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Significance of the D-Serine-Deaminase and D-Serine Metabolism of *Staphylococcus saprophyticus* for Virulence

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Staphylococcus saprophyticus is the only species of *Staphylococcus* that is typically uropathogenic and possesses a gene coding for a D-serine-deaminase (DsdA). As D-serine is prevalent in urine and toxic or bacteriostatic to many bacteria, it is not surprising that the D-serine-deaminase gene is found in the genome of uropathogens. It has been suggested that D-serine-deaminase or the ability to respond to or to metabolize D-serine is important for virulence. For uropathogenic *Escherichia coli* (UPEC), a high intracellular D-serine concentration affects expression of virulence factors. *S. saprophyticus* is able to grow in the presence of high D-serine concentrations; however, its D-serine metabolism has not been described. The activity of the D-serine-deaminase was verified by analyzing the formation of pyruvate from D-serine in different strains with and without D-serine-deaminase. Cocultivation experiments were performed to show that D-serine-deaminase confers a growth advantage to *S. saprophyticus* in the presence of D-serine. Furthermore, *in vivo* coinfection experiments showed a disadvantage for the Δ*dsdA* mutant during urinary tract infection. Expression analysis of known virulence factors by reverse transcription-quantitative PCR (RT-qPCR) showed that the surface-associated lipase Ssp is upregulated in the presence of D-serine. In addition, we show that *S. saprophyticus* is able to use D-serine as the sole carbon source, but interestingly, D-serine had a negative effect on growth when glucose was also present. Taken together, D-serine metabolism is associated with virulence in *S. saprophyticus,* as at least one known virulence factor is upregulated in the presence of D-serine and a Δ*dsdA* mutant was attenuated in virulence murine model of urinary tract infection.

U rinary tract infections (UTIs) are common and affect mainly women; it has been estimated that more than 50% of all women will contract a urinary tract infection at least once in their lifetimes (1, 2). Although many bacterial species may cause urinary tract infections in patients predisposed by anatomical or functional abnormalities, indwelling catheters or neurological disorders, only a subset of species/strains may cause urinary tract infections in patients without predisposing conditions (3). These organisms/strains have mostly been found to express virulence factors allowing them to adhere to the uroepithelium (4–6), to invade cells of the urinary tract (7, 8), or to degrade urea (9, 10), a component highly prevalent in urine.

In staphylococci, most species do not cause urinary tract infections in the absence of predisposing factors, and *Staphylococcus saprophyticus* is the only species that typically causes these infections (11).

We have described a number of virulence factors, such as urease (12, 13), the adhesins Aas (14) and SdrI (15, 16), and the lipase Ssp (17, 18). A role for Ssp and SdrI in virulence was described in a murine model of UTI (19). Since similar proteins are present in many other staphylococci (20, 21), a factor distinguishing *S. saprophyticus* from other staphylococci has been sought. Interestingly, of all sequenced staphylococcal genomes, only that of *S. saprophyticus* possesses a gene coding for D-serine-deaminase (22), an enzyme that catabolizes D-serine to pyruvate and ammonia. This enzyme is also present in uropathogenic *Escherichia coli* (UPEC) but not in enterohemorrhagic *E. coli* (23). The amino acid D-serine is relatively prevalent in urine (3 to 115 µg/ml) (24, 25) and is toxic or bacteriostatic to many bacteria, probably by inhibiting the synthesis of pantothenic acid (26–29). Therefore, the presence of D-serine may be used as a cue for the presence within the urinary tract of uropathogenic microorganisms. Strains of *E.*

coli expressing the deaminase DsdA can utilize this amino acid as a carbon and energy source (30). The operon *dsdCXA* comprises the regulator gene, *dsdC*, a specific transporter gene, *dsdX*, and the deaminase gene, *dsdA* (31, 32). In *S. saprophyticus*, such an operon does not exist. The *dsdA* gene apparently is under the control of its own promoter, and genes in the vicinity are not related to serine catabolism. Three genes in the genome are annotated as coding for D-serine/D-alanine/glycine transporters but are not located next to the *dsdA* gene. In UPEC, mutation of the deaminase somewhat surprisingly led to enhanced colonization of bladders and kidneys in experimentally infected mice and caused the mutants to be hyperflagellated and more motile (23). However, only wild-type (WT) UPEC strains possessing *dsdA* were able to grow on minimal medium containing D-serine as the sole carbon and energy source (23, 30). Later, it was shown that the high intracellular concentration of D-serine caused expression of the hypercolonization phenotype (23, 47). Regulation seems to involve the amino acid, since mutants defective in D-serine uptake showed wild-type coloniza-

TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Species or vector	Size (kb)	Description[a]	Reference or source
Strains				
DH5α	E. coli		F⁻ φ80 d$lacZ\Delta$M15 Δ(lacZA-argF)U169 deoR recA1 endA1 hsdR17(r_K^- m_K^-) phoA supE44 λ⁻ thi-1 gyrA96 relA1; cloning host	36
M15(pREP4)	E. coli		nalS strS rifS lac ara gal mtl F⁻ recA⁺ uvr⁺ (pREP4 lacI^q Kan^r)	Qiagen
TM300	S. carnosus		Cloning host	40
7108	S. saprophyticus		Wild-type isolate, fibronectin binding	33–35
TM300(pMB1406)	S. carnosus		Cm^r, contains pMB1406, expresses enzyme DsdA	This study
7108 ΔdsdA	S. saprophyticus		dsdA::erm(B) isogenic knockout mutant of 7108	This study
7108 ΔdsdA(pMB1406)	S. saprophyticus		Complemented dsdA knockout mutant of 7108 containing pBM1406	This study
7108(pMB2200)	S. saprophyticus		Tet^r	41
Plasmids				
pBT2		6.97	Staphylococcal shuttle vector, temp-sensitive replicon of pE194, Cm^r of pC194, Ap^r of pUC18	38
pPS44		4.38	Contains replicon and cat gene of pC194	
pUC18		2.69	Ap^r	
pEC1	pUC18	4.14	Em^r Ap^r donor of erm(B) cassette containing XbaI and PstI sites, tetracycline resistance plasmid from a clinical isolate of S. saprophyticus	38
pMB1400	pUC18	5.69	A 3-kb fragment containing dsdA gene with own promoter	This study
pMB1401	pUC18	3.39	Contains 0.7-kb fragment upstream of dsdA gene	This study
pMB1402	pUC18	4.84	Contains insert from pMB1401 ligated with a 1.45-kb erm(B) cassette	This study
pMB1403	pUC18	5.44	Containing insert from pMB1402 ligated with a 0.6-kb fragment downstream of dsdA gene	This study
pMB1404	pBT2	9.72	Contains insert (2.75 kb) of pMB1403	This study
pMB1406	pPS44	3	Contains dsdA gene with its own promoter	39

[a] Cm^r, chloramphenicol resistance; Tet^r, tetracycline resistance; Ap^r, ampicillin resistance; Em^r, erythromycin resistance.

tion capabilities, and mutants not expressing the regulator dsdC showed the same phenotype as dsdA mutants (47).

