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## SlyA Is a Transcriptional Regulator Involved in the Virulence of *Enterococcus faecalis*<sup>∇</sup>

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**Phylogenetic analysis of the crystal structure of the *Enterococcus faecalis* SlyA (EF\_3002) transcriptional factor places it between the SlyA and MarR regulator subfamilies. Proteins of these families are often involved in the regulation of genes important for bacterial virulence and stress response. To gather evidence for the role of this putative regulator in *E. faecalis* biology, we dissected the genetic organization of the *slyA*-EF\_3001 locus and constructed a *slyA* deletion mutant as well as complemented strains. Interestingly, compared to the wild-type parent, the  $\Delta$ *slyA* mutant is more virulent in an insect infection model (*Galleria mellonella*), exhibits increased persistence in mouse kidneys and liver, and survives better inside peritoneal macrophages. In order to identify a possible SlyA regulon, global microarray transcriptional analysis was performed. This study revealed that the *slyA*-EF\_3001 locus appears to be autoregulated and that 117 genes were differentially regulated in the  $\Delta$ *slyA* mutant. In the mutant strain, 111 were underexpressed and 6 overexpressed, indicating that SlyA functions mainly as an activator of transcription.**

*Enterococcus faecalis* is a natural member of the intestinal tracts of humans and animals. This ubiquitous Gram-positive bacterium is abundant in various ecological niches and is a highly versatile bacterium. *E. faecalis* can transit from a commensal relationship with the host to being causative agent of invasive infection, and it is now a leading cause of nosocomial infections in the United States and Europe (36). These infections include urinary tract infections, endocarditis, and surgical wound infection and are especially common in immunocompromised patients (14). Because of its innate and acquired resistance to many antibiotics, *E. faecalis* infections are increasingly difficult to treat (32). Therefore, the virulence of the organism is being examined for new insights into infection prevention and treatment.

A number of *E. faecalis* putative virulence factors have been identified, including a cytolytic toxin (Cyl), a cell surface adhesion termed aggregation substance (Agg), an antiphagocytic surface capsule (Cps), an enterococcal polysaccharide antigen (Epa), gelatinase (GelE), serine protease (SprE), collagen binding protein (Ace), enterococcal surface protein (Esp), pilus subunits (Ebp), and a general stress protein (Gls24) (12, 15, 20, 21, 29, 31, 33). Enterococcal virulence is subtle and complex, involving both pathogen and host factors, and all of these factors rarely occur in a single strain. In other organisms, virulence genes are controlled by environmental stresses and involve secondary RNA polymerase  $\sigma$  factors and specific transcriptional regulators. *E. faecalis* lacks a

$\sigma^B$ -like general stress  $\sigma$  factor, but about 10 regulators have been shown to be involved in virulence and stress response in *E. faecalis* (6, 13, 25).

In 2003, the crystal structure of an *E. faecalis* SlyA-like (EF\_3002) transcriptional factor (a member of the MarR/SlyA family) was determined by Wu and collaborators (37). SlyA was found to possess the same winged-helix DNA binding motif observed in the *Escherichia coli* MarR repressor and in the MexR repressor of *Pseudomonas aeruginosa*. Phylogenetic analysis placed it between the SlyA and MarR regulators based on sequence similarity to members of both subfamilies (37).

No functional studies have been conducted to define the role of this regulator. Proteins of the MarR/SlyA family regulate expression of virulence genes. Examples include the following: SlyA from *Salmonella enterica* serovar Typhimurium, which is involved in the modification of the bacterial surface that protects cells from the toxic compounds produced by the host; RovA from enteropathogenic *Yersinia enterocolitica*, which regulates *inv*, encoding a surface protein important for the invasion; PecS from the phytopathogen *Erwinia chrysanthemi*, which downregulates genes encoding pectate lyases involved in virulence; and Hor from *Erwinia carotovora*, AphaA from *Vibrio cholerae*, and MgrA from *Staphylococcus aureus*, which directly or indirectly regulate processes related to infection (3, 5, 7, 16, 17, 34).

In the present study, we report a functional analysis of the SlyA regulator (EF\_3002) of *E. faecalis*. Using different infection models, we found that SlyA plays a role in the virulence of *E. faecalis* as well as in its persistence inside the host. In addition, our global transcriptional analysis revealed that the lack of SlyA up- and downregulated more than 100 loci.

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant properties	Reference or source
<b>Strains</b>		
<i>E. faecalis</i>		
V19	Strain V583 (clinical isolate), without plasmid	38
V19 $\Delta$ slyA	V19 isogenic derivative slyA mutant	This study
V19 $\Delta$ slyA pCU1-slyA	V19 isogenic derivative slyA mutant with pCU1 carrying slyA	This study
<i>E. coli</i>		
XL1Blue	<i>recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac</i> [F' <i>proAB lacI<sup>q</sup> Z</i> $\Delta$ M15] Tn10 (Tet <sup>r</sup> )	Stratagene
DH5 $\alpha$	<i>recA endA1 gyrA96 thi-1 hsdR17</i> [ <i>r<sub>K</sub><sup>-</sup> m<sub>K</sub><sup>+</sup> supE44 relA1 lac</i> Z $\Delta$ M15]	15
<b>Plasmids</b>		
pMad	ori <sub>pE194</sub> <sup>ts</sup> , Em <sup>r</sup> , Amp <sup>r</sup> , <i>bgab</i>	1a
pMad- $\Delta$ slyA	pMad carrying slyA deletion	This study
pCU <sub>1</sub>	Cm <sup>r</sup> shuttle vector for <i>E. coli</i> and <i>E. faecalis</i>	2
pCU <sub>1</sub> -slyA	pCU1 carrying slyA	This study

