, the synthesis of which is positively regulated by RpoS, via the *glgS* (glycogen synthesis protein) gene (34). The intensity of the brown color depends on the amount of RpoS present (35). Stained patches were photographed with an EOS 500D digital reflex camera (Canon). The images obtained were processed with a computer, and a percent color intensity (relative intensity [RI]) was attributed to each spot, on the basis of correction with respect to two controls (a negative control corresponding to an *E. coli* K-12 MG1655 strain, which remained light yellow [0%], and a positive control, corresponding to an *E. coli* natural isolate known to express high levels of RpoS, which was stained dark brown [27] [100%]). The mean red intensity (I_{red}) and the mean green intensity (I_{green}) of the pixels within the patch were determined. Patch light intensity was defined as $I = \max(I_{red}, I_{green})$. The positive-control intensity ($I_{positive}$) and the negative-control intensity ($I_{negative}$) were calculated as described above. The RI of a patch was thus calculated as $(I - I_{positive})/(I_{negative} - I_{positive})$. Experiments were repeated twice.

H_2O_2 stress resistance. Strains were grown overnight in 10 ml of LB medium in 50-ml flasks at 37°C with shaking at 180 rpm. We then centrifuged 3 ml of culture to collect the cell pellet, which was washed in PBS. A suspension of the tested strain was prepared in PBS at an OD_{600} of 0.1. This suspension corresponded to time zero and was plated in duplicate at a dilution of 1 in 10^{-6} on LB agar plates to obtain an initial bacterial count. We then added H_2O_2 to a concentration of 5 mM and incubated the culture at 37°C with shaking for 60 min. Serial dilutions were then plated on LB agar plates, and the surviving bacteria were counted. Positive and negative controls identical to those used in the iodine staining assay were included. H_2O_2 resistance is expressed as a percentage, corresponding to the number of CFU at 60 min divided by that at time zero.

***rpoS* sequencing.** The complete coding sequence of the *rpoS* gene was amplified by PCR with flanking primers (primers *rpoS* forward ext [5′-ACAAGGGGAAATCCGTAAACC-3′] and *rpoS* reverse ext [5′-AGCCTCGCTTGAGACTGGCC-3′]) and was sequenced by the classical Sanger sequencing technique.

Statistical analysis. Results are presented as the median and range or the number and percentage of individuals. Associations between variables (MGRs in four media, competition in two media, iodine staining, H_2O_2 resistance, *rpoS* mutations categorized as wild type or nontruncating or truncating mutations, number of VFs, phylogenetic groups [A, B1, B2, D, and others], conditions of isolation, number of mice killed of the 10 inoculated) were assessed by determining Spearman's rank correlation coefficient (rho) and carrying out Wilcoxon or Kruskal-Wallis tests. We compared the presence/absence of microcin between strains that became winners in LB-DPY and strains for which no difference in competition phenotype was observed between the media with and without DPY by Wilcoxon's test.

Principal component analysis (PCA) was performed with the princomp function of the R statistical package (32). PCA is a mathematical procedure in which an orthogonal transformation is used to convert a data set of possibly correlated variables into a set of values for linearly uncorrelated variables called principal components. This transformation is defined in such a way that the first principal component accounts for the largest possible proportion of the variance, with each successive component in turn accounting for the highest possible proportion of the variance under the constraint that it is orthogonal to (i.e., uncorrelated with) the previous components. PCA generated a simultaneous display of 82 observations (the strains) and 10 variables (as described above, except for phylogenetic groups, the *rpoS* mutations, and the conditions of isolation, which are qualitative variables) on the same two-dimensional plane.

Uni- and multivariate analyses were performed on the determinants (conditions of isolation, phylogroups, MGRs, competition, iodine staining, H_2O_2 resistance, *rpoS* mutations, number of VFs) of the intrinsic virulence of the IAI collection of *E. coli* strains studied. Intrinsic virulence was assessed by determining the number of mice killed by each strain in a murine model of septicemia (7), with strains classified accordingly as killer or nonkiller strains (see above). Univariate analyses of associations between intrinsic virulence and determinants were performed with Fisher's exact tests for discrete variables and Wilcoxon tests for continuous variables. Determinants associated with intrinsic virulence and with P values of <0.20 in univariate analyses were entered in a multivariate adjusted logistic regression model with a backward selection procedure and a significance level of P equal to 0.05. Adjusted odd ratios (ORs) and 95% confidence intervals (CIs) were estimated. The discrimination obtained with the final multivariable model was assessed by use of the C statistic and its 95% CI, and the calibration was assessed with the Hosmer-Lemeshow goodness-of-fit test. All tests were two-tailed, and a significance level of 0.05 was used. All statistical analyses were carried out with R software (32).