Here we describe that the D-serine-deaminase of S. saprophyticus contributes to virulence within the urinary tract and that, in contrast to E. coli, the ΔdsdA mutant is attenuated in virulence.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1. S. saprophyticus strain 7108, a hemagglutinating, fibronectin- and collagen-binding clinical isolate has been described previously (33–35). E. coli DH5α (36) was the host for expression experiments and the intermediate host during construction of the plasmids for allelic replacement. The shuttle plasmid pBT2 (37) contains the temperature-sensitive replicon of pE194, the chloramphenicol resistance of pC194 and the multiple cloning site of pUC18. Plasmid pEC1 (38) was used as the erm(B) source. The pPS44 vector (39) was used for cloning experiments involving Staphylococcus carnosus strain TM300 (40) and for complementation experiments. The plasmid pMB2200, conferring tetracycline resistance, had been isolated from a clinical isolate of S. saprophyticus (41).

Bacterial growth media and antibiotics. E. coli strains harboring plasmids were grown in lysogeny broth (LB) or on LB agar plates. S. saprophyticus strain 7108 was grown in peptone-yeast extract (PY) broth or tryptic soy broth (TSB) (Oxoid, Wesel, Germany) or on agar plates. Bacteria were usually incubated at 37°C, but in some experiments, temperatures of 30°C and 42°C were also used. Ampicillin (100 μg/ml) was used for the selection of plasmids in E. coli. For selection of plasmids or chromosomal markers in S. saprophyticus, 10 μg/ml chloramphenicol, 5 μg/ml erythromycin, or 10 μg/ml tetracycline was used.

DNA preparation. Plasmid DNA was prepared using the Qiagen plasmid Midi kit (Qiagen Hilden, Germany) and genomic DNA with the Qiagen DNeasy kit (Qiagen, Hilden, Germany). For DNA preparations from staphylococci, pelleted bacteria were resuspended in lysis buffer (20 mM Tris [pH 8.0], 2 mM EDTA, 1.2% [vol/vol] Triton X-100). To lyse the bacteria, we added 100 μg/ml Amibicin L (Wak Chemie Medici GmbH, Steinbach, Germany) to the lysis buffer.

Construction of an insertion mutant by allelic replacement. The dsdA gene was deleted by the insertion of the erm(B) resistance gene, and pBT2 (37) was used as the replacement vector. To this end, a 700-bp PCR

amplificate of the chromosomal DNA upstream of the dsdA gene generated with the primers Ssa.dsd1seq/BamHI and Ssa.dsd2/2rev/XbaI (Table 2) was digested with BamHI and XbaI and was cloned into pUC18. This plasmid was designated pMB1401. For the construction of pMB1402, we inserted the erm(B) cassette cut from pEC1 into plasmid pMB1401 digested with the enzymes XbaI and PstI. A further 600-bp DNA amplificate downstream of the dsdA gene was generated with the primers Ssa.dsd1rev/HindIII and Ssa.dsd2seq/PstI and was ligated into pMB1402 cut with these enzymes. The resultant plasmid (pMB1403) contained the upstream and downstream flanking regions of the dsdA gene with an erm(B) cassette in the center. The insert of pMB1403 was excised with BamHI and HindIII and ligated into the temperature-sensitive replacement vector pBT2, yielding pMB1404.

The pMB1404 constructs were purified from E. coli DH5α and transformed into S. saprophyticus strain 7108 by protoplast transformation, as previously described (12). Chloramphenicol- and erythromycin-resistant clones were grown in the presence of erythromycin (5 μg/ml at 30°C for 24 h) and used to inoculate 1,000 ml of prewarmed (42°C) broth containing erythromycin. After overnight incubation, appropriate dilutions were plated onto P-agar containing erythromycin. Clones that grew on erythromycin but not on chloramphenicol had lost the plasmid, and correct insertion of the erm(B) cassette was checked by PCR and sequencing. Absence of the cat gene was verified by PCR.

Complementation of the ΔdsdA mutant. The vector part of pPS44 (39) was amplified by inverse PCR with the primers pPS44BamHI and pPS44HindIII. The dsdA gene with its own promoter was amplified with the primers Ssa.dsdA4seq and Ssa.dsdA4rev, and both were digested with BamHI and HindIII. Ligation and transformation of S. carnosus TM300 (40) were performed as described elsewhere (42). The resulting plasmid (pMB1406) was purified from this strain and introduced into the ΔdsdA mutant by protoplast transformation (12).

Animal experiments. Eight-week-old female C3H/HeN mice (Harlan) were infected by transurethral catheterization as previously described (43). Static bacterial cultures were started from freezer stocks, grown at 37°C for 18 h in brain heart infusion (BHI) broth and then subcultured at 1:250 or 1:100 into fresh medium. These subcultures were then grown statically at 37°C for 18 h, pelleted, and resuspended in phosphate-buffered saline (PBS), to yield 50-μl inocula containing 1×10^7 to 2×10^7 CFU. For competition experiments, bacteria were diluted appropriately