**MATERIALS AND METHODS**

**Bacterial strains, plasmids, and growth conditions.** The parental *E. faecalis* strain used in this work was strain V19, a plasmid-cured derivative of the vancomycin-resistant clinical isolate V583 (38). *E. coli* XL1Blue was used as the recipient for cloning. *Lactococcus lactis* IL1403 and *E. coli* DH5 $\alpha$  served as controls for assays of *Galleria mellonella* infection and survival within macrophages. *E. faecalis* V19 and its derivatives were grown without shaking at 37°C in M17 medium supplemented with 0.5% glucose (GM17), and when required, erythromycin (150  $\mu$ g/ml) was added. *E. coli* strains were cultured with shaking at 37°C in LB medium with ampicillin (100  $\mu$ g/ml), chloramphenicol (20  $\mu$ g/ml), or erythromycin (150  $\mu$ g/ml) when required. *L. lactis* was cultured without shaking at 30°C in GM17. Plasmids used in this study are listed in Table 1.

**General molecular methods.** PCR was performed with GoTaq polymerase (Promega, Madison, WI). The primers used for this work are listed in Table 2. PCR products and plasmids were purified using the Nucleospin plasmid kit (Macherey-Nagel, Düren, Germany). Restriction endonucleases, alkaline phosphatase, and T4 DNA ligase were purchased from Amersham Biosciences (Piscataway, NJ), Promega, and Roche Applied Science (Indianapolis, IN), respectively, and used according to the manufacturers' instructions. Genomic DNA extraction and other standard techniques were carried out as described by Sambrook et al. (30).

Using RNA extracted from strain V19, the transcriptional start point of *slyA* was determined by the rapid amplification of cDNA ends (RACE) (Roche, Mannheim, Germany) according to the manufacturer's instructions.

For reverse transcriptase PCR (RT-PCR) experiments, two micrograms of RNA was reverse transcribed with random hexamer primers and QuantiTect enzyme (Qiagen, Valencia, CA) according to the manufacturer's recommenda-

tions. cDNA and genomic DNA (positive control) were then used as templates for PCRs using primers listed in Table 2.

**Construction and complementation of  $\Delta$ slyA.** For the construction of a *slyA* deletion mutant ( $\Delta$ slyA) strain, allelic replacement was used essentially as previously described (19). Seventy-nine percent of the *slyA* gene was deleted in the mutant strain, starting 42 bp after the GTG start codon and stopping 51 bp before the TAA stop codon. Briefly, a DNA fragment (obtained using chromosomal DNA of *E. faecalis* V19 as a template) containing ligated upstream (1,011-bp) and downstream (1,020-bp) sequences of the desired deletion were cloned into plasmid pMAD (Table 1), and 1  $\mu$ g of recombinant plasmid was used to transform competent cells. Single-crossover transformants (dark blue and erythromycin-resistant colonies) were used for temperature shifts in order to release the plasmid. Candidate clones resulting from a double-crossover event were isolated on GM17 agar with or without erythromycin. Antibiotic-susceptible clones were analyzed for the presence of a deleted *slyA* gene and verified by sequencing.

To complement the *slyA* gene in *trans*, a PCR fragment containing *slyA* and its promoter (which was inferred from the nucleotide sequence and verified by RACE-PCR) were cloned into the plasmid pCU1 (Cm<sup>r</sup>) (2). The resulting vector (pCU1-slyA) was then used to transform the  $\Delta$ slyA mutant. In order to compare the phenotype, of the complemented strain and the wild type, empty pCU1 was also introduced into strain V19 and the  $\Delta$ slyA mutant strain.

**Infection and survival experiments.** Infection of *G. mellonella* larvae with *E. faecalis* was accomplished as previously described by Lebreton et al. (20). Briefly, using a syringe pump (KD Scientific, Holliston, MA), larvae (about 0.3 g and 3 cm in length) were infected subcutaneously with washed *E. faecalis* from an overnight culture in GM17, with  $6 \times 10^6 \pm 0.6 \times 10^6$  CFU per larva adminis-