RESULTS

Maximum growth rates of strains in various media. We first assessed the individual fitness of the strains in four different environments: three complex media—a reference rich medium (LB medium), LB medium with iron limitation due to the addition of 2-2′ dipyridyl (LB-DPY), and human urine—and a minimal medium containing gluconate. The aim was to test various media mimicking pathophysiological conditions. Iron is essential for bacterial growth, particularly during infection (36). Urine is a limiting, stressful medium (37); 53 of the 67 pathogenic strains of the collection were isolated from patients with urinary tract infections. Finally, gluconate has been identified as a major carbon source in the intestine (19). We used the assay that we developed to estimate the fitness of the strains from their MGRs in these various media. The MGR in a given environment varied considerably between strains (Fig. 1B; see Table S1 in the supplemental material), with this variability being the greatest for the gluconate medium. The MGRs of the strains were the highest in LB medium (median, 1.81 h^{-1}; range, 1.2 to 2.08 h^{-1}), followed by LB-DPY (1.73 h^{-1}; range, 1.17 to 1.9 h^{-1}) and urine (1.42 h^{-1}; range, 0.87 to 1.78 h^{-1}); the gluconate minimal medium gave the lowest MGRs (0.49 h^{-1}; range, 0.01 to 0.99 h^{-1}). MGRs in the three complex media were significantly correlated (for the correlation for LB medium and LB-DPY, rho = 0.47; for the correlation for LB medium and urine, rho = 0.42; for for the correlation LB-DPY and urine, rho = 0.74; $P < 0.0001$), but they were not correlated with the MGR in the minimal medium. Fitness thus depended on genotype-by-environment interactions.

We compared the MGRs of virulent and nonvirulent strains, i.e., mouse killer and nonmouse killer strains, respectively, in the mouse model of septicemia (7). The growth rate in urine

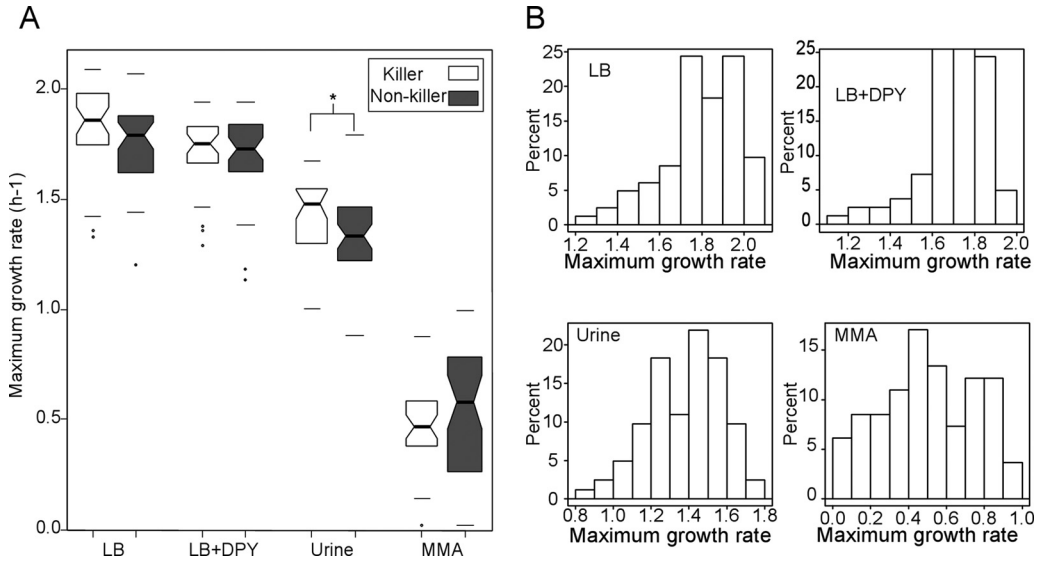

FIG 1 MGR of 82 *E. coli* strains in four different media. MGR is expressed in h^{-1}. The four media are LB broth, LB broth supplemented with 2-2′ dipyridyl (LB-DPY), urine, and minimal medium supplemented with gluconate (MMA). (A) Results are presented as box plots representing the distributions of MGRs calculated from three independent culture assays. The black bars within each box plot represent the median values. The upper and lower limits of the box correspond to the upper and lower quartiles, respectively. Bars above and below the box correspond to 1.5 times the interquartile range. Dots located at some distance outside the box correspond to outliers lying more than 1.5 times beyond the interquartile range. Strains were grouped into two categories (killer strains and nonkiller strains), on the basis of the number of mice killed (see Materials and Methods). *, significant difference between the two groups of strains (Wilcoxon test, $P < 0.05$). (B) Results are presented as histograms representing the distributions of MGRs calculated from three independent culture assays in the four media tested.

