Streptococcus agalactiae imports spermidine by a member of the amino acid/polyamine antiporter family to endure citric acid stress at the vaginal pH

Sarah Khazaal, Rim Al Safadi, Dani Osman, Aurélia Hiron, Philippe Gilot

MICROBIOLOGY

RESEARCH ARTICLE

Khazaal *et al.*, *Microbiology* 2022;168:001219

DOI 10.1099/mic.0.001219

MICROBIOLOGY SOCIETY

OPEN ACCESS

Streptococcus agalactiae imports spermidine by a member of the amino acid/polyamine antiporter family to endure citric acid stress at the vaginal pH

Sarah Khazaal[1,2], Rim Al Safadi[2], Dani Osman[2], Aurélia Hiron[1] and Philippe Gilot[1,*]

Abstract

Polyamines bind to various cellular components, such as nucleic acids, phospholipids, proteins and nucleotides. They are involved in the virulence and protection against physiological stresses of several bacterial species. *Streptococcus agalactiae* is able to colonize the vaginal tract of asymptomatic pregnant women and to resist, by an as yet poorly characterized mechanism, pH 4.0, the low physiological pH of this environment. We identified a transporter of the amino acid/polyamine antiporter family (SAK_1604 in strain A909) that shares 39.8% similar amino acids with CadB and 34.7% with PotE, two transporters implicated in acid resistance in *Escherichia coli*. We found that *sak_1604* is overexpressed in the presence of spermidine and during citric acid stress at the vaginal pH, but not during lactic acid or HCl stresses at the same pH or during a sodium citrate stress at pH 7.4. Dihydrogen citrate is the predominant form of citric acid at pH 4.0. Using a deletion mutant, we proved that SAK_1604 is involved in the survival of *S. agalactiae* during citric acid stress at pH 4.0 in the presence of spermidine, and we showed by TLC analysis that it is involved in spermidine transport in these conditions. Our data open new perspectives on the comprehension of the molecular mechanisms allowing *S. agalactiae* to survive at the physiological pH of the vagina and on the unsuspected role of an ionic form of citric acid.

INTRODUCTION

Polyamines (putrescine, spermidine and spermine) play a key role in normal cell growth for most prokaryotic and eukaryotic cells [1]. Their contents in cells are sustained by the coordination of biosynthesis, degradation processes and transport [2]. They are considered as the most abundant polycations in cells, together with Mg^{2+} and Ca^{2+}. Polyamines, as well as Mg^{2+}, are able to bind intracellular polyanions such as DNA, RNA and ATP to regulate their functions [3]. By their ability to stabilize nucleic acids and their effects on transcription and translation, they are also involved in bacterial resistance to acidic and oxidative stresses and, notably, to the harsh conditions imposed by phagocytic cells [4–8]. Consequently, polyamines and polyamine transport systems are also implicated in the pathogenesis and virulence of several human bacterial pathogens [4].

Streptococcus agalactiae, an important human pathogen, is a Gram-positive and β-haemolytic bacterium. It is able to colonize the vaginal tract of asymptomatic pregnant women, as well as the gastrointestinal tract of humans [9, 10]. It is one of the most common agents responsible for neonate infections, leading to pneumonia, septicaemia and meningitis, and is an emerging pathogen in immunocompromised non-pregnant and elderly adults. *S. agalactiae* is also able to infect animals, such as bovines and fish, and to contaminate food products [10–13]. The ability of *S. agalactiae* to colonize a large scale of environments, and to resist the acidity of the intestine and the vagina, indicates the existence of molecular mechanisms conferring this large capability for adaptation.

We previously reported that *S. agalactiae* lacks polyamine biosynthesis capability [14]. However, we found that *S. agalactiae* is able to import spermine, spermidine and putrescine from the growth medium. All strains of *S. agalactiae* possess the genes encoding the polyamine ATP-binding cassette (ABC) transporter, PotABCD. Transcription of the *potABCD* operon is induced

Received 14 March 2022; Accepted 14 June 2022; Published 03 August 2022

Author affiliations: [1]ISP, Bactéries et Risque Materno-Foetal, Université de Tours, INRAE, 37032 Tours, France; [2]LBA3B, AZM Center for Research in Biotechnology and its Applications, EDST, Lebanese University, Tripoli 1300, Lebanon.

***Correspondence:** Philippe Gilot, philippe.gilot@inrae.fr

Keywords: AdiC; CadB; dihydrogen citrate; polyamine; PotE; vagina.

Abbreviations: CDM, chemically defined medium; KEGG, Kyoto Encyclopedia of Genes and Genomes; qRT-PCR, quantitative reverse transcriptase PCR; RT-PCR, reverse transcription PCR.

Six supplementary figures are available with the online version of this article.

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or description	Source or reference
***E. coli* strain**		
XL1-blue	*endA1 gyrA96* (Nal^R) *thi-1 recA1 relA1 lac glnV44 hsdR17* (r_{K-} m_{K+}) F′ [::Tn*10* (Tet^R) *proAB*$^+$ *lacI*^{q} *ZΔM15*]	Stratagene
***S. agalactiae* strains**		
A909	Isolated from a septic human neonate in 1934 (ST 7, CC 7)	[45]
A909-pTCV-*lacZ*	A909 strain transformed with plasmid pTCV-*lacZ*	This study
A909Δ*sak_1604*	Isogenic *sak_1604* deletion mutant of A909	This study
A909Δ*potABCD*Δ*sak_1604*	Isogenic *potABCD* (*sak_1196*, *sak_1195*, *sak_1194 and sak_1193) and sak_1604* deletion mutant of A909	This study
A909Δ*sak_1604* –pTCV ::*sak_1604*	*sak_1604* plasmid complementation of A909Δ*sak_1604*	This study
Plasmids		
pG+host1^{TS}	Replication-thermosensitive shuttle $^{(TS)}$ plasmid, Ery^R	[25]
pTCV-*lacZ*	Promoter probe plasmid carrying the *ermB* gene (Ery^R) and a *lacZ* gene devoid of a promoter	[26]

by peroxide-induced oxidative stress, but not by acidic stress. Spermidine and spermine were found to be inducers of *potABCD* transcription at pH 7.4, whereas putrescine induced this expression only during peroxide-induced oxidative stress. However, we were unable to show that the PotABCD transporter has an influence on the growth of *S. agalactiae* at pH 7.4 and pH 5.5 or on the survival capacity of this bacteria at pH 4.0 or during peroxide-induced oxidative stress, probably due to the existence of one or more transporters with a redundant action [14].

In *Escherichia coli*, beside the PotABCD system, the intracellular level of polyamines is regulated by the PotE and CadB transporters of the basic amino acid/polyamine antiporter (APA) family [transport class (TC) no. 2.A.3.2]. PotE and CadB catalyse both the uptake at neutral pH and excretion at acidic pH of putrescine and cadaverine, respectively [15]. The PotE membrane protein is a putrescine : ornithine antiporter for putrescine export and a putrescine : H^+ symporter for uptake [16]. The CadB protein is a cadaverine : lysine antiporter for cadaverine export and a cadaverine : H^+ symporter for uptake [17]. As they are also functioning as exporters, PotE and CadB allow the bacteria to avoid the toxic effect of a high concentration of polyamines. PotE and CadB, as well as AdiC, one other member of the APA family catalysing the transport of agmatine – an intermediary in the biosynthesis of polyamines, also constitute an acid resistance system [18, 19]. In acidic conditions, these antiporters facilitate the transport of amino acids inside the bacteria and export them as a polyamine after their proton-consuming decarboxylation by a cytoplasmic decarboxylase. By extruding a cytoplasmic proton to the extracellular environment, this mechanism counteracts intracellular acidification [20]. The genes encoding the antiporters and decarboxylases are located in close proximity (*adiC* and *adiA*, *cadB* and *cadA*, *potE* and s*peF*).

In this study, we analysed the genomes of *S. agalactiae* strains available in the National Center for Biotechnology Information (NCBI) database to search for the presence of genes encoding homologues of PotE and CadB. Thus, we identified a member of the basic amino acid/polyamine antiporter family and determined its role in the resistance of *S. agalactiae* to citric acid at the physiological pH of the vagina (pH 4.0), in the presence of spermidine.

METHODS

Plasmids, bacterial strains and routine growth conditions

Plasmids and bacterial strains used in this study are listed in Table 1. *E. coli* strains were routinely grown in liquid LB medium (MP Biomedicals) or on LB agar plates (1.5% agar). Liquid cultures of *E. coli* were agitated at 200 r.p.m. at 37 °C. *S. agalactiae* strains were cultured on 5% horse blood trypticase soy (TSH) agar plates (1.5% agar) (bioMérieux) or on Todd Hewitt (TH) agar plates (BD Bacto). Liquid cultures of *S. agalactiae* were grown at 37 °C without agitation in TH broth (BD Bacto) adjusted to pH 7.4 with HCl. When necessary, *E. coli* and *S. agalactiae* strains were grown with erythromycin (150 µg ml^{-1} for *E. coli* or 10 µg ml^{-1} for *S. agalactiae*).