TABLE 2. Primers used in this study

Primer name <sup>a</sup>	Sequence (5'→3') <sup>b</sup>		Use
	Forward	Reverse	
EF3002 ( <i>slyA</i> )	GTGCGCGAAAATCAACACT	CATTTTTACGCATCCGGACTA	RT-PCR
EF3002R1		CCTTAAATTCAATATTACTG	5'RACE-PCR
EF3002R2		CTTTTCTTGGATAATCCCAGG	5'RACE-PCR
EF3002U	CCGGTGCAGATGAGTTTCTAGAGGAG TTCCTA	CGCGGATCCGGCCCGCGCAATCATGCCAAT TTCTC	Cloning in pMad, RT-PCR
EF3002D	CGCGGATCCGTCAGCGAAGATTGGGA ATTTGT	GAAGATCTCGATTATTTGTTCACTTTGAACG	Cloning in pMad, RT-PCR
EF3002int	GAGAAATTGGCATGATTGCGCGGGCC	TCTGTGGCATAAATCCGCTTA	RT-PCR
EF3001int	TAAATCGTGAGACGGCACAA	AAAGGAAAGAAGCTGGGGAC	RT-PCR
EF3002tot	GACGCTATAACTAATTTTATCTTAC	TTAATAATTTTCGAGTGTTC	Cloning in pCU1
PU/PR	GTA AACGACGGCCAGT	CAGGAAACAGCTATGAC	Cloning verification

<sup>a</sup> From the annotated sequence available at <http://www.tigr.org>. EF3002U and EF3002D are primers used to clone upstream (U) and downstream (D) sequences of *slyA* in order to construct the deletion mutant by a double-crossover, and EF3002tot is for the in *trans*-complemented strain.

<sup>b</sup> The underlined sequences are restriction enzyme recognition sites.

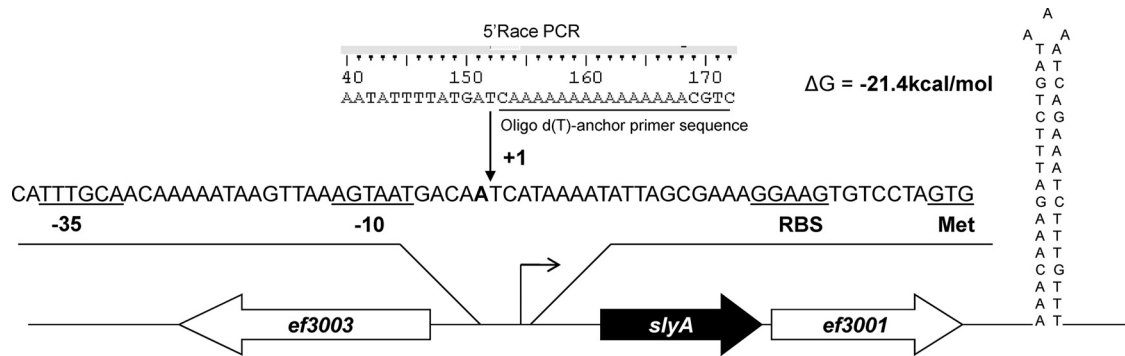


FIG. 1. Genetic organization of the *slyA* (EF\_3002) chromosomal region of *E. faecalis*. Large arrows represent the genes which compose the ORFs, and their orientation shows the transcriptional direction. The nucleotide sequence of the *slyA* promoter region is shown. The transcriptional initiation nucleotide (+1) identified by 5'RACE is in bold and marked by an arrow, and a putative ribosome binding site sequence, -10 and -35 motifs, and the transcriptional initiation codon are underlined.

tered in 10  $\mu$ l of sterile saline buffer. In each test, 30 insects were infected, and the experiments were repeated at least three times. *L. lactis* IL1403 was also tested under the same conditions as an avirulent control. Larval killing was then monitored at 20 h postinfection.

The virulences of V19 and the *slyA* deletion mutant were also assessed in an intravenous infection model, essentially as described by Gentry-Weeks et al. (11). Briefly, overnight cultures of the strains grown in brain heart infusion broth (BHI) supplemented with 40% heat-inactivated horse serum were centrifuged, and the resulting pellets were resuspended in sterile phosphate-buffered saline (PBS) to achieve final concentrations of  $1 \times 10^9$  bacteria/ml. Aliquots of 100  $\mu$ l from each strain were used to inject each of 10 female BALB/c mice (10 weeks old; Harlan Italy S.r.l., San Pietro al Natisone, Udine, Italy) through the tail vein. Infection experiments were repeated three times. Mice were monitored twice daily and were euthanized by CO<sub>2</sub> asphyxiation 7 days after infection. Kidneys and livers were then removed aseptically, weighed, and homogenized in 5 ml of PBS using a stomacher (model 80; Pbi International, Milan, Italy) for 120 s at high speed. For CFU determination, serial homogenate dilutions were plated onto *Enterococcus* selective agar (Fluka Analytical, Switzerland) to avoid environmental contamination, and plating efficiency was comparable to that with a nonselective medium. CFU counts were analyzed by the unpaired *t* test.