was significantly higher for the killer strains (median, 1.47 h^{-1}; range, 1 to 1.67 h^{-1}) than for the nonkiller strains (1.33 h^{-1}; range, 0.87 to 1.78 h^{-1}) ($P = 0.02$) (Fig. 1A). The MGR in urine did not differ significantly between the 53 strains originating from urinary tract infections and the other strains. The MGRs also did not differ between commensal and pathogenic strains (data not shown).

Competition assay and inhibitory interactions. We then assessed the fitness of the strains in the presence of a competitor of the same species. We developed an assay for this purpose to make it possible to make multiple flow cytometry measurements with an engineered fluorescent strain, Venus. *E. coli* strains in the intestine have to compete with the other *E. coli* strains present (38), and there is growing evidence that a single extraintestinal infection can be caused by several different strains of *E. coli* (27).

The mean relative growth rate per generation in competition differed strongly between strains in this assay, especially when DPY was added, with a few strains outcompeting Venus very strongly (median, 0.023 [range, −0.15 to +0.84] in LB medium; median, 0.08 [range, −0.13 to +0.94] in LB-DPY) (Fig. 2; see Table S1 in the supplemental material). In LB medium, 47 and 35 strains were classified as winners and losers, respectively, whereas in LB-DPY, 62 and 20 strains were classified as winners and losers, respectively. The addition of an iron chelator modified the shape of the distribution, generating a second peak, resulting in a switch from a unimodal to a bimodal distribution (Fig. 2B and C). The results obtained in these two media were correlated (rho = 0.5, $P < 0.0001$).

The result of the competition between two strains depends on the metabolic properties of the strains, which may confer a higher growth rate, and/or the inhibitory interactions between the strains. We tried to optimize our interpretation of the results of the competition assays by assessing interactions between strains due to phage and bacteriocin production. We did this by assessing (i) lysis of the Venus strain by the collection strain considered and (ii) lysis of the collection strain by the Venus strain (see Table S2 in the supplemental material). We also carried out PCR to detect the principal genes encoding bacteriocins (see Table S2 in the supplemental material). A factorial analysis of correspondence (see Fig. S1 in the supplemental material) showed that the results of our competition assay reflected a mixture of the metabolic properties of the strains and inhibitory interactions due to bacteriocins and/or phages. Furthermore, the six strains with the highest levels of fitness in competition in LB medium lysed the Venus strain, and five of these strains had at least one bacteriocin (see Tables S1 and S2 in the supplemental material).

The distribution of winner and loser strains was independent of mouse killer category, whatever the medium used (Fig. 2A). This distribution was also independent of commensal or pathogenic strain category (data not shown).

RpoS activity of collection strains. We investigated the activity of the general stress regulator RpoS, using a mixture of phenotypic and genotypic assays. This approach had the advantage that it took into account all levels of regulation of RpoS, from gene transcription to proteolysis (20). We first used a phenotypic assay which determined the amount of glycogen in the cell, indirectly reflecting RpoS activity, with the synthesis of glycogen being dependent on a gene, *glgS*, positively regulated by RpoS (34, 35). The amount of glycogen in the collection strains, as determined by iodine staining, was highly diverse, ranging from an absence of activity (0%), as in the Δ*rpoS* strain, to levels of activity higher than those of the positive control (133%) (Fig. 3A and C; see Table S1 in the supplemental material). We then assessed the stress resistance potential of the strains directly by exposing them to H_2O_2.