Liquid chemically defined medium (CDM) for growth of *S. agalactiae*

The liquid CDM used to grow *S. agalactiae* was adjusted to pH 7.4 with HCl, as described previously [21]. For some experiments, CDM was buffered to pH 7.4 with 100 mM HEPES, to pH 5.5 with 100 mM MES (ACROS Organics) and to pH 4.0 by

the addition of 33.5 mM sodium citrate dihydrate and 66.5 mM citric acid, 22.7 mM sodium acetate and 77.3 mM acetic acid, or 33.5 mM sodium lactate and 66.5 mM lactic acid. Final adjustments of the pH were made with HCl, citric acid, acetic acid or lactic acid. Spermidine (ACROS Organics), putrescine (ACROS Organics) or spermine (ACROS Organics) were also added for some experiments.

Measurement of bacterial growth

For measuring bacterial growth in TH medium, *S. agalactiae* strains were first grown overnight without agitation in TH broth at pH 7.4. These overnight cultures were then diluted to an OD_{600} of 0.05 in TH broth adjusted to the pH of interest. These last cultures were incubated at 37 °C for 12 h in microtitre plates (Greiner Bio-One; Cellstar) (300 µl culture volume per well) in an Eon thermoregulated spectrophotometer plate reader (BioTek Instruments). For measuring bacterial growth in the CDM, *S. agalactiae* strains were first cultured in TH broth at pH 7.4 (without agitation) to the stationary phase of growth. These cultures were centrifuged and the cells washed with CDM at pH 7.4. They were then suspended to an OD_{600} of 0.005 in the same medium and grown overnight at 37 °C, without agitation. These last cultures were finally diluted to an OD_{600} of 0.05 in CDM adjusted to the pH and polyamine concentration of interest, and incubated as described above. The OD_{600} was measured every hour after double orbital shaking of the plate for 5 s. The reported OD_{600} is the mean OD_{600} of three wells inoculated with the same culture. Three independent experiments were performed for all strains and tested conditions.

Survival assay

To measure the capability of *S. agalactiae* to survive in CDM at pH 4.0, the bacteria were cultured at 37 °C (without agitation) in non-buffered TH broth at pH 7.4 to the beginning of the stationary phase of growth. This culture was then centrifuged, the cells washed with non-buffered CDM at pH 7.4 and suspended to an OD_{600} of 0.005 in flasks containing 40 ml of the same medium. When necessary, antibiotics were added to the above cultures. After an overnight incubation at 37 °C without agitation, 10 ml aliquots of these cultures were transferred to Falcon tubes, which were centrifuged for 5 min at 5000 ***g***. Bacterial pellets were suspended in 1 ml CDM buffered at pH 4.0 by the addition of 33.5 mM sodium citrate dihydrate and 66.5 mM citric acid, without the presence of polyamines, or in 1 ml of the same medium also containing 1 mM spermidine, 1 mM spermine or 1 mM putrescine. For some experiments, bacterial pellets were also suspended in 1 ml CDM either supplemented with 1 mM spermidine and 100 mM Na citrate, and adjusted to pH of 7.4 with HCl, supplemented with 1 mM spermidine and adjusted at pH 4.0 with HCl, or supplemented with 1 mM spermidine and buffered at pH 4.0 by the addition of 33.5 mM sodium lactate and 66.5 mM lactic acid. All these suspensions, which contained no antibiotics, were then incubated at 37 °C (without agitation) for 6 h. Viable cell counts of the bacteria were assessed immediately after the suspension of the pellets (t_0) and at suitable time intervals thereafter. To this end, serial dilutions were performed in TH broth at pH 7.4. One hundred microlitres of each of these dilutions was immediately spread onto TH agar plates in triplicate, which were incubated at 37 °C for 24 h. All survival experiments were performed at least three times. Results are expressed as the percentage of survivors [(number of viable bacteria at the tested condition divided by the number of viable bacteria at t_0) multiplied by 100].

Expression of *sak_1604* in the presence of polyamine and during stress conditions

To determine the expression of *sak_1604* in the presence of polyamines, *S. agalactiae* A909 was grown at 37 °C to an OD_{600} of 0.6 (exponential phase) or to an OD_{600} of 1.2 (stationary phase) in a CDM buffered at pH 7.4 with 100 mM HEPES and supplemented with 1 mM spermidine, 1 mM spermine or 1 mM putrescine. Bacteria were harvested (10 ml samples) at each growth phase and each condition and centrifuged at 5000 ***g***. Samples were stored at −80 °C until RNA extraction.

To test the expression of *sak_1604* during stress conditions, *S. agalactiae* strain A909 was grown in CDM at pH 7.4 at 37 °C to an OD_{600} of 0.6 (exponential phase). Bacteria were harvested (10 ml samples) and centrifuged at 5000 ***g***. Bacterial pellets were then suspended in CDM either adjusted to pH 7.4 with HCl, supplemented with 100 mM sodium citrate and adjusted at pH 7.4 with HCl, adjusted at pH 4.0 with HCl, adjusted at pH 4.0 with 33.5 mM sodium lactate and 66.5 mM lactic acid, adjusted at pH 4.0 with 33.5 mM sodium citrate dihydrate and 66.5 mM citric acid, or supplemented with 1 mM spermidine and adjusted at pH 4.0 with 33.5 mM sodium citrate dihydrate and 66.5 mM citric acid. After an incubation of 30 min at 37 °C, samples were then centrifuged and stored at −80 °C until RNA extraction.

Quantitative reverse transcriptase PCR (qRT-PCR) of *sak_1604* transcripts was performed on these RNA extracts. The level of transcripts was normalized in relation to the *recA* transcript levels. Gene expressions are presented as fold change with regards to the level of *sak_1604* transcripts during growth of *S. agalactiae* in the absence of polyamines. Results are presented as means±standard deviations of three independent experiments.

Nucleic acid manipulations

Standard nucleic acid manipulation techniques were carried out as described by Sambrook and Russell [22]. *S. agalactiae* genomic DNA and RNA purifications were performed as previously described [21]. Plasmids were purified from *E. coli* with a NucleoSpin plasmid kit (Macherey-Nagel), according to the manufacturer's instructions. Nucleic acid concentrations were measured with

Table 2. Primers used in this study

Name*	Sequence†	Location‡
Primers used for the deletion of *sak_1604*		
$SK21_{fw}$	**TGGTCTCGCAAC**AATATCTCCATTTCTACTG	nt −1 to −19 upstream of *sak_1604*
$SK20_{rv}$	**AAGGATGGATCC**TAGGTAGTAGGCGATGC	nt 704 to 720 of *sak_1605*
$SK22_{fw}$	**TGGTCTC**GGTTGACTTTGTAGACTGAAGTG	nt 1353 of *sak_1604* to nt −151 upstream of *sak_1603*
$SK23_{rv}$	**AAGCTTGGTACC**CTAAGGGCTTCATAAACA	nt 489 to 471 of *SAK_1603*
Primers used for routine PCR		
$SK24_{fw}$	ACATGGTCTACGGCAATACG	nt 1036 to 1056 of *sak_1605*
$SK25_{rv}$	TGAACCGCCTTCCATTTTAT	nt 71 to 52 of *sak_1603*
Primers used for qRT-PCR		
OAH 326_{fw}	TTGGGTGTCAGGTCTGGTAC	nt 591 to 610 of *sak_1604*
OAH 327_{rv}	CGCTGGACCAATGACAAAGG	nt 714 to 695 of *sak_1604*
$OLM321_{fw}$	CTGGTGGTCGTGCTTTGAAA	nt 551 to 570 of *recA*
$OLM322_{rv}$	TATGCTCACCAGTCCCCTTG	nt 634 to 615 of *recA*
Primers used uniquely for sequencing		
$SK24_{fw}$	ACATGGTCTACGGCAATACG	nt 1036 to 1055 of *sak_1605*
$SK25_{rv}$	TGAACCGCCTTCCATTTTAT	nt 72 to 52 of *sak_1603*
OAH 326_{fw}	TTGGGTGTCAGGTCTGGTAC	nt 591 to 610 of *sak_1604*
OAH 327_{rv}	CGCTGGACCAATGACAAAGG	nt 714 to 695 of *sak_1604*
$Vlac\text{-}1_{fw}$	GTTGAATAACACTTATTCCTATC	nt −63 to −41 upstream of the *Eco*RI site of the pTCV-*lac* polylinker
$Vlac\text{-}2_{rv}$	CTTCCACAGTAGTTCACCACC	nt 60 to 40 downstream of the *Bam*HI site of the pTCV-*lac* polylinker
Primers used for the complementation of *sak_1604*		
Apa_{fw}	**ACCTCCGAATTC**AAACTCTATGATACCTT	nt −189 to −173 upstream of *sak_1604*
Apa_{rv}	**CTCATCGGATCC**TTCTAGAACTCATTCT	nt −13 to −29 upstream of of *sak_1603*

*fw, Forward primer; rv, reverse primer.
†Tails containing a restriction site (in bold) are underlined.
‡Nucleotide (nt) position with respect to the first coding nt of the gene of interest or to a polylinker restriction site.

a NanoDrop Lite spectrophotometer (Thermo Fisher Scientific). The ratio of absorbance at 260 nm and 280 nm was used to check the purity of nucleic acids. Bacteria were transformed by electroporation with the Micropulser apparatus (Bio-Rad) and Ec2 conditions (2.5 kV), as described by Dower and colleagues for *E. coli* and by Ricci and colleagues for *S. agalactiae* [23, 24].