Survival of *E. faecalis* in mouse peritoneal macrophages was also tested as described previously (10). Briefly, the  $\Delta slyA$  mutant and V19 were grown as standing cultures at 37°C in BHI for 16 h. The bacteria were then pelleted and resuspended in 5 ml of PBS for injection. Mice were infected with  $10^7$  to  $10^8$  cells of each strain (estimated by CFU determination) by intraperitoneal injection of 200  $\mu$ l of the PBS-enterococcus suspension. After 6 h of infection, the peritoneal macrophages were collected by two peritoneal washes, each using 5 ml of PBS. The cell suspension was dispensed into 24-well tissue culture plates and incubated at 37°C under 5% CO<sub>2</sub> for 2 h. After exposure to antibiotics (vancomycin at 10  $\mu$ g/ml and gentamicin at 150  $\mu$ g/ml) to kill extracellular bacteria (verified by the absence of CFU in supernatant fluids), the infected macrophages were washed, and triplicate wells of macrophages were lysed with detergent to quantify viable intracellular bacteria. No difference in the MICs of these antibiotics was observed for the  $\Delta slyA$  mutant and wild-type strains. The procedure was repeated three times, and results were analyzed using one-way analysis of variance with a Bonferroni correction posttest with SPSS statistical software (SPSS, Chicago, IL).

All statistical analyses were performed using Prism software (version 5.00) for Windows (GraphPad Software, San Diego, CA). For all comparisons, a *P* value of less than 0.05 was considered significant.

**Sample preparation for microarray analysis.** *E. faecalis* V19 and  $\Delta slyA$  strains were cultured in GM17 to mid-exponential phase (optical density [OD] of 0.5). RNA was extracted using the RNeasy Midiprep kit (Qiagen). RNA samples were treated with DNase, and DNA elimination was verified by PCR amplification. Microarray hybridizations were performed three times with three independent RNA preparations. cDNA preparation, fragmentation, labeling, and hybridization were performed as described in the Affymetrix manual (1). After washing, hybridization was assessed at 570 nm with a resolution of 1.56  $\mu$ m using an Affymetrix GeneChip Scanner 3000 7G. Results were analyzed and compared using GeneChip Operating Software (GCOS) version 1.4. Correlation coeffi-

cients (*r* values) between experiments were from 0.934 to 0.99. RNA species that varied in abundance by greater than 2-fold were included in the analysis.

**Microarray data accession number.** Microarray data have been deposited at the EMBL-EBI database, and the accession number is E-MEXP-2903.

## RESULTS

**Genetic characterization of EF\_3002, encoding the SlyA regulator of *E. faecalis*.** Five potential transcriptional regulators have been annotated in the genome of *E. faecalis* V583 as being members of the MarR family, and they share moderate sequence identity at the amino acid level (49 to 54% identity). As noted by Wu and collaborators (37), EF\_3002 was the most homologous to SlyA-like proteins identified in other species of bacteria and archaea that are involved in virulence and/or stress response. Examination of the currently available sequences of 26 different genomes of *E. faecalis* shows that the *slyA* gene is present in each and is part of the core genome of *E. faecalis* (4, 27, 28).

The *slyA* open reading frame (ORF) appears to begin with a GTG initiator codon and encodes a protein of 150 amino acids with a predicted molecular mass of 17.4 kDa and a pI of 9.5. The start codon is preceded by seven nucleotides by a potential ribosome binding site sequence (RBS), GGAAG. Using 5' rapid amplification of cDNA ends (5'RACE), a transcriptional start point of the *slyA* gene was mapped (Fig. 1). We concluded that the A at 31 bp upstream of the GTG codon corresponds to the nucleotide +1 of the transcript. A putative -10 box (AGTAAT) located 5 nucleotides upstream from the transcriptional start point and a sequence near the -35 box consensus TTTGCA separated by 15 bp were identified. Furthermore, as shown in Fig. 1, the *slyA* locus (including EF\_3001) is followed by an inverted repeat ( $\Delta G = -21.4$  kcal/mol) that may function as a transcriptional terminator.

Based on comparison between *slyA* of *E. faecalis* and other homologous genes in Gram-positive genomes, as well as our RT-PCR results (see below), we suspected that *slyA* and downstream gene EF\_3001 (encoding a protein homologous to PaiA of *Bacillus subtilis*, which is an N<sup>1</sup>-acetyltransferase) were in an operon structure. To test this possibility, we performed RT-PCR using primers able to (i) amplify parts of *slyA* and

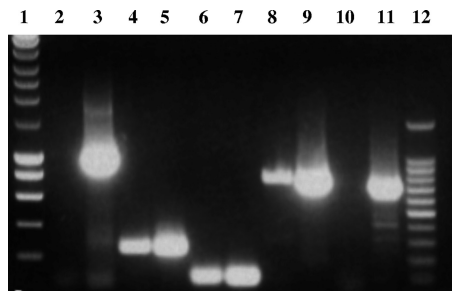


FIG. 2. Reverse transcriptase PCR (RT-PCR) analysis of *slyA*-EF\_3001 transcription. The primers used are listed in Table 2. PCRs were performed on chromosomal DNA (lanes 3, 5, 7, 9, and 11) or cDNA (lanes 2, 4, 6, 8, and 10) as a template. To demonstrate the absence of contaminating DNA in the cDNA preparation, sequences upstream of *slyA* (EF3002U primers, 1,011 bp) (lanes 2 and 3) and downstream of EF\_3001 (EF3001int5' and EF3002D3' primers, 728 bp) (lanes 10 and 11), which would not be predicted to be cotranscribed, were amplified. The presence of *slyA* and EF\_3001 transcripts was shown by amplifications of internal fragments of *slyA* (EF3002int primers, 249 bp) (lanes 4 and 5) and of EF\_3001 (EF3001int primers, 100 bp) (lanes 6 and 7). To determine whether transcription can proceed from *slyA* into EF\_3001, amplifications of part of *slyA*-EF\_3001 including the 15-bp intergenic region (EF3002int5' and EF3001int3' primers, 778 bp) (lanes 8 and 9) were performed. Lanes 1 and 12 correspond to the 10-kb and 100-bp DNA ladders, respectively.