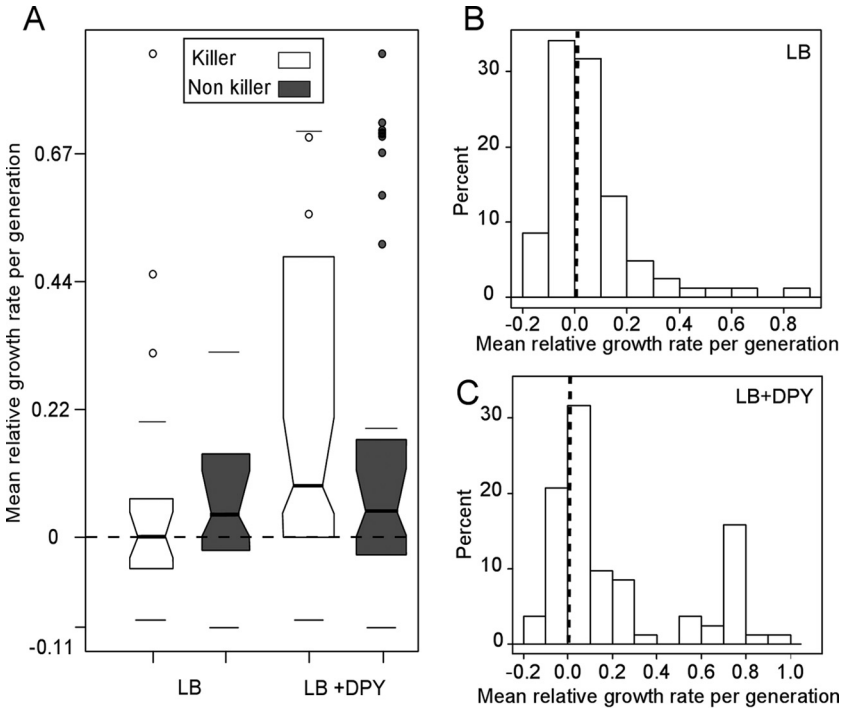

FIG 2 Relative growth rate of 82 *E. coli* strains in competition with the *E. coli* Venus strain. (A) The mean relative growth rate per generation of each strain of the IAI collection in competition with the fluorescent Venus strain is shown on the *y* axis. A 0 indicates the absence of a change in the proportion of the two competing strains after 48 h (black dotted line), with the winner and loser strains having positive and negative values, respectively. Box plots show the distribution of the median values obtained following the application of a linear model to the results of three independent competition assays. On the *x* axis, strains are classified as a function of the competition medium (LB medium and LB-DPY). The black bars within each box plot indicate the median values. The upper and lower limits of the box correspond to the upper and lower quartiles, respectively. Bars above and below the box indicate 1.5 times the interquartile range. Dots located at some distance outside the box correspond to outliers lying more than 1.5 times outside the interquartile range. Strains were grouped into two categories (killer strains and nonkiller strains), on the basis of the number of mice killed (see the Materials and Methods). The results are also presented as histograms representing the distributions of the mean relative growth rate per generation of the strains of the IAI collection in LB medium (B) and LB-DPY (C). Black dotted lines, absence of a change in the proportions of the two competing strains after 48 h.

The percentage of bacteria surviving this challenge also differed considerably between collection strains (Fig. 3B and E). A U-shaped distribution was observed, with many strains being either highly sensitive or resistant to H_2O_2. Finally, we sequenced the *rpoS* coding region. Comparisons with the K-12 *rpoS* sequence showed that 39 strains had a mutation, 15 of which were nontruncating (missense mutations and in-frame insertions/deletions); in the other 24 strains, the mutations (nonsense mutations and frameshift mutations) resulted in the production of a truncated protein (see Table S3 in the supplemental material). We found that 93% of the nontruncating mutations affected residues in functional domains (39). None of these mutations had displayed phylogenetic inertia (i.e., clustering within a phylogenetic group), and for two codons (F79 and R141), convergence was observed with different molecular defects at the same amino acid (see Table S3 in the supplemental material). All these data indicate that such mutations resulted from selection rather than phylogenetic drift. The results of the two phenotypic assays were correlated (rho = 0.35, $P = 0.005$), and significant differences were observed for both iodine staining (Fig. 3D) and H_2O_2 resistance (Fig. 3F) between strains with and without mutations. Thus, the results of our mixture of phenotypic and genotypic assays may be considered a good proxy for RpoS activity.

Winner strains in the competition assay had a significantly higher iodine staining intensity than loser strains in LB medium (median, 34% [range, 0 to 133.4%] versus 18% [range, 0 to 96.7%]). Winner strains also displayed significantly higher levels of H_2O_2 resistance than loser strains in LB-DPY (38% [range, 0 to 100%] versus 8% [range, 0 to 100%]). Moreover, strains with wild-type *rpoS* genes had significantly higher mean relative growth rates per generation in competition than strains with nontruncating or truncating mutations of the *rpoS* gene. No significant difference was observed between the killer and nonkiller strains in terms of iodine staining (Fig. 3A), H_2O_2 resistance (Fig. 3B), and mutations (data not shown). No significant difference was also observed between commensal and pathogenic strains (data not shown).