Amplification of nucleic sequences by PCR, by reverse transcription PCR (RT-PCR) and by qRT-PCR

PCRs were carried out with the Applied Biosystem 2720 thermal cycler using Q5 High-Fidelity DNA polymerase (New England Biolabs) for cloning or sequencing or OneTaq polymerase (New England Biolabs) for analytical PCR. For cloning or sequencing, the resulting PCR fragments were further purified with a NucleoSpin gel and PCR clean-up kit (Macherey-Nagel) or with a NucleoSEQ kit (Macherey-Nagel), according to the manufacturer's instructions. Oligonucleotides (Sigma-Aldrich) used in this study are listed in Table 2.

For RT-PCR and qRT-PCR, the RNAs were reverse transcribed as previously described [21]. For RT-PCR, cDNAs were amplified by PCR with appropriate primers (Fig. 1, Table 2), as described above for PCR amplification of DNA. Control RT-PCRs, omitting reverse transcriptase, were performed to check for DNA contamination of the RNA preparation.

For qRT-PCR, primers were selected with Primer3web software (https://bioinfo.ut.ee/primer3/) in order to generate 100 to 300 bp amplicons (Table 2). qRT-PCRs were performed in a 15 µl reaction volume containing 40 ng cDNA, 0.5 µl gene-specific primers (10 µM) and 7.5 µl LightCycler 480 SYBR Green I master mix (2×) (Roche). PCR amplification, detection and analysis were performed with the Bio-Rad CFX Connect real-time PCR detection system and Bio-Rad CFX Maestro software. PCR conditions included an initial denaturation step at 95 °C for 5 min, followed by a 40 cycle amplification (95 °C for 10 s, 60 °C for 20 s and

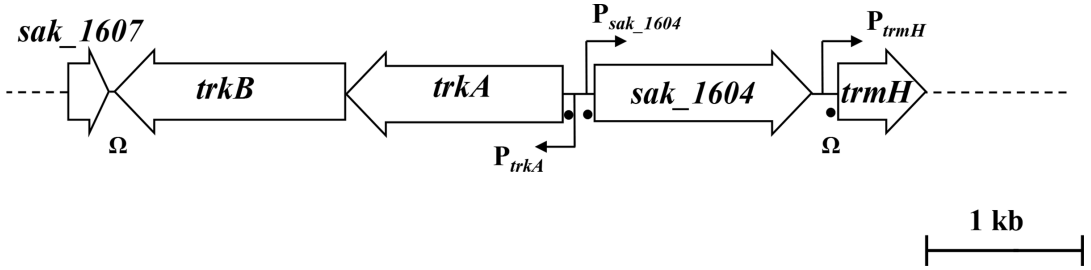

Fig. 1. Schematic representation of the *sak_1604* region of *S. agalactiae*. Putative transcriptional promoters (→) and rho-independent terminators (Ω) were identified *in silico* by BPROM and Arnold software, respectively. ORFs (large open arrows) and ribosome binding sites (●) are indicated. *trmH*, *sak_1603*; *trkA*, *sak_1605*; *trkB*, *sak_1606*.

72 °C for 20 s). The specificity of the amplified product and the absence of primer dimer formation were verified by generating a melting curve (65–98 °C, continuous increase). The cycle threshold (C_t) was defined for each sample. The expression levels of the tested genes were normalized using the *recA* gene of *S. agalactiae* (primers OLM321 and OLM322), whose transcript levels did not vary under our experimental conditions (Table 2). The fold change in the transcript level was calculated using the following equations: $\Delta C_t = C_t$ (target gene) – C_t (*recA* gene); $\Delta\Delta C_t = \Delta C_t$ (reference condition) – ΔC_t (test condition); relative quantification (RQ)=$2^{-\Delta\Delta Ct}$. Each assay was performed in triplicate and repeated with at least three independent RNA samples.

Bioinformatics analysis

To identify σ^{70} transcriptional promoters, the sequence of the intergenic regions between *sak_1604* and *trkA* and between *sak_1604* and *trmH* of strain A909 were analysed with the BPROM software from the SoftBerry suite (http://www.softberry.com/berry.phtml?topic=bprom&group=programs&subgroup=gfindb). Rho-independent transcriptional terminators were searched with the Arnold program (http://rssf.i2bc.paris-saclay.fr/toolbox/arnold/) with the sequences of all intergenic regions and with the entire coding sequence of *sak_1604*, *trkA*, *trkB* and *sak_1607*. Multiple sequence alignment was performed by the Clustal Omega program (https://www.ebi.ac.uk/Tools/msa/clustalo/). Prediction of transmembrane helices in protein was performed by the TMHMM server of the Center for Biological Sequence Analysis at the Technical University of Denmark (https://services.healthtech.dtu.dk/service.php?TMHMM-2.0).

DNA sequencing

PCR products were sequenced on both strands using the BigDye Terminator v3.1 cycle sequencing kit from Applied Biosystems and the ABI Prism 310 Genetic Analyzer.

Construction of *potABCD* and *sak_1604* deletion mutants

The *S. agalactiae* A909Δ*sak_1604* mutant is a non-polar mutant of strain A909. It was deleted of the complete coding strand of the *sak_1604* gene by allelic exchange. Upstream and downstream regions of the deleted sequence were amplified by PCR with primers $SK21_{fw}$/$SK20_{rv}$ and $SK22_{fw}$/$SK23_{rv}$, respectively (Table 2). These amplified fragments were cut by *Bsa*I and a recombination cassette, consisting of a fusion between these two regions, was obtained by splicing-by-overlap-extension PCR with primers $SK20_{rv}$ and $SK23_{rv}$ (Table 2). To carry out chromosomal gene inactivation, the overlap-extension fragment was hydrolysed by *Bam*HI and *Kpn*I, and cloned into the *Bam*HI/*Kpn*I sites of the thermosensitive shuttle plasmid pG+host1 [25]. The recombinant plasmid was electroporated into *E. coli* for amplification, purified and finally electroporated into strain A909. Allelic exchange was performed as described by Biswas and collaborators [25]. Deletion of the *sak_1604* gene of *S. agalactiae* A909 was confirmed by sequencing with the above primers $SK20_{rv}$, $SK21_{fw}$, $SK22_{fw}$ and $SK23_{rv}$, and with primers $SK24_{fw}$ and $SK25_{rv}$ (Table 2). *S. agalactiae* A909Δ*potABCD*Δ*sak_1604* is a non-polar deletion mutant of strain A909Δ *potABCD* created as described above for the *S. agalactiae* A909Δ*sak_1604* mutant but using the A909Δ *potABCD* strain constructed previously [14].

Complementation of the *S. agalactiae* A909Δ*sak_1604* mutant

To complement the *S. agalactiae* A909Δ*sak_1604* mutant, the entire coding sequence of *SAK_1604*, with its own promoter and terminator, was amplified by PCR with primers Apa_{fw} and Apa_{rv}, and inserted into the *Eco*RI/*Bam*HI restriction sites of plasmid pTCV-*lacZ* [26]. After being electroporated into *E. coli,* the recombinant plasmid was purified and the sequence of the cloned fragment was verified (with primers $Vlac\text{-}1_{fw}$, $Vlac\text{-}2_{rv}$, $OAH\ 326_{fw}$ and $OAH\ 327_{rv}$) before being electroporated into strain A909Δ*SAK_1604*.