TABLE 2 Cloning and PCR primers used in this study

Primer	Sequence (5′→3′)[a]	Use (bp location)[b]
Ssa.dsd1seq/BamHI	CACGGATCCGAAATTGCTGAAGATGCG	Cloning
Ssa.dsd1rev/HindIII	GTGAAGCTTTGTACGATGATCGTAAGG	Cloning
Ssa.dsd2seq/PstI	CAGCTGCAGTTTACTTTAGTTCGGTAGAG	Cloning
Ssa.dsd2/2rev/XbaI	GTGTCTAGAGTTCCTGACTCTTTGTGG	Cloning
Ssa.dsd4rev/HindIII	GCGAAGCTTCTATAAGCAAGATTTACC	Cloning
Ssa.dsd4seq/BamHI	GTAGGATCCAACGATTTAGCAACACTT	Cloning
Ssa.dsd10rev	TCACCTCGCTATATCTGG	PCR
Ssa.dsd10seq	TTTCTTCCACTGACGTGC	PCR
catseq	GTTACAGTAATATTGACT	PCR, DIG probe synthesis (183–201)
catrev	CATAAACAATCCTGCATG	PCR, DIG probe synthesis (872–890)
ermB	TCTAGAACTAGTGGATCCC	PCR, DIG probe synthesis
ermB	TATTGTCTGCAGccgagagtgattggtctt	PCR, DIG probe synthesis
CAT473seq	CAGCAAACTACGTATAGC	PCR, DIG probe synthesis
CAT473rev	CAAGGAATCATTGAAATCG	PCR, DIG probe synthesis
pPS44rev/SstI	ATAGAGCTGGTGACCACTTGTGATAACG	PCR, cloning
pPS44seq/XbaI	TGCTCTAGAACTCGTGTGCATAATTCACGC	PCR, cloning

[a] Restriction sites in the 5′ extensions of primers are underlined. Lowercase letters indicate nonhomologous sequences with added restriction sites.
[b] DIG, digoxigenin.

to achieve 10^7 CFU each of the wild type and *dsdA* mutant together in a total volume of 50 µl. To quantify bacteria present in mouse organs, bladder and kidneys were aseptically harvested at the indicated times postinfection, homogenized in phosphate-buffered saline, serially diluted, and plated onto BHI agar plates for total CFU enumeration and with erythromycin for mutant CFU (CFU_m) enumeration. Wild-type CFU (CFU_w) were calculated as total CFU − mutant CFU. In samples where zero CFU were recovered, the limit of detection of the protocol was used. Competition indices (CI) were calculated similarly to the method described by Freter et al. (44) by using the wild type as the reference strain, as follows: CI = (CFU_m/CFU_w recovered from mice)/(CFU_m/CFU_w present in the initial inoculum).

Significance was calculated using the nonparametric Wilcoxon signed-rank test (two tailed) comparing the theoretical median CI to 1 (GraphPad Prism 5). All animal studies were performed in accordance with the Committee for Animal Studies at Washington University School of Medicine.

Cocultivation experiments. Cocultivation experiments were carried out in lysogeny broth (LB) (Invitrogen, Karlsruhe, Germany) supplemented with 2.5, 5, or 10 mg/ml D-serine (AppliChem, Darmstadt, Germany) or L-serine (AppliChem) or without any supplements. Bacteria were precultured overnight in 10 ml medium at 37°C with a flask-to-medium ratio of 100:10 and with shaking at 100 rpm. Cells from overnight cultures were then resuspended in 0.9% NaCl, and the optical density at 600 nm (OD_{600}) was adjusted to 0.5. For inoculation of the coculture, 100 µl of each strain was added to 10 ml of medium. Cultures were then incubated for 24 h at 37°C and 100 rpm. Every 24 h, the mixed populations were diluted 1:100 into fresh medium. Growth was checked by determining CFU every 48 h by serial dilutions (10^{-3} to 10^{-9} in 0.9% NaCl). Each dilution was plated onto three LB agar plates (LB agar powder; AppliChem): one without and two with different antibiotics for differentiation of the strains. Erythromycin (10 µg/ml; Sigma-Aldrich) was used for selection of the mutant, chloramphenicol (20 µg/ml; AppliChem) for selection of the complemented mutant, and tetracycline (10 µg/ml; AppliChem) for positive selection of the wild type, which had been transformed with a tetracycline resistance plasmid (pMB2200) isolated from a clinical isolate of *S. saprophyticus*. Agar plates were incubated overnight at 37°C, and bacterial counts were determined.

Chemically defined medium. Chemically defined medium contained the following (per liter of water): 7 g $K_2HPO_4 \cdot 3H_2O$, 2 g KH_2PO_4, 0.4 g Na_3 citrate $\cdot 2H_2O$, 0.05 g $MgSO_4$, 1 g $(NH_4)_2SO_4$, 20 mg thymine, 0.5 g sodium thiosulfate, 50 mg L-arginine, 100 mg L-glutamine, 50 mg L-glycine, 20 mg L-histidine, 90 mg L-leucine, 50 mg L-lysine, 3 mg L-methio-

nine, 40 mg L-phenylalanine, 80 mg L-proline, 30 mg L-threonine, 10 mg L-tryptophan, 80 mg L-valine, 5 mg $FeSO_4 \cdot 7H_2O$, 10 mg $CaCl_2$, 7 mg $ZnCl_2$, 13 mg $NiSO_4 \cdot 6H_2O$, 10 mg $MnCl_2 \cdot 4H_2O$, 0.5 mg thiamine hydrochloride, 0.6 mg nicotinic acid, 0.125 mg D-pantothenic acid calcium salt, and 0.125 mg biotin, adjusted to pH 7.0. Before use, the medium was filter sterilized. To exclude contaminating constituents from residues in reusable glassware, only new plastic materials were used for preparation and growth. Also, bacterial cultures were grown in plastic tubes (10-ml culture in a 50-ml Falcon tube). Without addition of the carbon and energy source (glucose or D-serine), bacteria were not able to grow.

Pyruvate assay. The assay was conducted basically as described by McFall (45). It measures the concentration of a colored α-keto acid that is formed as the product of a reaction of pyruvate with 2,4-dinitrophenylhydrazine under alkaline conditions. The strains used in this assay were cultured in 100 ml peptone-yeast extract (PY) broth (10 g/liter peptone, 5 g/liter yeast, 1 g/liter glucose, 5 g/liter NaCl, 1.25 g/liter $Na_2HPO_4 \cdot 2H_2O$ [pH 7.3]) overnight (37°C and 100 rpm). The medium was supplemented with D-serine (1 g/liter) to ensure that the D-serine-deaminase was expressed. Twenty-five milliliters of the overnight culture was pelleted by centrifugation at 4,000 rpm for 10 min. The bacteria were then washed with 0.07 M PBS (pH 7.4) and resuspended in 2 ml of this buffer. The following lysis was done by addition of 10 µl lysostaphin (5 mg/ml; WAK-Chemie, Steinbach, Germany), incubation at 37°C for 15 to 20 min, and subsequent sonication (6 × 30 s; Branson sonifier W185, level 4). Cell debris was removed by centrifugation (13,000 rpm, 1 min), and the lysate was used for the following reaction. Four hundred microliters of lysate was incubated with 100 µl D-serine (10 mg/ml) for 60 min at 37°C. 2,4-Dinitrophenylhydrazine (Acros; 500 µl; 0.3 mg/ml in 1 N NaCl) was then added, and incubation continued at room temperature for 20 min. The reaction was stopped by addition of 1 ml 2.8 N NaOH. The optical density was read at 450 nm in a spectrophotometer against an assay blank to which all components had been added except D-serine. Additionally, a standard curve with pyruvate from 0.5 µg/ml to 10 µg/ml was measured. Analysis of pyruvate generation was done per mg total protein, which was determined by the method of Markwell (46).