Determinants associated with the intrinsic virulence of the strains. We carried out PCA to obtain a global overview of the relationships between the different genotypes and phenotypes of the strains (Fig. 4). When considering the plane defined by the first component (23.88% of the variance) and the third component (17.07% of the variance), the virulence vector (number of mice killed by the septicemia caused by each strain) and the VF vector (number of VFs detected in each strain) were very similar. They were projected together onto the positive values of the first and third components, indicating their strong correlation. Such a correlation was also observed for the LB medium and LB-DPY vectors located on the positive values of the first component and the negative values of the third component and the competition, com-

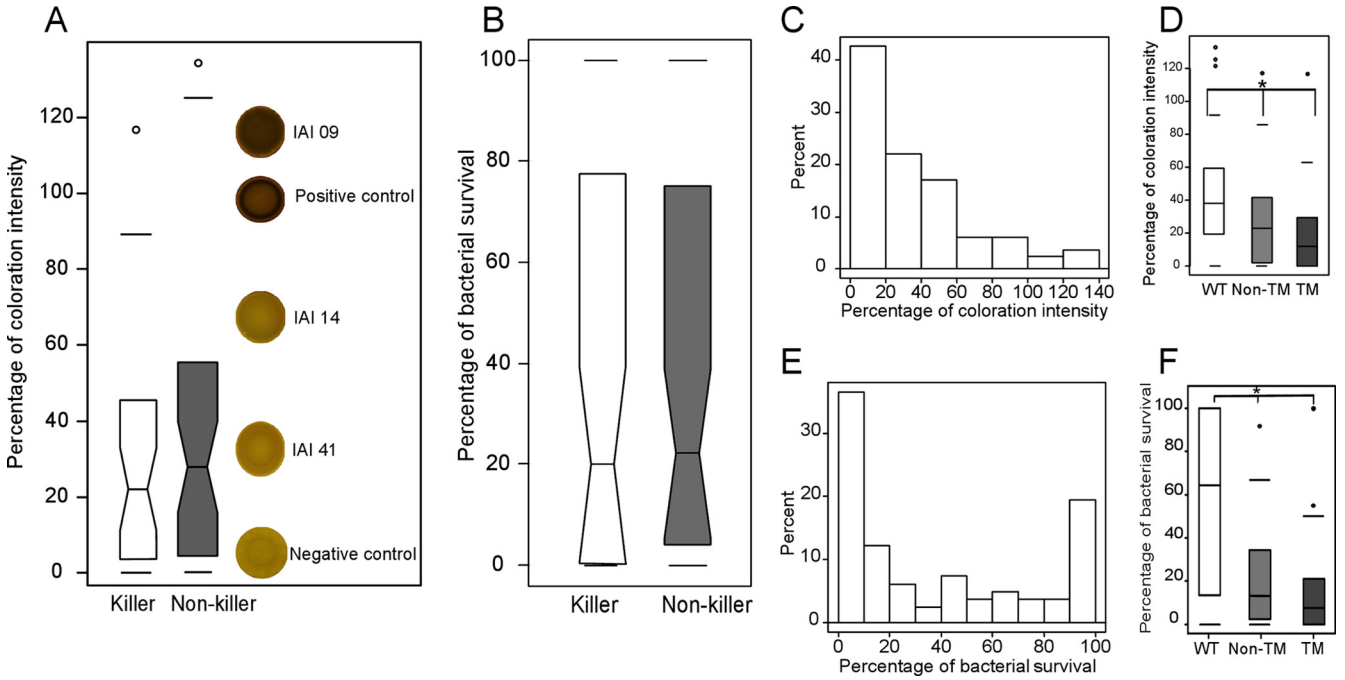

FIG 3 RpoS activity phenotypes (iodine staining and H_2O_2 resistance) of the 82 strains of *E. coli*. (A and C) Iodine staining of patches of bacteria from the IAI collection. (A) The *y* axis represents the percent staining of each bacterial patch with iodine with respect to the positive (100%; strain with high levels of RpoS activity [27]) and negative (0%; *E. coli* K-12 MG1655 Δ*rpoS*) controls. Box plots show the distribution of median values obtained in two independent experiments. The black bars within each box plot correspond to the median values. The upper and lower limits of the box correspond to the upper and lower quartiles, respectively. Bars above and below the box correspond to 1.5 times the interquartile range. Dots located at some distance from the box correspond to outliers lying beyond 1.5 times the interquartile range. Strains were grouped into two categories (killer strains in white and nonkiller strains in gray), on the basis of the number of mice killed (see the Materials and Methods). Patches for the various strains tested and for the two control strains stained with iodine are shown on the right of panel A. (C) The distribution of percent staining intensity is presented as a histogram. (B and E) Bacterial survival after incubation for 60 min with 5 mM H_2O_2. The results are presented in box plots (B), as described for panel A, whereas the distribution is given as a histogram (E). (D and F) Percent staining intensity (D) and bacterial survival after H_2O_2 challenge (F) presented as box plots according to the *rpoS* sequence. WT, Non-TM, and TM, wild-type *rpoS*, nontruncating mutations of *rpoS*, and truncating mutations of *rpoS*, respectively. *, significant differences between groups of strains (Wilcoxon test, $P < 0.05$).