Determination of the intracellular polyamine content

Intracellular polyamine content of *S. agalactiae* strains was visualized as described elsewhere [14, 27]. In brief, aliquots from bacterial cultures were pelleted. Then, 200 mg (wet weight) of wild-type or mutant bacteria were washed four times with PBS and suspended in 1 ml of 0.2 M perchloric acid. They were subsequently disrupted by sonication and centrifuged for 10 min at 12 000 ***g*** (4 °C). The supernatant was collected and 200 µl of the extract was dansylated by the addition of 0.4 ml of a solution of dansylchloride (30 mg dansylchloride ml^{-1} in acetone) and 50 mg $Na_2CO_3.10H_2O$. After incubation for 16 h in the dark (ambient temperature), 0.1 ml of a proline solution was added (100 mg ml^{-1} in Milli-Q H_2O) and the extract was incubated again for 30 min. Dansylated polyamines were extracted with 700 µl toluene. Dansylated toluene extracts were then separated by TLC on silica gel 60 G plates (Merck). Preliminary TLC experiments were made with different amounts of the dansylated toluene extracts (10 to 40 µl). As a 40 µl extract gave a strong signal when the wild-type strain was grown in the presence of polyamine and no signal at all when the wild-type strain was grown in the absence of polyamines, this volume of extract was used for all experiments. The dansylated polyamines were separated by development in ethylacetate/cyclohexane (2:3, v/v) followed immediately by spraying the TLC plate with triethanolamine/cyclohexane (1:4, v/v) to enhance and stabilize fluorescence. Similarly, 200 µl of a 1 mM solution of spermine, spermidine or putrescine in Milli-Q water was dansylated, extracted with toluene and 40 µl of each extract was assessed by TLC. After drying, spots were visualized under a Wood's light and photographed.

Statistical analysis

Data are presented as the mean±standard deviation for three independent experiments. An unpaired Student's *t*-test was used to determine the significance of the differences between means (*, $0.01<P<0.05$; **, $0.001<P<0.01$) [28].

RESULTS

Identification of a member of the basic amino acid/polyamine antiporter family putatively involved in polyamine transport in *S. agalactiae*

To identify potential *S. agalactiae* polyamine transporters other than PotABCD, we performed a BLAST analysis of the proteins encoded within the genome of strain A909 with the PotE and CadB proteins of *E. coli*. We found four proteins, one of which, the SAK_1604 protein, is annotated as a potential basic amino acid/polyamine antiporter in the Kyoto Encyclopedia of Genes and Genomes (KEGG) (https://www.genome.jp/dbget-bin/www_bget?sak:SAK_1604). SAK_1604 shares 39.8% similar amino acids with CadB and 34.7% with PotE. This protein is encoded by a gene that, contrary to the APA transporters, PotE, CadB or AdiC of *E. coli*, is not adjacent to a decarboxylase-encoding gene. Nevertheless, two genes (*trkA* and *trkB*) adjacent to *sak_1604* could also be involved in a mechanism aimed to counteract intracellular acidification (Fig. 1). The *trkA* and *trkB* genes encode homologues of components of the Trk potassium transport system of *E. coli* (https://www.genome.jp/entry/T00278:SAK_1605). In *E. coli*, this system was described to be involved in the maintenance of the electrochemical membrane potential and in the regulation of the cytoplasmic pH homeostasis [15, 19, 20, 29]. A nucleotide BLAST analysis with the sequences of *sak_1604*, *trkA* and *trkB* showed the presence of this region in all the completely sequenced genomes of *S. agalactiae* (135 strains at the time of our analysis).

We compared the characteristics of the SAK_1604 amino acid sequence with those of the PotE, CadB and AdiC proteins. Similarly to PotE, CadB and AdiC, SAK_1604 is composed of 12 predicted transmembrane helices (TMs) positioned at a similar location (I to XII; Fig. 2). TM1 and TM6 of acid resistance antiporters are disrupted by short, nonhelical, Gly-containing loops containing highly conserved amino acids [18]. The signature loop 'GSG' in TM1 is conserved in SAK_1604. It was found that the S26K mutation of AdiC in the GSG loop led to abrogation of transport activity. Although the 'GVESA', nonhelical Gly-containing loop in TM6 is not conserved in SAK-1604, the glycine at the beginning of this motif is also present in SAK_1604. The E208A mutation of AdiC in this motif also abrogates its transport activity. We also identified a motif in TM5 and at the junction of TM6 and TM7 conserved in all four transporters (framed in Fig. 2). Their roles are currently unknown. Furthermore, some of the amino acids important for the function of the PotE, CadB or AdiC transporters of *E. coli* are conserved at the same position in SAK_1604 (S^{27}, Y^{81}, E^{83}, Y^{102}, Y^{244}, Y^{379}, Y441; grey boxes in Fig. 2) [15, 18, 30].

In silico analysis of the *sak_1604* region of *S. agalactiae* A909

We analysed the *sak_1604* genomic region of strain A909 (Fig. 1). *sak_1604* is transcribed in the same direction as 13 ORFs, the first of which (*trmH*) encodes a tRNA (cytidine/uridine-2′-*O*-)-methyltransferase of the TrmH family. The *trkA* and *trkB* genes upstream of *sak_1604* are transcribed in the opposite direction. *sak_1604* is separated from *trkA* and from *tmrH* by an intergenic region of 205 and 172 nt, respectively. The length between *trkA* and *trkB* is 4 nt. Correctly conserved and positioned ribosome binding sites were identified upstream of *trkA*, *sak_1604* and *trmH*. Putative σ^{70} transcriptional promoters were identified upstream of *trmH* (P_{trmH}: −10 box, TGTTATGAT; −35 box, TTGTTA), of *sak_1604* (P_{sak_1604}: −10 box, GGTTATAAT; −35 box, GTGACA) and of *trkA* (P_{trkA}: −10 box, TGTTATTTT; −35 box, TTGTCA). A potential Rho independent transcriptional terminator was also identified in the intergenic regions downstream of *sak_1604* (position −91 to −29 upstream of *trmH*; ΔG=−7.0 kcal) and of *trkB* (position 222 of *sak_1607* to +20 downstream of *sak_1607*; ΔG=−7.70 kcal).

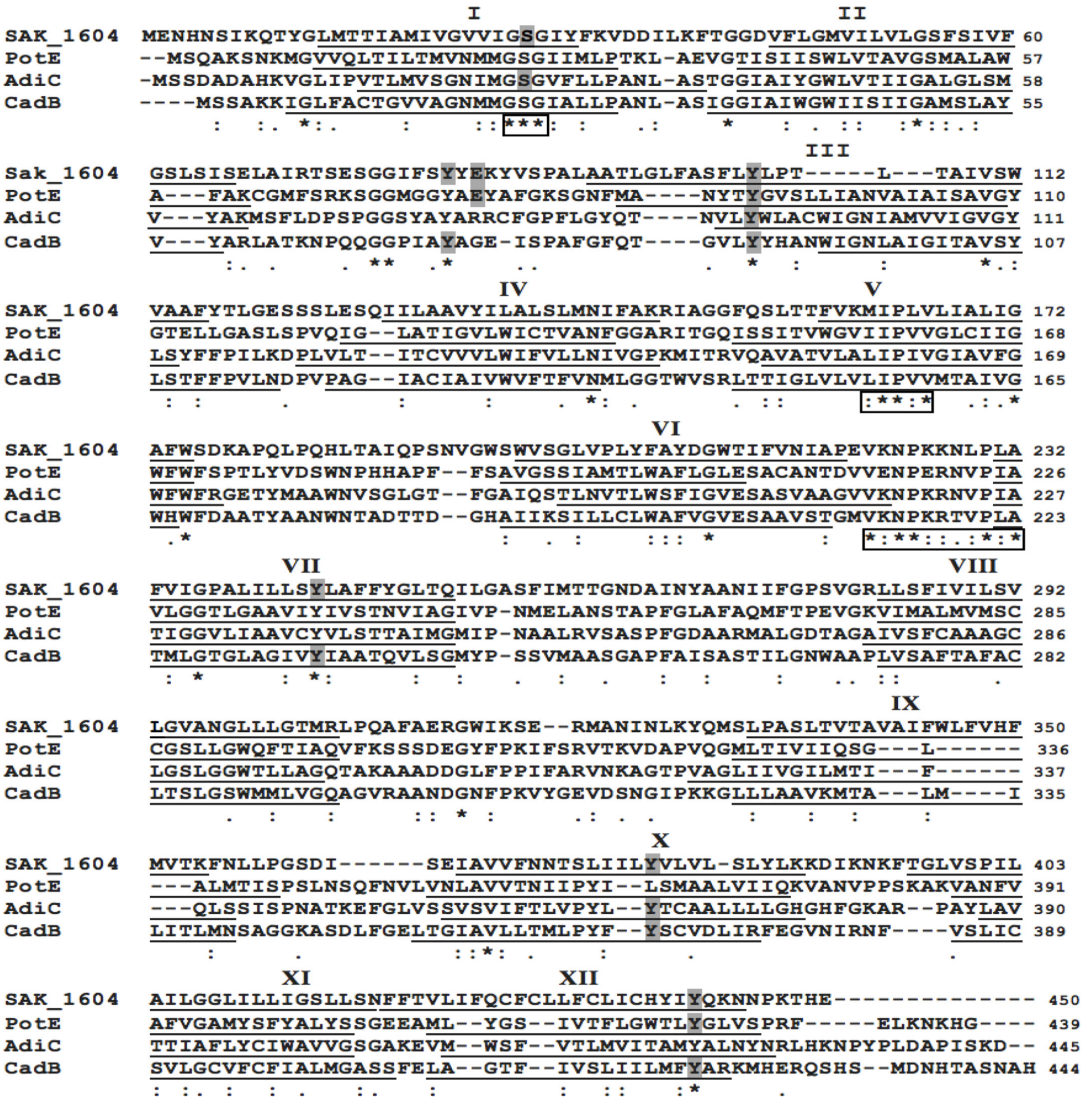

Fig. 2. Amino acid alignment of SAK_1604, PotE, AdiC and CadB. Multiple sequence alignment was performed by the Clustal Omega program (asterisk, identical amino acid residues in all four sequences; colon, highly conserved amino acid; dot, similar amino acid; blank, dissimilar amino acid or gaps in sequences). Predicted transmembrane helices are underlined and numerated. Amino acids of PotE, CadB or AdiC proved to be involved in the function of the transporter and conserved in SAK_1604 are highlighted in grey. A Gly-containing loop and two motifs conserved in all four transporters are boxed.