RNA preparation from *Staphylococcus saprophyticus*. For RNA preparation, wild-type *S. saprophyticus* or the Δ*dsdA* mutant was grown in the chemically defined medium supplemented with glucose or glucose and D-serine until an OD_{600} between 0.450 and 0.500 was reached. For each strain and growth condition, RNA was extracted from three biological replicates. Before harvesting, 4 ml Bacteria Protect reagent (Qiagen) was added to 2 ml bacterial culture, and the mixture was incubated for 5

min at room temperature. By a subsequent centrifugation (5,000 rpm, 10 min, room temperature), bacteria were pelleted, and pellets were stored for not longer than 2 weeks at −20°C. RNA was prepared using the RNeasy minikit (Qiagen). The pelleted bacteria were resuspended in 100 μl TE buffer (30 mM Tris, 1 mM EDTA [pH 8.0]), and 10 μl lysostaphin (5 mg/ml) and 10 μl proteinase K (Applichem) were added. After undergoing vortexing and incubation (10 min, 37°C, 100 rpm), bacteria were disrupted mechanically following the manufacturer's instructions with the modification that a Power Vortex was used instead of a TissueLyser. DNase digestion was done on-column using the Qiagen RNase-free DNase set following the manufacturer's instructions. After elution, aliquots of RNA were frozen in liquid nitrogen and stored at −80°C. The integrity and purity of RNA were checked by gel electrophoresis, and absorbance was measured using a Nanodrop 1000. The ratio of A_{260} to A_{280} was between 1.9 and 2.1 for all RNA samples used.

RT-qPCR. Real-time reverse transcription-quantitative PCR (RT-qPCR) was carried out by using the QuantiFast SYBR green RT-PCR kit (Qiagen) for one-step real-time PCR following the manufacturers' instructions and the LightCycler 1.2 (Roche). The reaction volume was 20 μl. RNA was used as the template at a final concentration of 2.5 ng/reaction. Primers were added to a final concentration of 0.5 pmol/each. After RT for 10 min at 50°C, the following temperature protocol was used: an initial activation step for 5 min at 95°C (temperature transition, 20°C/s), followed by a two-step cycling PCR (40 cycles) consisting of denaturation at 95°C for 10 s (temperature transition of 20°C/s), a combined annealing and extension at 60°C for 30 s (temperature transition of 20°C/s), and fluorescence acquisition at 60°C in single mode. Melting curve analysis was performed at 60°C to 95°C (temperature transition, 0.1°C/s). The specificity of the products was checked by melting point analysis and gel electrophoresis.

Calculation of virulence factor expression. For each strain and growth condition, RNA was extracted from three biological replicates. Each replicate was analyzed in triplicate. The test genes where either normalized to *gyrB* or to *dpol*. Calculations were done by using the formula $2^{\Delta CT\ test\ gene}/2^{\Delta CT\ reference\ gene}$, in which the change in cycle threshold (ΔC_T) is the difference of the C_T under glucose conditions − the C_T under D-serine conditions. Values of >2.0 were defined as upregulated, and values of <0.5 were defined as downregulated if observed in all three independent biological replicates.

RESULTS

The D-serine-deaminase catabolizes D-serine to pyruvate and ammonia. To show that in *S. saprophyticus* the D-serine-deaminase is functional and catabolizes D-serine to pyruvate, we constructed an isogenic mutant of strain 7108 by interrupting the gene with an erythromycin resistance cassette. In addition, the gene was cloned into a staphylococcal vector and expressed in *S. carnosus* TM300. We prepared cell extracts from the wild-type *S. saprophyticus* strain, the Δ*dsdA* mutant, and *S. carnosus* TM300 as well as *S. carnosus* TM300(pMB1406) containing the *dsdA* gene on a plasmid. The extracts were incubated with D-serine, and generation of a keto acid—pyruvate in this case—was measured with the dinitrophenylhydrazone assay (45). The *S. saprophyticus* wild-type strain 7108 generated 4.6 (standard deviation [SD], 0.9) μg mg^{-1} min^{-1} pyruvate, the mutant 0.4 (SD, 0.06) μg mg^{-1} min^{-1}, the complemented strain 80.2 (SD, 2.8) μg mg^{-1} min^{-1}, *S. carnosus* TM300 0.5 (SD, 0.25) μg mg^{-1} min^{-1}, and *S. carnosus* TM300(pMB1406) 36.1 (1.04) μg mg^{-1} min^{-1}. The presence of the D-serine-deaminase led to degradation of D-serine to pyruvate, whereas no degradation was detectable in strains not containing *dsdA*.

The D-serine-deaminase-negative mutant has a significant disadvantage against the wild type in coinfection experiments. The wild-type strain, 7108, and its Δ*dsdA* mutant were used in
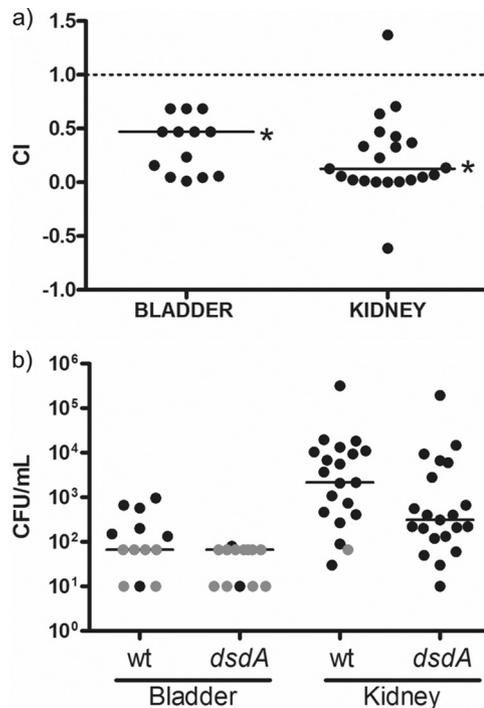