EF\_3001 individually to demonstrate the presence of transcripts of each in the cell and (ii) amplify from *slyA* to EF\_3001, including the 15-bp intergenic region, to determine whether transcription can proceed from *slyA* into EF\_3001 as in an operon. Additionally, to demonstrate the absence of contaminating DNA in the preparation, sequences upstream of EF\_3002 and downstream of EF\_3001, which would not be predicted to be cotranscribed, were amplified as well (Fig. 2). As shown in Fig. 2, an amplicon could be generated across *slyA*-EF\_3001, indicating that readthrough does occur.

**SlyA is involved in virulence and bacterial persistence within mouse organs and peritoneal macrophages.** Based on the literature and despite the lack of phenotype for the  $\Delta$ *slyA* mutant toward stresses likely encountered during infection, such as H<sub>2</sub>O<sub>2</sub>, acidity, lysozyme, urine, or serum (data not shown), SlyA may nevertheless be involved in the virulence of *E. faecalis*. In order to verify this, we compared the abilities of the wild-type and the  $\Delta$ *slyA* mutant strains to kill *G. mellonella* larvae in infection experiments. As shown in Fig. 3, the rate of killing was significantly higher in larvae infected with the  $\Delta$ *slyA* mutant strain than in those infected with wild-type V19. After 20 h of infection, more than 26% of the V19-infected larvae were still alive, whereas fewer than 6% of the animals infected with  $\Delta$ *slyA* strain had survived ( $P = 0.001$ ). The nonpathogenic Gram-positive bacterium *L. lactis* IL1403 was used as a control; all of the larvae infected with this organism survived. Experiments conducted with the complemented  $\Delta$ *slyA* mutant strain, in which virulence was restored to the wild-type level, confirmed that the observed phenotype was due to the deletion of *slyA* (Fig. 3). In addition, experiments with the strains containing empty pCU1 were performed. Wild-type and mutant phenotypes were not modified by the presence of this vector (data not shown). *slyA* deletion mutants have been also created in two other backgrounds of *E. faecalis* (JH2-2 and OG1) and

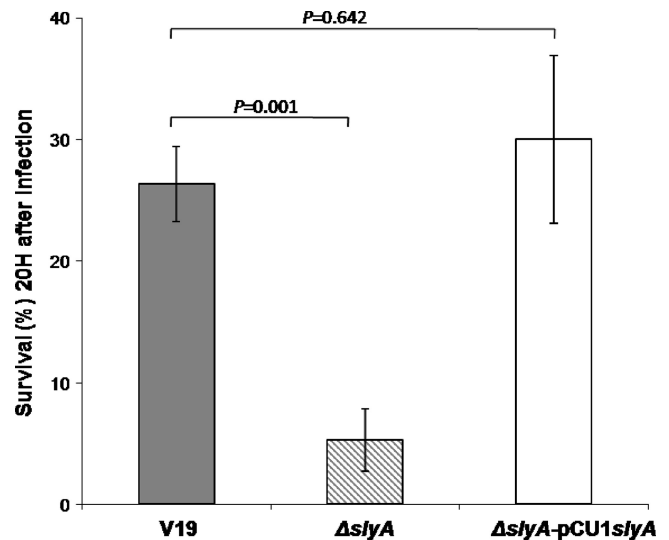


FIG. 3. Effect of *slyA* inactivation on virulence. Percent survival of *G. mellonella* larvae at 20 h after infection with around  $1.2 \times 10^5$  CFU of *E. faecalis* V19, the  $\Delta$ *slyA* mutant, and the complemented  $\Delta$ *slyA* mutant strain ( $\Delta$ *slyA* pCU1-*slyA*) is shown. Experiments were repeated at least three times, and the results represent the mean  $\pm$  standard deviation for live larvae.

their virulence tested using the *G. mellonella* model. In both cases, the mutants were 2- to 3-fold more virulent than the corresponding wild-type strains (data not shown).

We then examined the fates of the mutant and wild-type strains in the murine systemic infection model, specifically, tissue burdens in kidneys and livers of infected mice. As shown in Fig. 4, the  $\Delta$ *slyA* mutant strain showed statistically significant increases of tissue burden in both organs. The  $\Delta$ *slyA* mutant exhibited an increase of 1.6 log units ( $P < 0.0001$ ) in kidney and of 1 log unit ( $P = 0.0412$ ) in liver compared to the V19 parental strain, showing that SlyA is involved in the survival of *E. faecalis* within the host.