No requirement of the SAK_1604 transporter for the growth of *S. agalactiae* at pH 7.4 or pH 5.5

As SAK_1604 belongs to a family of transporters involved in polyamine transport and acid resistance, we tested the role of SAK_1604 and of polyamines for the growth of *S. agalactiae* at acidic pH. To this end, we constructed a non-polar deletion mutant of the *sak_1604* gene and a double deletion mutant of the *sak_1604* and *potABCD* genes. These two mutants, the wild-type and the Δ*sak_1604* complemented strains have a similar growth in media not supplemented with polyamines, both at pH 7.4 and at pH 5.5 (Fig. S1, available with the online version of this article.). We then compared the growth of the two deletion mutants, the Δ*sak_1604* complemented strain and the wild-type strain at pH 5.5 (pH of the intestine), in the absence or in the presence of 1 mM spermine, 1 mM spermidine or 1 mM putrescine (a previously found non-inhibitory concentration of polyamine for *S. agalactiae*) [14]. The addition of each of these polyamines slightly improved the growth of all strains similarly, indicating that the SAK_1604 and the PotABCD transporters are not involved in this effect (Fig. S2). We previously showed that the wild-type strain and the Δ*potABCD* mutant transport spermine, spermidine and putrescine similarly [14]. TLC analysis of the intracellular content of the Δ*sak_1604* and Δ*sak_1604*Δ*potABCD* mutants show that both mutants are also not affected in the transport of these polyamines during growth at pH 7.4 or pH 5.5 (Fig. S3). An aspecific crossing of these compounds through the membrane

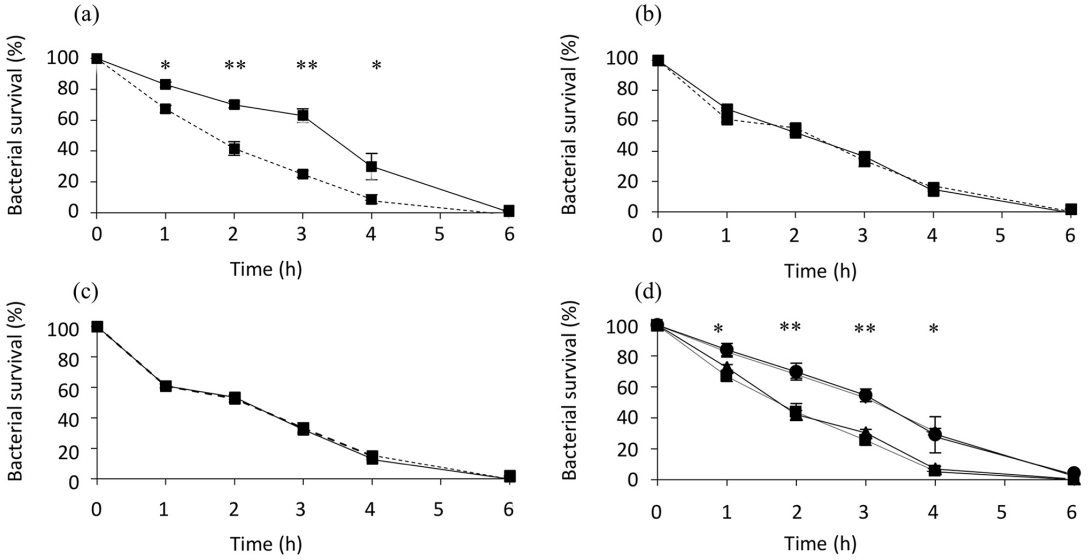

Fig. 3. Survival capacity of *S. agalactiae* in an acidic environment containing sodium citrate and polyamines. In (a–c), strain A909 was suspended either in 1 ml of a CDM buffered at pH 4.0 with a 100 mM mix of sodium citrate and citric acid in the absence of polyamines (a–c, dashed lines) or in the presence of 1 mM spermidine (a, plain line), 1 mM spermine (b, plain line) or 1 mM putrescine (c, plain line). In (d), strains A909 (●) and A909Δ*sak_1604*-pTCV::*sak_1604* (♦) (upper curves) or A909Δ*sak_1604* (▲) and A909Δ*potABCD*Δ*sak_1604* (■) (lower curves) were incubated in a CDM buffered with a 100 mM mix of sodium citrate and citric acid at pH 4.0, with the presence of 1 mM spermidine. All these suspensions were then incubated at 37 °C for 6 h. The proportion of surviving bacteria of each strain was monitored by plating diluted suspensions on TH agar plates, at suitable time intervals. Results are expressed as percentage of survivors [(number of viable bacteria at the tested condition divided by the number of viable bacteria at t_0) multiplied by 100]. They are presented as the means±standard deviations for three independent cultures. The asterisks indicate *P* values obtained using an unpaired Student's *t*-test to compare the proportion of the strain with the presence or not of spermidine at the indicated times (a–c) or the proportion of the A909 or the A909Δ*sak_1604*-pTCV::*sak_1604* strains to the proportion of the A909Δ*sak_1604* or the A909Δ*potABCD*Δ*sak_1604* strains at the indicated times. *, 0.01<*P*<0.05; **, 0.001<*P*<0.01; ***, 0.0001<*P*<0.001.

or by a not yet identified transporter should occur. In conclusion, *S. agalactiae* does not require the transport of polyamines by the PotABCD or SAK_1604 transporters for growth at pH 7.4 or pH 5.5.

SAK_1604 transporter is involved in the survival of *S. agalactiae* in a citric acidic environment at pH 4.0 in the presence of spermidine

We previously found that *S. agalactiae* strains do not grow in the CDM at pH 4.0 (the pH of the vagina), either in the absence or in the presence of polyamines [14]. Similar results were obtained with strains Δ*sak_1604* and Δ*potABCD*Δ*sak_1604* (results not shown). However, as polyamines are very basic molecules, the growth media were buffered at pH 4.0 with a 100 mM mix of sodium citrate and citric acid to avoid modifications of their pH, after the addition of polyamines. We suspected that citrate could be responsible for this above effect. Thus, we tested the growth of *S. agalactiae* in the presence of various concentrations of citrate buffer at pH 5.5. No growth of the bacteria could be obtained with a concentration of citrate buffer as low as 15 mM. Lower concentrations of citrate buffer were sub-inhibitory at pH 5.5 but unable to buffer the medium at pH 4.0 (results not shown). Similarly, we tested whether acetate could be used as a buffer for that experiment. Again, we found that *S. agalactiae* is unable to grow at pH 5.5 in the presence of a concentration of acetate buffer as low as 15 mM. Furthermore, acetate buffer concentrations below 30 mM were unable to buffer the medium at pH 4.0. As we were not able to use a buffer for testing the influence of polyamines on the growth of *S. agalactiae* at pH 4.0, we tested the growth of the bacteria in the CDM and in the rich TH medium, both adjusted to pH 4.0 with HCl, without the use of a buffer. *S. agalactiae* grew very slightly in the TH medium but not at all in the CDM (Fig. S4). These data indicate that although citrate and acetate have an inhibitory effect on the growth of *S. agalactiae*, pH 4.0 itself prevents the growth of *S. agalactiae* in our CDM. As the TH medium contains beef heart infusion, it probably contains a small quantity of polyamine or other components that enable *S. agalactiae* to cope with acid stress.