FIG 1 (a) Competitive indices of coinfection experiments. Mice were infected by instillation with a mixture of cells of the wild type and the mutant, and competitive indices (CIs) were determined for bladders and kidneys. The dashed line represents equal numbers of CFU recovered from bladder and kidney. Median CIs, indicated by horizontal lines, were significantly lower than 1 (0.47 in bladders and 0.13 in kidneys), indicating a disadvantage of the mutant in these experiments. $n = 13$ for bladders and 21 for kidneys. *, $P < 0.05$ by Wilcoxon signed-rank test. (b) CFU data from coinfection experiments. $n = 13$ for bladders and 21 for kidneys. Horizontal lines represent the geometric mean titer of each sample ($n = 13$ for bladders and 21 for kidneys). Gray circles indicate samples in which no CFU were detected and for which the limit of detection of the protocol has been entered.

coinfection experiments in a murine model of urinary tract infection ([Fig. 1](#)). The animals were infected transurethrally with a suspension containing $\sim 1 \times 10^7$ CFU of each strain. After 48 h, the animals were sacrificed, bladders and kidneys were aseptically removed, and CFU ml^{-1} tissue were determined. The competitive index (CI) of the two strains was calculated as follows: CI = (CFU · ml^{-1} mutant at 0 h/CFU · ml^{-1} wild type at 0 h)/(CFU · ml^{-1} of mutant at 48 h/CFU · ml^{-1} of wild type at 48 h). Competitive indices significantly less than 1 indicate an advantage for the wild type. The CI in the bladders was 0.47, and that for the kidneys was 0.13; both values were statistically different from 1 ($P < 0.05$, Wilcoxon signed-rank test). These experiments showed that in contrast to *E. coli*, the Δ*dsdA* mutation did not lead to increased colonization but conferred a disadvantage.

The D-serine-deaminase-negative mutant is at disadvantage in cocultivation experiments in the presence of D-serine. Since we wanted to know if the disadvantage of the mutant in animal experiments is solely related to its inability to catabolize D-serine, we used *in vitro* competition experiments in the presence and absence of D-serine. To allow for selection of the wild type, we transformed this strain with plasmid encoding tetracycline resistance (pMB2200) that had been isolated from a clinical strain of *S. saprophyticus* (41). When this strain and the Δ*dsdA* mutant were
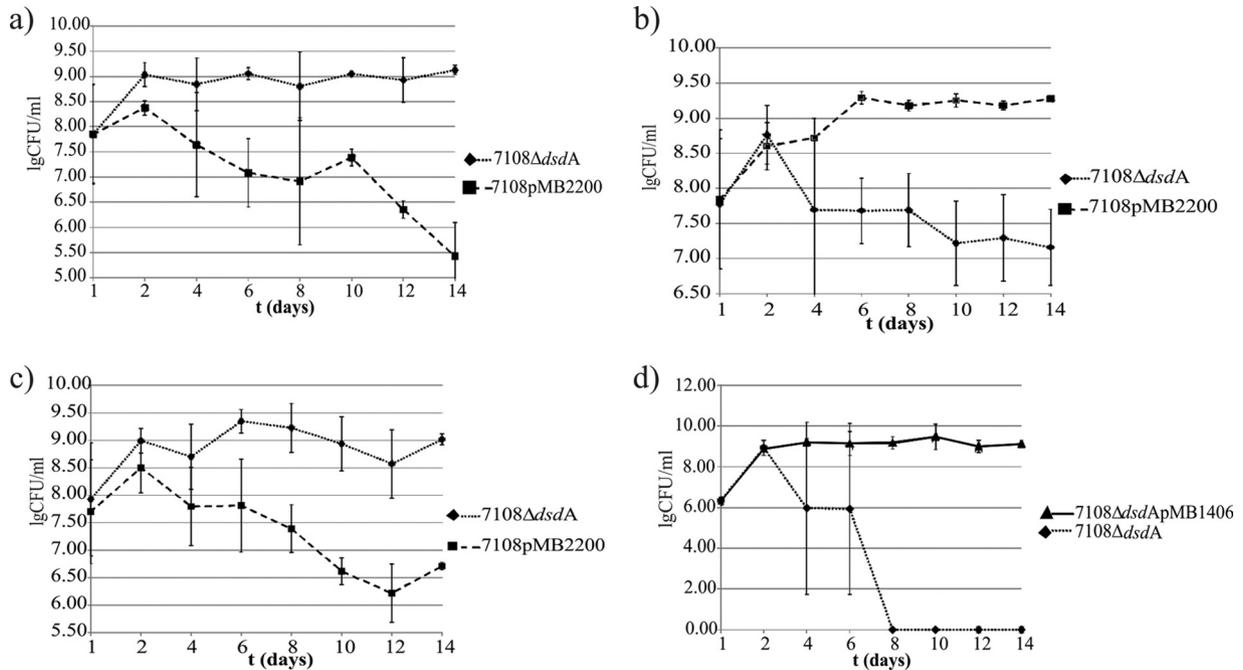
FIG 2 (a to d) The ability to degrade D-serine confers a growth advantage. Pairs of strains were grown in full medium in the presence or absence of D-serine. (a) Wild type and mutant grown without D-serine. The mutant outcompetes the wild type. (b) Wild type and mutant in the presence of D-serine. The wild type outcompetes the mutant. (c) Both strains in the presence of L-serine. The mutant again has an advantage. (d) Mutant and the complemented mutant in the presence of D-serine. The complemented mutant outcompetes the mutant. Values are the means of at least three experiments and SD (log CFU/ml).

cocultivated in broth without D-serine, the mutant outcompeted the wild type (Fig. 2a). In cultures containing D-serine, however, the mutant was at disadvantage (Fig. 2b). When the L-enantiomer of serine was used for supplementation, however, the mutant again had an advantage (Fig. 2c). These experiments show that the mutant was only at a disadvantage if D-serine was present, excluding the possibility that the resistance gene on the plasmid caused slower growth. When the Δ*dsdA* mutant and the complemented mutant were cocultivated in the presence of D-serine, the mutant was not detectable after 8 days (Fig. 2d). The effects were observed with D-serine concentrations as low as 2.5 mg/ml in both media (data not shown).