petition-DPY, iodine staining, and H_2O_2 resistance vectors located on the positive values of the third component. The urine vector is located on the positive values of the first component, between the virulence/VF and LB-DPY/LB medium vectors. The MMA vector is poorly represented on this plane. The B2 pathogenic strains were mostly projected onto the positive values of the first component. These PCA results provide support for the previously observed link between the number of virulence genes and virulence in mice (7, 12). They also demonstrate the association between MGR in urine and virulence in mice (Fig. 1) and the association between the results of the competition assay and RpoS activity (iodine staining and H_2O_2 resistance).

Multivariate analysis identified the number of virulence factors as the only independent factor accounting for virulence in mice, with strains classified as mouse killer and nonkiller strains (OR = 1.95; 95% CI, 1.55 to 2.67; $P < 0.0001$) (see Table S4 in the supplemental material). For a given strain, each VF carried by the strain increases by a factor of 1.95 the risk of the strain being classified as a mouse killer strain. Discrimination was very good (C statistic = 0.97; 95% CI, 0.94 to 0.99) and calibration was good (Hosmer-Lemseshow goodness-of-fit test, $P = 0.98$) for this model. Similar results were obtained when the multivariate analysis was performed on the quantitative data of the mouse assay, i.e., the number of killed mice (data not shown).

DISCUSSION

One of the most interesting findings of this study was the high level of diversity observed among the strains with a continuum of fitness and stress response phenotypes. The MGRs obtained in complex and minimal media were not correlated, and we found no evidence of a correlation between MGR and the mean relative growth rate in competition in the same medium, even after the elimination from the analysis of the 49 IAI strains displaying phage/bacteriocin inhibitory interactions. Bacteria therefore make use of several strategies, rather than a single strategy, to optimize their fitness, depending on ecological interactions. Furthermore, no correlation between these phenotypes and the lifestyle of the strains has been found. More specifically, we found no correlation between the MGR of the strains in urine and their urinary origin or between the MGR in medium containing gluconate, a major carbon source in the intestine, and a commensal origin. Such intraspecies diversity of the growth rate in different media (40, 41) and of the RpoS level (42, 43) has been reported in other sets of *E. coli* strains, with no link to the commensal or pathogenic origin of the strains concerned being found. Studies of growth yield on 95 carbon sources in 168 *Escherichia* strains also demonstrated a high level of diversity that was not structured into groups but that was continuous, with the lifestyle of the strains having almost no impact on growth yield (44). Natural isolates of