As the effect of polyamines on the growth of *S. agalactiae* at pH 4.0 is difficult to assess, we tested their effect on the survival capacity of the bacteria at this pH. To this end, *S. agalactiae* was incubated in a CDM buffered at pH 4.0 with a 100 mM mix of sodium citrate and citric acid, in the presence or absence of 1 mM spermidine, 1 mM spermine or 1 mM putrescine. The proportion of surviving bacteria (c.f.u.) was monitored with time. Fig. 3(a) shows that the addition of spermidine improves the survival capacity of *S. agalactiae* in these conditions, whereas spermine and putrescine have no effect (Fig. 3b, c).

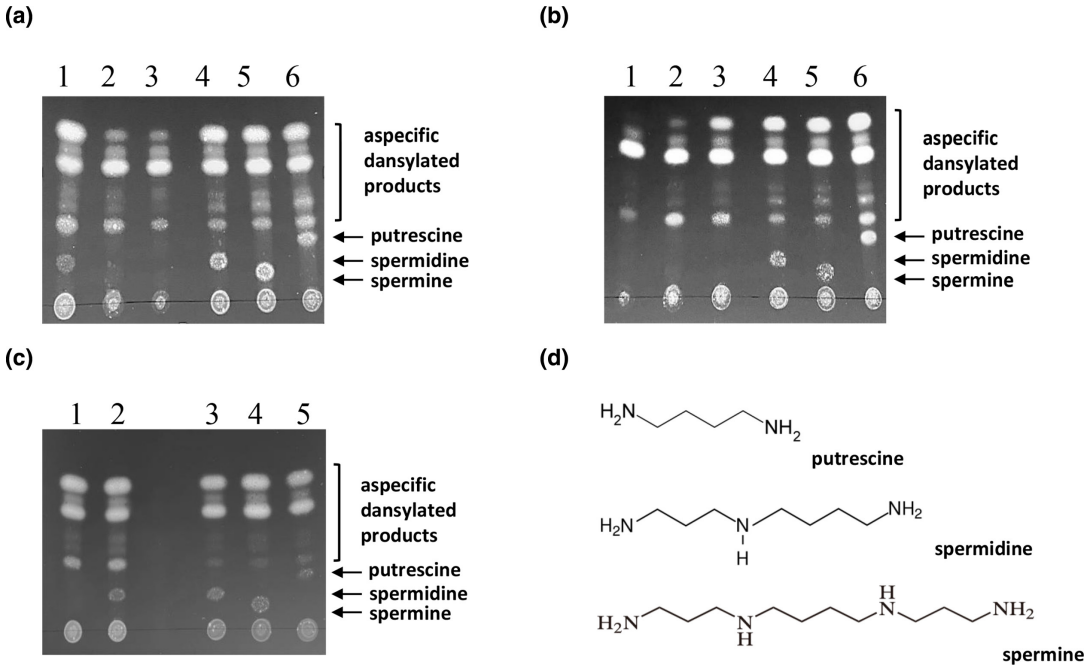

Fig. 4. Intracellular polyamine content of *S. agalactiae* at pH 4.0. *S. agalactiae* A909 (a), A909Δ*sak_1604* (b and c, lane 1) and A909Δ*sak_1604*-pTCV::*sak_1604* (c, lane 2) were grown to an OD_{600} of 0.6 (exponential growth phase) in a CDM at pH 7.4. After centrifugation, they were suspended in a CDM adjusted at pH 4.0 with 33.5 mM sodium citrate dihydrate and 66.5 mM citric acid in the presence of 1 mM spermidine (lanes a1, b1, c1 and c2), 1 mM spermine (lanes a2 and b2) or 1 mM putrescine (lanes a3 and b3). Bacterial extracts were dansylated, separated by TLC and photographed under a Wood's light. Dansylated standards of spermidine (0.2 µg; lanes a4, b4 and c3), spermine (0.2 µg; lanes a5, b5 and c4) and putrescine (0.1 µg; lanes a6, b6 and c5) were deposited in each TLC plate. Non-specific dansylated products that are always visualized correspond to products produced under the conditions of dansylation. They correspond to dansylmethylamine, dansyldimethylamine, dansylammnonia and dansylhydroxide. Other dansylated amines could also appear [26]. The semi-developed formula of the three polyamines separated by TLC are represented in (d).

We next tested the role of the SAK_1604 transporter in the above identified phenotype by comparing the survival capacity at pH 4.0 of the wild-type strain, the Δ*sak_1604* mutant, the Δ*sak_1604*Δ*potABCD* double mutant and the Δ*sak_1604* complemented strain, in the presence of 1 mM spermidine. Fig. 3(d) shows that the SAK_1604 transporter is involved in this phenotype as the Δ*sak_1604* and Δ*potABCD*Δ*sak_1604* mutants survived less than the wild-type and the Δ*sak-1604* complemented strains. We also confirmed that the introduction of the empty plasmid used for complementation has no effect on the behaviour of strain A909Δ*sak_1604* (Fig. S5). Furthermore, TLC analysis of the bacterial intracellular content of these strains confirms the role of SAK_1604 in the survival improvement as spermidine is imported into the wild-type and the complemented strains but not into the Δ*sak_1604* mutant during incubation at pH 4.0 (Fig. 4).

We then tested whether the pH 4.0 itself or the sodium citrate used for buffering the medium is a prerequisite for the survival improvement of *S. agalactiae* in the presence of spermidine. Thus, we compared the survival capacity of the wild-type strain and of the Δ*sak_1604* mutant in a CDM containing 1 mM spermidine either supplemented with 100 mM sodium citrate and then adjusted to a pH of 7.4 with HCl or only adjusted to pH 4.0 with HCl without the addition of sodium citrate. In these two conditions, no difference in the survival capacity of the wild-type and of the mutant strains could be detected (Fig. S6a, b). Thus, the SAK_1604 transporter is involved in the increase in resistance of *S. agalactiae* in the presence of spermidine in an environment maintained at pH 4.0 with the organic citric acid but not with the mineral HCl acid. We wondered whether this property can be generalized to another organic acid. Thus, we repeated the above experiments after having adjusted the medium at pH 4.0 with a 100 mM mix of sodium lactate and lactic acid. Again, no significant difference in the survival rate of the wild-type and the mutant strains was observed (Fig. S6c).

The acidification of the medium by citrate is, thus, an obligate prerequisite for allowing SAK_1604 to improve the survival of *S. agalactiae* in the presence of spermidine. The calculation of species concentration for citric acid at pH 4.0 indicates that dihydrogen citrate (H_2A^-) is largely the predominant form of this organic acid in solution (80% H_2A^-, 10% HA^{2-} and 10 % H_3A), whereas it is completely absent at pH 7.4 (5% HA^{2-}, 95% A^{3-}) [31].

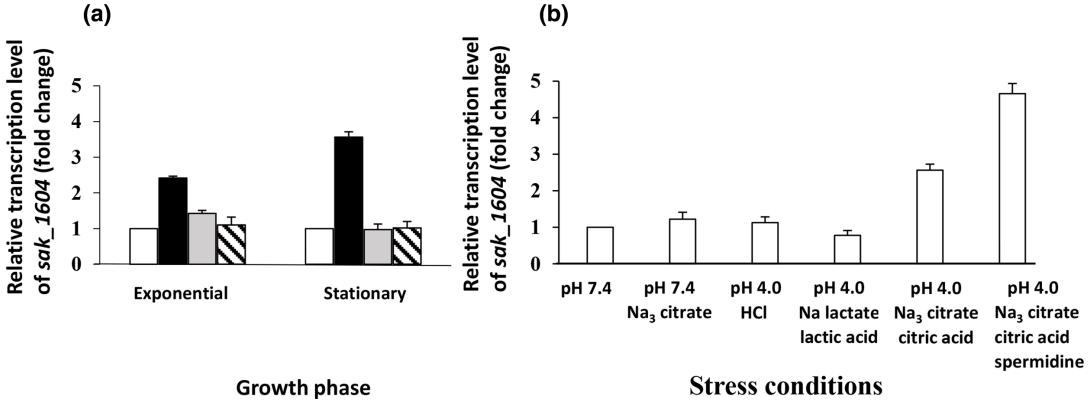

Fig. 5. Induction of the expression of *sak_1604* by spermidine and citric acid (pH 4.0). In (a), qRT-PCRs of *sak_1604* transcripts were performed on RNA extracts of *S. agalactiae* A909 grown at 37 °C to an OD_{600} of 0.6 (exponential phase) or to an OD_{600} of 1.2 (stationary phase) in a CDM buffered at pH 7.4 with 100 mM HEPES and supplemented with 1 mM spermidine (black bars), 1 mM spermine (grey bars) or 1 mM putrescine (striped bars). The level of transcripts was normalized in relation to the *recA* transcript levels. Gene expressions are presented as fold change with regards to the level of *sak_1604* transcripts during growth of *S. agalactiae* in the absence of polyamines (white bars). Results are presented as means±standard deviations of three independent experiments. In (b), qRT-PCRs of *sak_1604* transcripts were performed on RNA extracts of *S. agalactiae* A909 grown in CDM at pH 7.4 at 37 °C until OD_{600} of 0.6 (exponential phase), and then incubated for 30 min in CDM buffered at pH 4.0 with a 100 mM mix of sodium citrate and citric acid in the absence or in the presence of 1 mM spermidine, buffered at pH 4.0 with a mix of 100 mM sodium lactate and lactic acid, adjusted at pH 4.0 with HCl, or adjusted at pH 7.4 with HCl in the presence of 100 mM sodium citrate. The level of transcripts was normalized in relation to the *recA* transcript levels. Gene expressions are presented as fold change with regards to the level of *sak_1604* transcripts during growth of *S. agalactiae* at pH 7.4. Results are presented as means±standard deviations of three independent experiments.