The *Staphylococcus saprophyticus* wild-type strain is able to use D-serine as the sole carbon and energy source. To analyze if *S. saprophyticus* is able to grow with D-serine as the sole carbon and energy source, like *E. coli* (30), we conducted growth experiments in a chemically defined medium supplemented with 10 mM D-serine. Whereas the Δ*dsdA* mutant was not able to grow with D-serine as the sole carbon source, wild-type *S. saprophyticus* started growing after an extended lag time (~168 h), and the complemented mutant also grew after a shorter lag time (48 h) compared to the wild type (Fig. 3). Since D-serine is catabolized to pyruvate and ammonia, we also supplemented the medium with 10 mM pyruvate instead of D-serine. In this medium, the wild type, the Δ*dsdA* mutant, and the complemented mutant grew immediately (lag phase of about 8 h) and reached high cellular densities (data not shown). If no carbon source was added, none of the strains was able to grow.

D-Serine is not only an additional nutrient for *Staphylococcus saprophyticus*. To analyze if the growth advantage of wild-type *S. saprophyticus* in cocultivation experiments was due to the

utilization of D-serine as an additional nutrient, we analyzed the influence of D-serine on growth in the *S. saprophyticus* wild-type strain and the Δ*dsdA* mutant. To this end, we cultivated *S. saprophyticus* in our chemically defined medium supplemented with 2.5 mM glucose or 2.5 mM glucose and 5 mM D-serine. Both the wild type and the Δ*dsdA* mutant grew more slowly in the presence of D-serine (Fig. 4). This indicates that D-serine has an inhibitory effect on growth of *S. saprophyticus*.

Regulation of known virulence factor in the presence of D-serine. For uropathogenic *E. coli*, it was shown that a Δ*dsdA* mutant is hyperflagellated and more motile than the wild type (23).
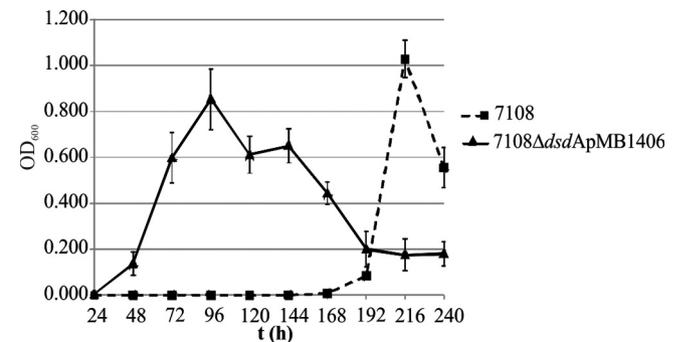
FIG 3 *Staphylococcus saprophyticus* is able to use D-serine as the sole carbon source. The *S. saprophyticus* 7108 wild-type strain and the complemented Δ*dsdA* mutant strain [7108 Δ*dsdA*(pMB1406)] are able to grow in chemically defined medium supplemented with D-serine (DS) (10 mM) as the sole carbon source. The Δ*dsdA* mutant was not able to grow. Without any additional supplements, none of the strains could grow. Values are means of at least three experiments and SD.

FIG 4 Influence of D-serine on growth of *S. saprophyticus*. D-Serine (DS) leads to growth impairment of the *dsdA*-knockout mutant as well as the wild-type strain, 7108, when the chemically defined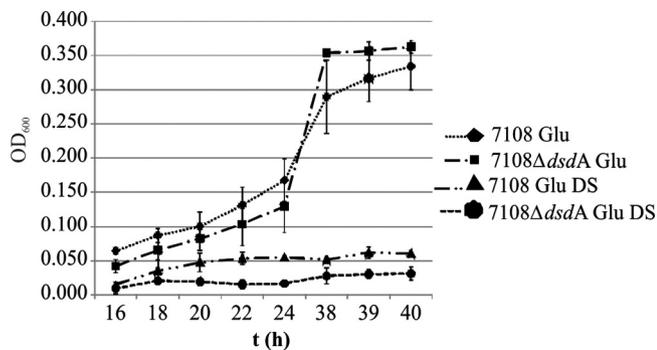 medium was supplemented with glucose (2.5 mM) and D-serine (5 mM). Values are means of at least three experiments and SD.

This hyperflagellation leads to hypercolonization; therefore, the Δ*dsdA* mutant of *E. coli* is more virulent than the wild type. D-Serine acts as a signal for upregulation of genes encoding fimbriae and some other virulence factors (47, 48). In *S. saprophyticus*, the Δ*dsdA* mutant is attenuated in virulence, suggesting that the influence of D-serine and D-serine metabolism on virulence gene expression is different from that in *E. coli*. To investigate if there is also a connection between virulence and D-serine in *S. saprophyticus*, we analyzed the expression of known virulence factors in the presence and absence of D-serine in the wild-type *S. saprophyticus* strain and the Δ*dsdA* mutant. Using chemically defined medium for these experiments, we were able to study the effect of D-serine without the influence of other, unknown components of the medium. Wild-type strain 7108 of *S. saprophyticus* and the Δ*dsdA* mutant were grown in chemically defined medium supplemented with 2.5 mM glucose or with 2.5 mM glucose and 5 mM D-serine to an OD_{600} between 0.450 and 0.500, which represents the late exponential phase. RNA was prepared, and the expression of known virulence factors was measured by RT-qPCR. The genes and primers used in this study are listed in Table 3.

We analyzed the expression of the genes by relative quantification using *gyrB* as an internal reference gene, as previously described for *Staphylococcus aureus* (49, 50). While it is common practice to use only one reference gene for relative quantification, this practice assumes that the reference gene is not altered under the conditions being examined. However, it has been shown that the conventional normalization strategy based on a single gene can lead to significant errors (51). Therefore, we decided to use a second reference gene, *dpol*, along with *gyrB* because their gene products, DNA polymerase and gyrase subunit B, respectively, are not involved in metabolism and therefore should be constantly expressed under the different conditions we were interested in. Accordingly, we observed only minute differences in the expression of both reference genes when strains were grown with glucose compared to D-serine. Fig. 5a to c present the fold changes of each gene under D-serine conditions compared to glucose conditions referenced either to *gyrB* or to *dpol* in wild-type *S. saprophyticus* (Fig. 5a) and the Δ*dsdA* mutant (Fig. 5b).