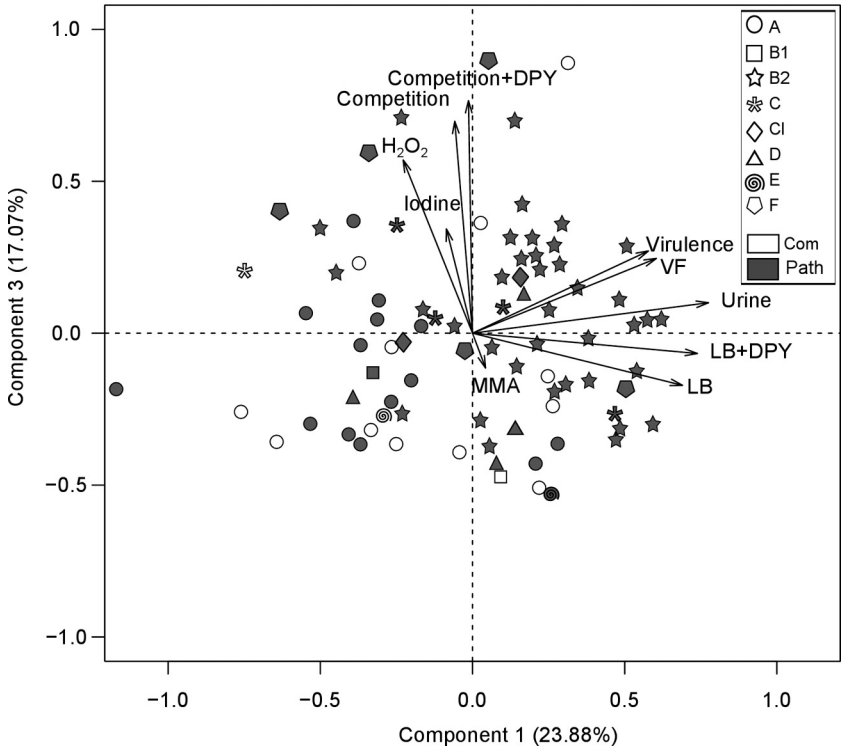

FIG 4 PCA for 82 *E. coli* strains, based on all the data. The first and third principal component scores of each variable are plotted against each other on the plane. Each arrow represents the projection of a variable subjected to PCA: VF, number of virulence genes detected in the strain; Virulence, number of mice killed per strain in the mouse model of septicemia; LB, LB-DPY, urine, and MMA, MGRs obtained for LB broth, LB broth supplemented with 2-2′-dipyridyl medium, urine, and minimal medium supplemented with gluconate, respectively; Competition and Competition-DPY, fitness in competition with the Venus strain in LB medium and LB-DPY, respectively; Iodine, percent staining intensity with iodine; H_2O_2, percent bacterial survival after 60 min of incubation with 5 mM H_2O_2. Only variables far from the center can be analyzed. If the variables are close to each other, they are significantly positively correlated; if they are orthogonal, they are not correlated; if they are on opposite sides of the center, they are significantly negatively correlated. The projections of the strains on the plane are represented with symbols (phylogenetic groups) and colors (commensal/pathogenic [Com/Path] isolation conditions).

E. coli have been shown to display various endogenous oxidative stresses when grown in urine, regardless of their origin or pathogenic potential (37). It remains unclear how there can be such a high level of heterogeneity of metabolic and stress responses within a single species with no apparent link to strain lifestyle. *E. coli* is a versatile species continually alternating between its primary (vertebrate gut) and secondary (water, soil, and sediment) habitats (45). It can also colonize new niches, such as urine, blood, and cerebrospinal fluid, during the extraintestinal infection process (14). Thus, strains undergo frequent and unpredictable ecological niche shifts. The high level of diversity observed may correspond to a bet-hedging strategy (46), with a continuum of phenotypes being rapidly generated in the bacterial population (47), enabling individuals to optimize their fitness in a variable and unpredictable environment (48). Species diversity can be maintained because there are many ecological strategies for ensuring survival, depending on the previous environments encountered.

Genes encoding proteins with metabolic functions confer a selective advantage on some pathogens (49), whereas the role of RpoS in virulence appears to be species or within species pathovar specific (21). The absence of a link between the pathogenic lifestyle and these metabolic and stress resistance phenotypes in *E. coli* species may result from (i) extraintestinal virulence being a by-product of commensalism (50) and (ii) strain pathogenicity depending on the host (3). The originality of our work is that it overcame the difficulty of assessing the pathogenic status of a given strain solely under the conditions of isolation by using a highly reliable mouse model of septicemia induced by subcutaneous injection of the bacterium, which characterizes the intrinsic extraintestinal virulence of the strain (7). Our findings suggest that neither the ability to grow in various media alone or in competition nor the general stress response is involved in the intrinsic virulence of the strains. The multivariate analysis confirmed the major role of virulence factors in this model (7, 9, 12, 51) and excluded the effect of any other phenotypes in the prediction of killer status. The MGR in complex media was higher in killer strains than in nonkiller strains (Fig. 1), but this difference was significant only for urine in univariate analysis and did not persist in the multivariate analysis (see Table S4 in the supplemental material).

In this work, we have developed a method for the simple and rapid analysis of growth curves reflecting the fitness of individual bacteria. Our approach is model and parameter free and is therefore not dependent on any particular assumption or prior knowledge. Many models (e.g., logistic, Gompertz, and Baranyi) (52) for the fitting of microbial growth curves have been proposed, but none can provide an accurate description of all growth curves. Moreover, the number of parameters required is specific to the model concerned. It is therefore difficult to compare strains best

described by such incompatible models. In addition, in all these parametric methods, it is essential to estimate the carrying capacity (or maximal yield or final optical density). We found this parameter to be highly biased, depending on the well position when growth was measured in microplates (data not shown). In addition, the lag time (i.e., the time required for the microbes to switch from the stationary to the exponential growth phase), which is generally estimated in these models, depends on the initial number of bacteria. Lag-time estimates are therefore highly variable, depending on the serial dilutions used and the initial intrinsic (i.e., strain- or medium-specific) differences in cell concentrations. In contrast, our method is not subject to any of these limitations. The maximal growth rate recorded was highly robust to initial conditions and well position, unlike that reported by Jasnos et al. (53), and it was also little affected by the exact form of the growth curve. The median values for maximum growth rate obtained for the collection strains studied correspond to median doubling times of 33, 34, 42, and 131 min in LB medium, LB-DPY, urine, and gluconate medium, respectively. These values are higher than those classically reported for *E. coli* (20 to 25 min in LB medium), due to the experimental conditions used, with cells cultured in small volumes with lower levels of oxygenation than those in flasks.