Spermidine and citric acid at the vaginal pH induce the expression of the *sak_1604* gene

As spermidine is able to improve the resistance of *S. agalactiae* to citric acid at the vaginal pH via the SAK_1604 transporter, we searched whether the transcription of *sak_1604* is induced by both components. We first tested by qRT-PCR whether the transcription of *sak_1604* is induced during growth of the bacteria at pH 7.4 in a medium containing spermidine, spermine or putrescine. As shown in Fig. 5a, only spermidine was able to induce the expression of *sak_1604*, both during the exponential and the stationary phase of growth. We then tested whether *sak_1604* is induced by an acid stress in a medium adjusted at pH 4.0 with HCl, citric acid or lactic acid. As shown in Fig. 5b, the transcription of *sak_1604* is increased 2.5-fold during an acid stress generated by citric acid but not by an acid stress caused by HCl or lactic acid. The addition of spermidine during the acid stress induced by citric acid increased again the expression of *sak_1604* to a fivefold level. On the contrary, a stress made by 100 mM citrate at pH 7.4 did not induce the expression of *sak_1604*. These expression data correlate with the phenotypic results presented above and again indicate the importance of particular ionic forms of citric acid in this stress resistance mechanism.

DISCUSSION

Citric acid is a weak carboxylic acid naturally occurring in fruits. Due to its ability to inhibit growth of bacteria, it has been used, for hundreds of years, as a food additive. It is one of the most common additives in a majority of food and drink products. Citric acid is also described as a characteristic component of seminal plasma and of vaginal secretion, and is one of the components of many treatments for vaginosis [32, 33]. Although its specific function is not known, it may be involved in activating prostatic acid phosphatase and in the coagulation and liquefaction of semen. The mechanism of action of citric acid on the growth and survival of bacteria is not completely understood [34, 35]. It was proposed that sodium citrate, by its binding to cell wall components, disrupts the outer cell membrane of Gram-negative bacteria, resulting in leakage of cytoplasmic components. It was also suggested that sodium citrate chelates important divalent cations, such as Ca^{2+} and Mg^{2+}, leading to growth inhibition [35]. We found that growth of *S. agalactiae* is inhibited or strongly reduced in mediums adjusted to pH 7.4, pH 5.5 or pH 4.0 with citric acid (results not shown). Citrate anions are able to complex divalent ions, which are important factors influencing the growth of Gram-positive bacteria. However, the chelation property of citric acid should not be the same at all pH values. At pH 5.5 or 7.4, two or three of the three COOH groups of citric acid have lost the hydrogen, respectively, and are negatively charged, thereby attracting strongly positively charged divalent ions [31]. The strong chelation of these ions could have impaired the growth and the survival of *S. agalactiae* rendering the polyamines, which are also polycations, unable to counteract this effect. At pH 4.0, dihydrogen citrate is largely the predominant form of citric acid in solution [36]. Although dihydrogen citrate has the possibility still to form a complex with certain positive divalent ions, it has a low complexing power because only one of the three COOH groups has lost the hydrogen (R. Jastrzab, personal communication; [37]). We found that at pH 4.0, spermidine is able to counteract in part the inhibitory effect of dihydrogen citrate (Fig. 3a). However, it is unlikely

that this effect is due to the counteraction of the complexing property of dihydrogen citrate as other polyamines, such as spermine and putrescine, are unable to counteract dihydrogen citrate and because dihydrogen citrate has a low complexity power (Fig. 3b, c).

Dihydrogen citrate (H_2A^-) and the non-ionic form of citric acid (H_3A) are the only forms of citric acid present at pH 4.0 and absent at pH 7.4 [33]. As the acidification of the medium by HCl and by lactic acid or the presence of citrate ions at pH 7.4 have no effect on the upregulation of *sak_1604* (Fig. S6), either dihydrogen citrate or the non-ionic form of citric acid should be inductor(s) of *sak_1604* expression. However, as dihydrogen citrate is largely the predominant form of citric acid at pH 4.0 (80% of the three forms present), it is probable that it has a major role in the resistance of *S. agalactiae* to low pH by allowing the import of spermidine via the SAK_1604 transporter. As shown in Fig. 4, our TLC analyses confirm the implication of SAK_1604 in spermidine uptake at pH 4.0. Spermidine is present in the normal vaginal metabolome. An increase in the vaginal concentration of polyamines is considered as a clinical feature of vaginosis [38]. This increase is due to the synthesis of polyamines by the various micro-organisms that proliferate during vaginosis. The vaginal environment contains, thus, both the dihydrogen citrate and spermidine molecules favouring the survival of *S. agalactiae* in this niche.

To our knowledge, genes encoding proteins involved in citrate uptake in *S. agalactiae* had not been described in the literature. However, we determined two genes encoding a two-component system of the CitA/CitB family (SAK_1880 and SAK_1881) in the KEGG database for strain A909. CitA/CitB has been identified in *E. coli* as the signal transduction component of a two-component system for citrate in which CitT acts as a citrate transporter [39]. In strain A909, SAK_1880 and SAK_1881 are adjacent to a gene encoding a protein of the citrate : cation symporter (CSS) family protein (SAK_1879). The citric acid cycle (TCA cycle) is nevertheless completely missing in *S. agalactiae*, depriving this bacterium of the ability to synthesize the precursors of most amino acids [40]. The molecular mechanism allowing *S. agalactiae* to internalize spermidine to better resist against citric acid stress at pH 4.0 is for the moment unknown. Nevertheless, studies made in *E. coli* can give certain clues. In conjunction with Mg^{2+}, spermidine forms complexes with RNA and stabilizes higher orders structures. Its binding to RNA can cause unique structural change, different of those enhanced by Mg^{2+}. Spermidine was described to be more crucial than Mg^{2+} for a maximum synthesis of polypeptides synthesis. This is due to its ability to stimulate the polypeptide synthesis at the level of aminoacyl-tRNA binding to ribosomes [41]. In addition, by its binding to ribosome, spermidine increases the fidelity of codon usage during protein synthesis [42]. Furthermore, by its binding to a G+C-rich double-stranded region near the Shine–Dalgarno region of the mRNA of the periplasmic substrate-binding protein of the oligopeptide uptake system, spermidine enhances the translation of this mRNA [43]. The interaction of spermidine with nucleic acids has also been shown to affect the stability of dsDNA and to protect it from oxidative stress, damaging agents, ionizing radiation and endonuclease digestion [44]. Thus, the role of spermidine in the resistance of *S. agalactiae* to acidic pH may be based on the protection of nucleic acids from damage, and/or to a stabilization of mRNA and ribosomes for a better transcription and translation under this harsh condition. However, further investigations are needed to better comprehend the mechanism of protection of spermidine in these conditions. In conclusion, our data open new perspectives on the comprehension of the molecular mechanisms allowing *S. agalactiae* to survive at the physiological pH of the vagina and on the unsuspected role of an ionic form of citric acid.

Funding information
S.K. was supported by PhD fellowships of the Lebanese University and AZM and SAAD, and of the Lebanese Association for Scientific Research (LASeR), and by funding from the Human Resources Department (International Mobility Service) of INRAE and from the University Foundation Rabelais (University of Tours, France).

Conflicts of interest
The authors declare that there are no conflicts of interest.