Phagocytic cells constitute an important part of the innate immunity against pathogens. To determine whether the  $\Delta$ *slyA* mutant was affected at this stage of infection, cells were tested in a macrophage infection model. The intracellular survival of the  $\Delta$ *slyA* mutant and wild-type strain V19 was monitored by determining the number of viable bacteria inside infected mouse peritoneal macrophages over a 72-h time course. No significant difference was observed between the cell counts at 8 h postinfection, suggesting that phagocytosis of the two strains by the macrophages was similar. However, as shown in Fig. 5, at 24 h ( $P < 0.007$ ), 48 h ( $P < 0.0019$ ), and 72 h ( $P < 0.0026$ ) postinfection, the  $\Delta$ *slyA* mutant was significantly more abundant intracellularly. At 72 h after infection, the survival rate of the  $\Delta$ *slyA* mutant in mouse macrophages was approximately 1 log unit higher than that of the wild-type strain (Fig. 5).

**Identification of the SlyA regulon in *E. faecalis*.** In order to identify candidate genes that contribute to increased persistence in organs and macrophages, microarray analysis was used to compare gene expression in wild-type and *slyA* mutant cells (22). Total RNA was isolated from cells harvested in mid-exponential growth phase. Genes whose expression differed by

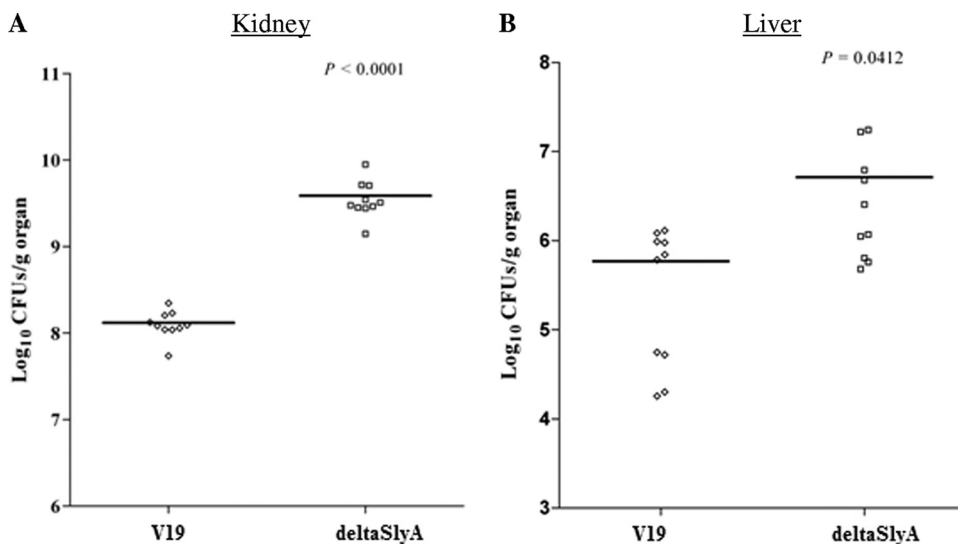


FIG. 4. Bacterial persistence within mouse organs. Enterococcal tissue burdens in kidneys and in livers from BALB/c mice infected intravenously with  $1 \times 10^8$  cells of *E. faecalis* wild-type V19 and the  $\Delta slyA$  mutant strain are shown. Kidney pair and liver homogenates were obtained from groups of 10 mice sacrificed and necropsied at day 7 postinfection. The results, expressed as  $\log_{10}$  CFU per gram of tissue, represent values recorded separately for each of the 10 mice. Horizontal bars represent the geometric means.  $P$  values of less than 0.05 were considered to be significant.

more than 2-fold were analyzed further and are listed as members of the SlyA regulon in Table 3. This study revealed that 117 genes were differentially regulated (with  $P$  values of  $<0.05$ ) in the  $\Delta slyA$  mutant. Most exhibited reduced expression in the  $\Delta slyA$  mutant strain (2- to 32.24-fold; 111 genes), with only 6 genes exhibiting increased transcript abundance in the mutant strain (2.04- to 16.62-fold). It is probable that genes with the most increased or decreased transcriptional deregulation have an important impact on the mutant cells. Among these, the transcript encoding EF\_3217, which is annotated as encoding a putative helicase, was 32-fold more abundant in the wild-type cells. Other gene products obviously expressed at lower levels in the absence of SlyA included transcriptional regulators of the ArgR family (EF\_0102-0103 operon, involved in arginine

metabolism) and energy and nucleotide metabolism enzymes (putative EF\_3137-3139 and EF\_1783-1786 operons, respectively). Expression of EF\_3001, the second member of the *slyA* operon, was more than 16-fold higher in the absence of SlyA. Two other clearly SlyA-repressed genes (overexpressed in the mutant) play roles in protein degradation (EF\_2997) and glycosis/glycogenesis reaction (putative EF\_0718-0719 operon) (Table 3). Major deregulation results were confirmed by quantitative RT-PCR (qRT-PCR), showing a decrease of 20-fold for EF\_3217 in the mutant strain while EF\_0718, EF\_2997, EF\_3203, and EF\_3001 expression levels in the  $\Delta slyA$  mutant were increased by 2.1-, 3.6-, 3.2-, and 124-fold, respectively (results not shown).