However, fitness is a complex trait that varies with environmental and competitive conditions. The maximum growth rate attained by a strain in a given medium is probably a good measure of its adaptation to this abiotic environment, but it does not convey the complexity of interactions and competition with other strains observed in nature under both commensal (38) and pathogenic (27) conditions. Competitive fitness is usually assessed in competition assays in which the studied strain is in competition with another strain. These assays are based on plating, incubation, and colony counting and are therefore time-consuming and fastidious, restricting their use to small numbers of strains. Several authors have developed flow cytometry-based methods that automatically count very large numbers of bacterial cells, saving time and increasing precision (54–57). Unlike classical competitive assays comparing wild-type and mutant strains that are otherwise isogenic, our method uses a fluorescently labeled reference *E. coli* K-12 strain which is placed in competition with all the studied strains. Hence, only the reference strain is genetically modified and the others are used without any further modification, in contrast to other methods (56, 57), which preclude the analysis of large numbers of strains and require extra care to check that no extra mutations are introduced when the fluorescent marker is added. Our approach made it possible to perform large-scale experiments in 96-well microplates. In our assay, fitness in competition is the result of the metabolic properties of the strains, optimization of resource use, and bacterial interactions, including inhibitory interactions due to phage and/or bacteriocin production (see Fig. S1 in the supplemental material).

In this study, most of the strains outcompeted the Venus competitor, with 57% winners in LB medium and 75% winners in LB-DPY. The distribution and magnitude of the positive competitive fitness values, particularly in LB-DPY (Fig. 2), raise questions about the fitness of the Venus strain used as the reference competitor. However, the Venus strain has good individual growth capacities, as its MGR in LB medium and in LB-DPY was equal to the median MGR of the strains of the IAI collection and its fluorescence expression had no fitness cost (data not shown). The greater competitive fitness of some strains under growth conditions with DPY (Fig. 2C; see Table S1 and Fig. S1 in the supplemental material) probably resulted from microcin induction under conditions of iron limitation (58). Thus, 15 of the 82 strains displayed an induction of Venus lysis under iron limitation conditions with DPY, and 7 of these strains were positive for at least one microcin gene (see Table S2 in the supplemental material). Strains that became winners in LB-DPY were found to be more likely to produce microcin than strains displaying no difference in competition category between media with and without DPY ($P < 0.004$).

We used a mixture of phenotypic (iodine staining of intracellular glycogen and H_2O_2 resistance) and genotypic (*rpoS* sequencing) assays to estimate RpoS activity, overcoming the problems of its assessment relating to the multiple levels at which regulation operates. The results of the three assays were correlated (Fig. 3D and F; see also Results), indicating that this approach provides a good proxy of RpoS activity. Strains with high levels of RpoS activity were better competitors, but RpoS activity was independent of MGRs in complex media (Fig. 4). This is probably due to the experimental conditions of the assays. Competition involves long periods in stationary phase, where RpoS is expressed, whereas the MGR corresponds to the exponential phase.

Our work has some potential limitations. The first one is the number of strains studied. We chose the strains for this study to be representative of the phylogenetic species diversity. The use of a larger number of strains might result in different conclusions. The second one is the storage history of our strains. Although the strains had suffered few subcultures (about 5) and were stored at −80°C, they had been passaged twice in stabs for short periods. It has recently been reported that storage in stabs selects for partial or complete loss-of-function mutations in *rpoS* (59, 60). The high level of *rpoS* mutations could be related to the storage conditions. However, we have retested more than 20 strains in the mouse model assay, some of them several times over the last 10 years, and obtained similar results (data not shown). The last limitation of this study is the mouse model of septicemia used, which does not perfectly reproduce the pathophysiology of the human disease, as it does not involve the classical entry portals in human disease: the urinary and digestive tracts. A clear role of the portal of entry in determining the severity of human septicemia has been demonstrated (3).

In conclusion, our work shows that a broad continuum of diversity in individual and competitive fitness and in the general stress response is observed within the *E. coli* species and that these features are not linked to pathogenic lifestyle. Our work also demonstrates for the first time that the fitness and stress responses of the strains do not correlate with virulence, as assessed in a mouse model of septicemia. These findings are not entirely intuitive and constitute a major step toward improvements in our understanding of pathogenicity.

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