Using our criteria for up- and downregulation, only *ssp* in wild-type *S. saprophyticus* was consistently induced in the presence of D-serine. The ability to metabolize D-serine is required for

ssp induction, as we did not observe this upregulation in the Δ*dsdA* mutant. Given the intersample variability in *ssp* induction, we confirmed the result in three freshly prepared biological samples (Fig. 5c). None of the other genes examined in *S. saprophyticus* displayed reproducibly altered transcription in the presence of D-serine. None of the genes analyzed in the Δ*dsdA* mutant was clearly regulated.

DISCUSSION

We have shown previously that *S. saprophyticus* is the only species of *Staphylococcus* that possesses a D-serine-deaminase and grows in the presence of high concentrations of D-serine (52). When the *dsdA* gene is transferred to *S. aureus* (36) or *S. carnosus* (this work), which do not naturally express the deaminase, these species can grow in the presence of the same concentration of D-serine as *S. saprophyticus*. We used the *dsdA* homologue cloned from *S. saprophyticus* into *S. carnosus* to show that its product indeed causes degradation of D-serine. In addition we showed that the *S. saprophyticus* wild type produces pyruvate from D-serine, whereas the Δ*dsdA* mutant and *S. carnosus* TM300 did not.

In uropathogenic *E. coli* (UPEC), D-serine is used as a cue for the organism's presence in the urinary tract, can be used as the sole carbon source, and regulates the expression of virulence factors (48). Curiously, a *dsdA* UPEC mutant expressed higher adherence capabilities as well as increased flagellation (23) and outcompeted the wild type in coinfection experiments, which indicates that serine metabolism is important but that there are functions of D-serine besides the pure generation of energy.

In order to analyze the role and relevance of the D-serine-deaminase for virulence of *S. saprophyticus*, we performed coinfection experiments in mice infected with the wild-type and Δ*dsdA* mutant strains. In these experiments, the mutant had a distinct disadvantage compared to the wild type. The effect was more pronounced in the bladders of the animals than in the kidneys, although the mutant had a significant disadvantage in both organs. These results clearly indicate that the D-serine-deaminase is important for *S. saprophyticus* during experimental infection.

To show that the disadvantage of the Δ*dsdA* mutant *in vivo* was

TABLE 3 Primer used for RT-qPCR in this study

| GenBank accession no.	Primer name	Sequence (5′→3′)	Product size (bp)
AF402316	RNA_sdrI_F1 RNA_sdrI_R1	GCAGACGCAGACGCAGAC CAGCATCTGCATCTGCATCTGAG	147
AY551101	RNA_ssp_F1 RNA_ssp_R1	TGGTGCTGCACATGCAGAAAG ACGGACAGTTTGTCCTCCCATAC	129
YP_300522	RNA_dsdA_F2 RNA_dsdA_R2	TTACTGAACCAACACATGCCCC ATTTGCCCGACTAAGCGAGATG	147
AJ000007	RNA_aas_F1 RNA_aas_R1	GCCGACTACGCAGCAACTAAC CCATGAGGGTCAGAGTGGTCAG	136
Q4A0V8	RNA_uafA_F1 RNA_uafA_R1	CGATTACCGTAACGGGTTATCCAG GAGAGCCAGAATTACCTCCGAATG	131
YP_300353	RNA_ureC_F1 RNA_ureC_R1	ACACATATCGGTGGCGGTACAG GGTTTACAGCTTGCCCTTTACCAG	121
YP_300095	RNA_gyrB_F1 RNA_gyrB_R1	GCAGAGTCACCCTCGACGATAAAG GTGAAGTTACGCGCCGTAAATCAG	118
YP_301165	RNA_dpoI_F1 RNA_dpoI_R1	TGCGTAGACAAGCGAAAGCAG GTTTCACACCAGGGAAACTATCGAG	140
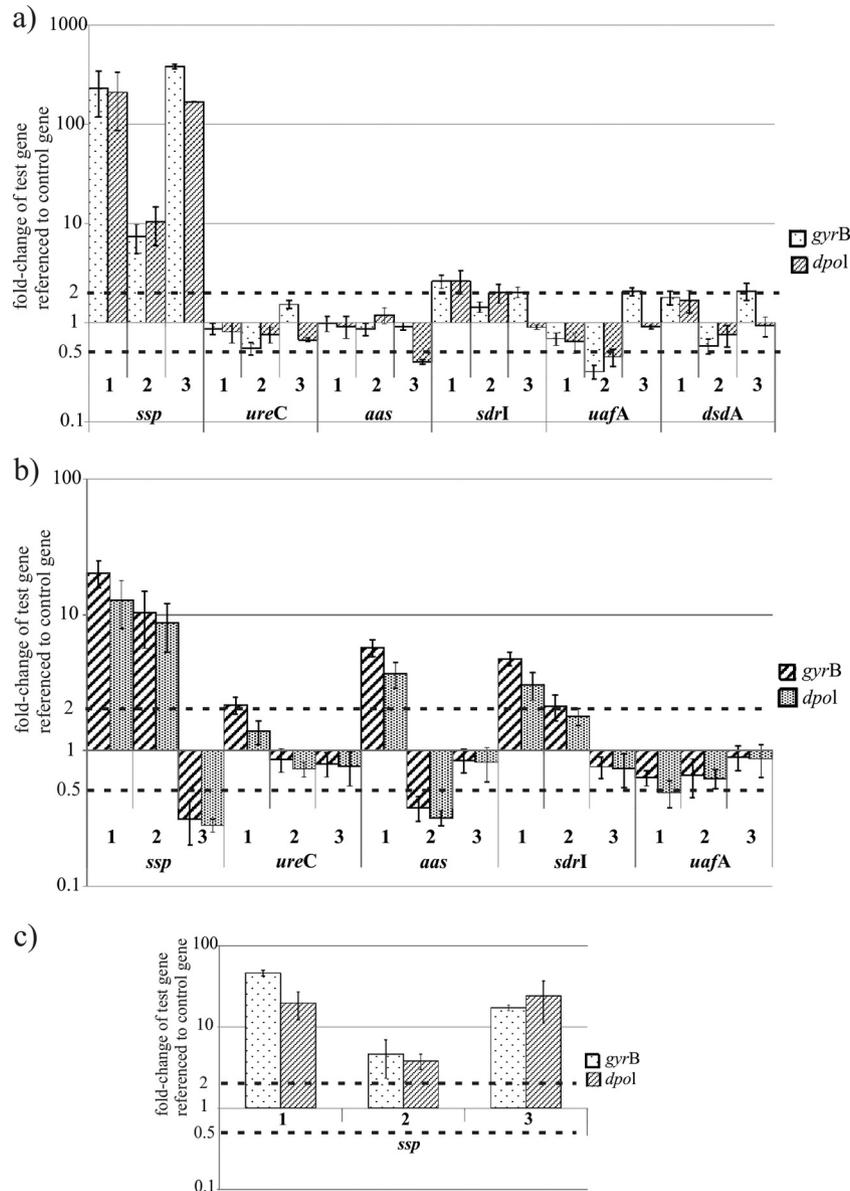
FIG 5 (a to c) Regulation of known virulence factors in the presence of D-serine. Each test gene was analyzed in three biological replicates and referenced to *gyrB* and *dpoI* as the control genes. The values of the biological replicates are means of two or three experiments and SD. Dashed lines represent the threshold values for up- and downregulation. (a) Regulation of known virulence in wild-type *S. saprophyticus*. (b) Regulation of known virulence genes in the Δ*dsdA* mutant. (c) Regulation of *ssp* in wild-type *S. saprophyticus* analyzed in three additional biological replicates.