## DISCUSSION

Members of the MarR/SlyA transcriptional factor family are widely distributed among prokaryotes. Some of them modulate the transcription of virulence and/or stress genes. That is the case for MarR of *E. coli*, PecS of *E. chrysanthemi*, HprR of *B. subtilis*, RovA of *Y. enterocolitica* and *Y. pseudotuberculosis*, SlyA of *S. enterica* serovar Typhimurium, MarR of *Bacillus anthracis*, and MgrA of *S. aureus* (7). From these observations, it appeared that the physiological characterization of this protein would be of interest to increase our understanding about regulators involved in the virulence in this opportunistic pathogen.

We characterized the *slyA* promoter region, and our results showed that *slyA* belongs to a likely bicistronic operon with EF\_3001, encoding an  $N^1$ -acetyltransferase enzyme (8). Such an organization is conserved in *Bacillus*, *Listeria*, *Lactobacillus*, and *Clostridium* genomes, and a gene homologous to EF\_3001 is also present near a putative MarR-encoding gene in *Streptococcus gordonii*.

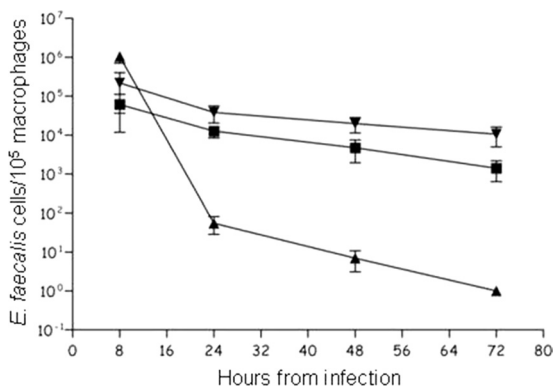


FIG. 5. Time course of intracellular survival of *E. faecalis* V19 (■) and the  $\Delta slyA$  mutant (▼) within murine peritoneal macrophages. Results for *E. coli* strain DH5 $\alpha$  (▲) are shown as a control of a nonvirulent strain in macrophages. The results represent the mean  $\pm$  standard deviation of viable intracellular bacteria per  $10^5$  macrophages from three independent experiments with three wells.

TABLE 3. Up- and downregulated genes in the  $\Delta$ slyA mutant

Category and locus	Gene product function	Fold change in expression
Protein synthesis EF_3203	Ribosomal protein L33	+2.04
Protein fate EF_2524 EF_2997	Sortase family protein Peptidase, M20M25M40 family	-2.55 +3.81
Phage EF_0309 EF_1455	Excisionase, putative Terminase, large subunit, putative	-2.27 -2.04
DNA metabolism EF_1435 EF_2546	Recombination protein U, putative Recombinase, phage integrase family	-2.15 -2.48
Cell envelope and cell division EF_2533	FtsK-SpoIIIE family protein	-2.24
Transport and binding protein EF_0758 EF_0893 EF_3137 EF_3138 EF_3139 EF_3208 EF_3209	Cadmium-translocating P-type ATPase Amino acid ABC transporter PTS system, IIB component PTS system, IID component PTS system, IIC component ABC transporter, permease protein ABC transporter, ATP binding protein	-2.16 -2.97 -2.17 -2.53 -3.06 -2.53 -2.18
Regulatory and DNA integration EF_0102 EF_0103 EF_0307 EF_0317 EF_0719 EF_1423 EF_1450 EF_2138 EF_2141 EF_2528	Transcriptional regulator, ArgR family Transcriptional regulator, ArgR family Transcriptional regulator, CroCI family Transcriptional regulator, CroCI family Transcriptional regulator, DeoR family Transcriptional regulator, CroCI family Positive control factor, putative Transcriptional regulator, CroCI family Transcriptional regulator, CroCI family Transcriptional regulator, CroCI family	-2.71 -3.49 -2.78 -2.16 +2.19 -2.25 -2.05 -2.53 -2.45 -2.35
Nucleotide metabolism EF_1783 EF_1784 EF_1785 EF_1786	Phosphoribosylformylglycinamide synthetase I Phosphoribosylformylglycinamide synthase, PurS protein Phosphoribosylaminoimidazole-succinocarboxamide synthase Phosphoribosylaminoimidazole carboxylase, ATPase subunit	-3.55 -3.76 -3.80 -2.39
Energy metabolism EF_0271 EF_0696 EF_0718 EF_1603	Glycosyl hydrolase, family 1 Tagatose 1,6-diphosphate aldolase 1-Phosphofructokinase Sucrose-6-phosphate dehydrogenase	-2.43 -2.03 +3.12 -2.07
Other metabolism EF_0312 EF_0759 EF_1434 EF_3001 EF_3217	Aspartate 1-decarboxylase domain protein SapB protein, putative DnaD domain protein Protease synthase and sporulation negative Helicase, putative	-2.54 -2.31 -2.06 +16.62 -32.24
Virulence EF_0149 EF_1426	Aggregation substance, putative VrII protein, putative	-2.10 -2.70
Unknown EF_0131/EF_1085/EF_1451 EF_0135/EF_0142/EF_0308/EF_0315/EF_0326/EF_0328/EF_0329/ EF_1081/EF_1456/EF_1459/EF_1466/EF_1467/EF_1470/EF_1471/ EF_1485/EF_1533/EF_2140/EF_2220/EF_2519/EF_2520/EF_2521/ EF_2529/EF_2534/EF_2536/EF_2951/EF_2996 EF_0132/EF_0134/EF_0310/EF_0311/EF_0313/EF_0314/EF_0316/ EF_0320/EF_0327/EF_1015/EF_1067/EF_1083/EF_1278/EF_1418/ EF_1424/EF_1425/EF_1427/EF_1428/EF_1429/EF_1431/EF_1439/ EF_1458/EF_1489/EF_1686/EF_1812/EF_1988/EF_2133/EF_2135/ EF_2136/EF_2139/EF_2523/EF_2526/EF_2530/EF_2531/EF_2532/ EF_2537/EF_2538/EF_2539/EF_2540/EF_2541/EF_2542/EF_2942/ EF_2944/EF_2945/EF_2949/EF_2950/EF_2952/EF_2953	Conserved domain protein Conserved hypothetical protein Hypothetical protein	-2.01 to 3.07 $\pm$ 2.00 to 2.83 -2.00 to 3.36

One important finding of this work is that *ΔslyA* mutants of three different strains were more virulent than the parental strains in the *G. mellonella* model. It has been shown that the *G. mellonella* immune response has a number of structural and functional similarities to the innate immune response of mammals, and this insect was shown to be an excellent infection model to evaluate the involvement of *E. faecalis* pathogenic factors (i.e., Fsr, GelE, and Ace) in virulence (9, 18, 20). Recently, it has been proved that group A *Streptococcus* virulence in these wax worms strongly correlates with data obtained in vertebrate models (mice and monkeys) (26). In addition, mutant strain bacterial counts recovered from macrophages and from liver and kidney after peritoneal and systemic infections were higher than those of the wild type. This phenotype does not seem to be due to a better tolerance to environmental stress, since growth was not increased in the presence of several stressing agents likely encountered during infection and was even somewhat more sensitive when bile salts were added to the medium (data not shown). Two *E. faecalis* mutants affected in the PerR and Err10 transcriptional regulators (a two-component system) also display better survival within macrophages than the parental wild-type strain (23, 35). In contrast to the *ΔslyA* mutant, a PerR-deficient strain of *E. faecalis* is more resistant to H<sub>2</sub>O<sub>2</sub>, and an *err10* mutant survived better with heat and bile salts challenges (23, 35). Nevertheless, we cannot exclude that SlyA may have a role in an as-yet-unidentified stress response encountered during host infection. These observations make SlyA a special case of this regulator family, since *marR/slyA* mutants of other studied bacteria are less virulent or have no role in infection (3, 5, 7, 16, 24, 34). Despite common folding and similar DNA binding properties of members of the MarR/SlyA family, structural analysis showed that local divergence in sequence allows them to bind to different gene targets (37).

It may be suggested that SlyA could be a repressor for the expression of virulence factors in *E. faecalis*. It is then tempting to speculate that among genes overexpressed in the *ΔslyA* mutant, one or more could play a role in the more virulent phenotype observed for the mutant. In this hypothesis, EF\_3001 may be an interesting candidate. It is the most induced gene in the *ΔslyA* mutant and encodes a protein similar to *B. subtilis* PaiA, which is an N<sup>1</sup>-spermidine/spermine acetyltransferase (52% identical and 71% similar) (8). This enzyme is involved in polyamine homeostasis, preventing toxicity due to an excess of this molecule. Overexpression of EF\_3001 should then be an advantage for the *ΔslyA* mutant during infection. In order to test this hypothesis, we tried to construct an EF\_3001 mutant strain using a double-crossover strategy with a thermosensitive replication plasmid. Unfortunately, all antibiotic-sensitive clones obtained after the second recombination harbored the wild-type gene. These results strongly suggest that EF\_3001 may be essential for *E. faecalis*. In addition, the more modest but significant overexpression of the putative fructose phosphotransferase system (PTS) operon EF\_0718-0719, encoding phosphofructokinase and a transcriptional regulator of the DeoR family, should also participate in the better survival of the *ΔslyA* mutant inside the host compared to the V19 parental strain. Transcription of EF\_3217, encoding a putative helicase, is strongly downregulated in the *ΔslyA* mutant. However, it is unlikely that this reduced expression has an impact in the more

virulent phenotype of the mutant, because EF\_3217 seems to be part of a mobile element and is present in only 5 of the 26 sequenced genomes (4, 27, 28).

It is of note that 11 loci encoding transcriptional regulators were deregulated in the mutant strain, and it is probable that some members of the SlyA regulon are under several transcriptional controls. Such a regulatory cascade has been shown for *S. aureus* and *Salmonella* (17, 24).

In conclusion, in this work we have characterized SlyA of *E. faecalis*, which is a transcriptional regulator involved in persistence in host cells as well as in virulence. Identification of the SlyA regulon by a global transcriptomic approach is a first step in the identification of new effectors of pathogenicity.

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