caused by its inability to metabolize D-serine, we conducted cocultivation experiments in the presence and absence of D-serine. To allow positive selection of the wild-type strain, 7108, we transformed it with a naturally occurring tetracycline resistance plasmid of *S. saprophyticus*, pMB2200, which we had isolated from a clinical strain (41). When the wild-type strain (Tc[r]) and the mutant were grown in LB without D-serine, the mutant outcompeted the wild type; in the presence of D-serine, the wild type, however, outcompeted the mutant. When we used the L-enantiomer of serine, the mutant again grew better than the wild type. The advantage of the mutant in the absence of D-serine can be explained by the additional burden the tetracycline plasmid represents; in the presence of D-serine, the advantage of the mutant does not compensate for the effects of D-serine. We therefore conclude that

D-serine is the factor that caused the disadvantage of the mutant in these experiments. This conclusion is corroborated by our finding that the wild type and the complemented mutant can grow when D-serine is the only carbon and energy source, whereas the mutant cannot. In these experiments, the lag phases were quite long. When the strains were grown with pyruvate instead of D-serine, all of them grew very fast, suggesting that the velocity of the DsdA enzyme was the limiting factor in these experiments. This is supported by the fact that the complemented mutant grew faster than the wild type. In the presence of glucose, D-serine had a negative effect on growth of the Δ*dsdA* mutant as well as on the wild type, mainly because of a prolonged lag phase of at least 45 h. Strains start to grow after the prolonged lag phase but apparently also replicate more slowly. We therefore conclude that the growth ad-

vantage of the wild type in the presence of D-serine cannot be explained just by an additional nutrient source and that the influence of D-serine on *S. saprophyticus* is more complex. Obviously, there is an inhibitory effect on growth in the presence of glucose; on the other hand, *S. saprophyticus* is able to grow with D-serine as the sole carbon and energy source.

DsdA catabolizes D-serine by forming pyruvate and ammonia. Bacteria not able to catabolize D-serine are usually inhibited by the compound, and it is thought that this effect is due to inhibition of the synthesis of pantothenic acid by D-serine, which functions as an analogue of β-alanine (29). In other species, D-serine inhibits pantothenic acid biosynthesis at different steps (26–28, 53). In *E. coli*, the target of D-serine inhibition is the pantoate-β-alanine ligase, encoded by *panC*, and it was shown that there is a second target of D-serine inhibition, the L-serine metabolism (29). For growth in our chemically defined medium, *S. saprophyticus* requires pantothenic acid or β-alanine; therefore, our chemically defined medium contains pantothenic acid. As we saw growth inhibition caused by D-serine in this medium, it is unlikely that growth impairment is caused by this classical mechanism.

In *E. coli*, D-serine regulates expression of virulence factors (47, 48). Because of our findings that wild-type *S. saprophyticus* has an advantage over the Δ*dsdA* mutant in coinfection experiments as well as in cocultivation experiments in the presence of D-serine, we hypothesized that D-serine metabolism affects *S. saprophyticus* virulence factor expression, albeit in a different way than in *E. coli*. To test this hypothesis, we analyzed the expression of known *S. saprophyticus* virulence factors in the presence and absence of D-serine in the wild type and the Δ*dsdA* mutant by RT-qPCR. Since we observed slight differences in expression of our chosen reference genes under both conditions, we decided to regard only those results as certain that were >2.0 or <0.5 in RNA preparations from three biological replicates referenced to both genes. Using these criteria, only *ssp* in wild-type *S. saprophyticus* was induced in the presence of D-serine. This result was confirmed by additional experiments (Fig. 5c). Therefore, we conclude that *ssp* is upregulated. It has been shown that an *ssp*-knockout mutant is less virulent in a murine model of UTI (19), although the role of the lipase during infection remains undefined. It has been suggested that lipases may be important for colonization, possibly in terms of nutrition or by release of free fatty acids which may promote adherence (54, 55). We conclude that the D-serine metabolism induces *S. saprophyticus* to produce greater amounts of lipase, which is needed during infection. Our hypothesis is that D-serine is used as a cue for the presence in the urinary tract and induces a different metabolism, including expression of Ssp in *S. saprophyticus*. It is not yet clear if D-serine directly or indirectly regulates *ssp*; these analyses will be the subjects of subsequent studies.

In conclusion, we have shown that *S. saprophyticus* is able to use D-serine as the sole carbon and energy source, like *E. coli* (30), but D-serine also has a negative effect on growth of *S. saprophyticus*. Coinfection experiments and cocultivation experiments showed that the D-serine-deaminase confers an advantage to the wild-type strain. Coinfection experiments demonstrated that the D-serine-deaminase is important for virulence of *S. saprophyticus* during urinary tract infection. In the presence of D-serine, the virulence-associated lipase Ssp is upregulated in the wild type, which may explain its advantage in experimental infections. We conclude that the D-serine-deaminase acts as a virulence factor in two different ways. First, it catabolizes D-serine, which is toxic or

bacteriostatic to many bacteria and other staphylococci. Only strains that expressed this enzyme are able to grow in the presence of D-serine and to catabolize this amino acid. Second, D-serine, the D-serine-deaminase, or D-serine metabolism affects the expression of at least one virulence factor, the lipase Ssp, suggesting that D-serine may serve as a cue to the bacteria for their presence in the urinary tract and to induce adaptation to this environment.

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