References

1. Cohen S. *A Guide to the Polyamines*. New York: Oxford University Press; 1998.
2. Tabor CW, Tabor H. Polyamines. *Annu Rev Biochem* 1984;53:749–790.
3. Miyamoto S, Kashiwagi K, Ito K, Watanabe S, Igarashi K. Estimation of polyamine distribution and polyamine stimulation of protein synthesis in *Escherichia coli*. *Arch Biochem Biophys* 1993;300:63–68.
4. Shah P, Romero DG, Swiatlo E. Role of polyamine transport in *Streptococcus pneumoniae* response to physiological stress and murine septicemia. *Microb Pathog* 2008;45:167–172.
5. Hou MH, Lin SB, Yuann JM, Lin WC, Wang AH, *et al*. Effects of polyamines on the thermal stability and formation kinetics of DNA duplexes with abnormal structure. *Nucleic Acids Res* 2001;29:5121–5128.
6. Lee YH, Kim BH, Kim JH, Yoon WS, Bang SH, *et al*. CadC has a global translational effect during acid adaptation in *Salmonella enterica* serovar Typhimurium. *J Bacteriol* 2007;189:2417–2425.
7. Moreau PL. The lysine decarboxylase CadA protects *Escherichia coli* starved of phosphate against fermentation acids. *J Bacteriol* 2007;189:2249–2261.
8. Shah P, Swiatlo E. A multifaceted role for polyamines in bacterial pathogens. *Mol Microbiol* 2008;68:4–16.
9. Lancefield RC. A serological differentiation of human and other groups of hemolytic streptococci. *J Exp Med* 1933;57:571–595.
10. Schuchat A. Epidemiology of group B streptococcal disease in the United States: shifting paradigms. *Clin Microbiol Rev* 1998;11:497–513.
11. Phares CR, Lynfield R, Farley MM, Mohle-Boetani J, Harrison LH, *et al*. Epidemiology of invasive group B streptococcal disease in the United States 1999-2005. *JAMA* 2008;299:2056–2065.
12. Verani JR, McGee L, Schrag SJ, Division of Bacterial Diseases, National Center for Immunization and Respiratory Diseases, Centers for Disease Control and Prevention (CDC). Prevention of perinatal group B streptococcal disease – revised guidelines from CDC, 2010. *MMWR Recomm Rep* 2010;59:1–36.

13. van der Mee-MarquetN, Domelier A-S, Salloum M, Violette J, Arnault L, *et al*. Molecular characterization of temporally and geographically matched *Streptococcus agalactiae* strains isolated from food products and bloodstream infections. *Foodborne Pathog Dis* 2009;6:1177–1183.
14. Khazaal S, Al Safadi R, Osman D, Hiron A, Gilot P. Investigation of the polyamine biosynthetic and transport capability of *Streptococcus agalactiae*: the non-essential PotABCD transporter. *Microbiology* 2021;167:001124.
15. Igarashi K, Kashiwagi K. Characteristics of cellular polyamine transport in prokaryotes and eukaryotes. *Plant Physiol Biochem* 2010;48:506–512.
16. Igarashi K, Kashiwagi K. Polyamine transport in *Escherichia coli*. *Amino Acids* 1996;10:83–97.
17. Soksawatmaekhin W, Kuraishi A, Sakata K, Kashiwagi K, Igarashi K. Excretion and uptake of cadaverine by CadB and its physiological functions in *Escherichia coli*. *Mol Microbiol* 2004;51:1401–1412.
18. Gao X, Zhou L, Jiao X, Lu F, Yan C, *et al*. Mechanism of substrate recognition and transport by an amino acid antiporter. *Nature* 2010;463:828–832.
19. Krammer E-M, Ghaddar K, André B, Prévost M. Unveiling the mechanism of arginine transport through AdiC with molecular dynamics simulations: the guiding role of aromatic residues. *PLoS One* 2016;11:e0160219.
20. Krammer E-M, Prévost M. Function and regulation of acid resistance antiporters. *J Membr Biol* 2019;252:465–481.
21. Khazaal S, Al Safadi R, Osman D, Hiron A, Gilot P. Dual and divergent transcriptional impact of IS1548 insertion upstream of the peptidoglycan biosynthesis gene murB of *Streptococcus agalactiae*. *Gene* 2019;720:144094.
22. Sambrook J, Russell DW. *Molecular Cloning: a Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory; 2001.
23. Dower WJ, Miller JF, Ragsdale CW. High efficiency transformation of *E. coli* by high voltage electroporation. *Nucleic Acids Res* 1988;16:6127–6145.
24. Ricci ML, Manganelli R, Berneri C, Orefici G, Pozzi G. Electrotransformation of *Streptococcus agalactiae* with plasmid DNA. *FEMS Microbiol Lett* 1994;119:47–52.
25. Biswas I, Gruss A, Ehrlich SD, Maguin E. High-efficiency gene inactivation and replacement system for gram-positive bacteria. *J Bacteriol* 1993;175:3628–3635.
26. Poyart C, Trieu-Cuot P. A broad-host-range mobilizable shuttle vector for the construction of transcriptional fusions to beta-galactosidase in gram-positive bacteria. *FEMS Microbiol Lett* 1997;156:193–198.
27. Pedrol N, Tiburcio F. A. Polyamines determination by TLC and HPLC. In: *Handbook of Plant Ecophysiology Techniques*. Kluwer: Dordrecht, 2001. pp. 335–363.
28. Swinscow TDV. *Statistics at Square One*, 4th edn. London: BMA; 1978.
29. Tomitori H, Kashiwagi K, Igarashi K. Structure and function of polyamine-amino acid antiporters CadB and PotE in *Escherichia coli*. *Amino Acids* 2012;42:733–740.
30. Kitko RD, Wilks JC, Garduque GM, Slonczewski JL. Osmolytes contribute to pH homeostasis of *Escherichia coli*. *PLoS One* 2010;5:e10078.
31. Elbagerma MA, Alajtal AI, Edwards HGM, Azimi GH, Verma KD, *et al*. Raman spectroscopic and potentiometric studies of acidity level and dissociation of citric acid in aqueous solution. *J Appl Chem Sci Int* 2015;2:1–11.
32. Gavella M. A simple automated method for determination of citric acid levels in semen. *Int J Androl* 1983;6:585–591.
33. Martin PD, Cheshire SK. A comparative study of the citrate and lactate concentrations in stains from semen, vaginal secretion and mixtures of the two using isotachophoresis. In: Brinkmann B and Henningsen K (eds). *11th Congress of the Society for Forensic Haemogenetics (Gesellschaft Für Forensische Blutgruppenkunde e.V.), Advances in Forensic Haemogenetics*. Berlin, Heidelberg: Springer; 1986. pp. 299–303.
34. Sinha DP, Drury AR, Conner GH. The *in vitro* effect of citric acid and sodium citrate on *Streptococcus agalactiae* in milk. *Indian Vet J* 1968;45:805–810.
35. Nagaoka S, Murata S, Kimura K, Mori T, Hojo K. Antimicrobial activity of sodium citrate against *Streptococcus pneumoniae* and several oral bacteria. *Lett Appl Microbiol* 2010;51:546–551.
36. Zhao B, Houry WA. Acid stress response in enteropathogenic gammaproteobacteria: an aptitude for survival. *Biochem Cell Biol* 2010;88:301–314.
37. Zabiszak M, Nowak M, Taras-Goslinska K, Kaczmarek MT, Hnatejko Z, *et al*. Carboxyl groups of citric acid in the process of complex formation with bivalent and trivalent metal ions in biological systems. *J Inorg Biochem* 2018;182:37–47.
38. Nelson TM, Borgogna J-L, Brotman RM, Ravel J, Walk ST, *et al*. Vaginal biogenic amines: biomarkers of bacterial vaginosis or precursors to vaginal dysbiosis? *Front Physiol* 2015;6:253.
39. Scheu PD, Witan J, Rauschmeier M, Graf S, Liao Y-F, *et al*. CitA/CitB two-component system regulating citrate fermentation in *Escherichia coli* and its relation to the DcuS/DcuR system in vivo. *J Bacteriol* 2012;194:636–645.
40. Glaser P, Rusniok C, Buchrieser C, Chevalier F, Frangeul L, *et al*. Genome sequence of *Streptococcus agalactiae,* a pathogen causing invasive neonatal disease. *Mol Microbiol* 2002;45:1499–1513.
41. Igarashi K, Sugawara K, Izumi I, Nagayama C, Hirose S. Effect of polyamines of polyphenylalanine synthesis by *Escherichia coli* and rat-liver ribosomes. *Eur J Biochem* 1974;48:495–502.
42. Ito K, Igarashi K. The increase by spermidine of fidelity of protamine synthesis in a wheat-germ cell-free system. *Eur J Biochem* 1986;156:505–510.
43. Yoshida M, Meksuriyen D, Kashiwagi K, Kawai G, Igarashi K. Polyamine stimulation of the synthesis of oligopeptide-binding protein (OppA). Involvement of a structural change of the Shine-Dalgarno sequence and the initiation codon AUG in OppA mRNA. *J Biol Chem* 1999;274:22723–22728.
44. Kabir A, Suresh Kumar G. Binding of the biogenic polyamines to deoxyribonucleic acids of varying base composition: base specificity and the associated energetics of the interaction. *PLoS One* 2013;8:e70510.
45. Tettelin H, Masignani V, Cieslewicz MJ, Donati C, Medini D, *et al*. Genome analysis of multiple pathogenic isolates of *Streptococcus agalactiae*: implications for the microbial "pan-genome" *Proc Natl Acad Sci USA* 2005;102:13950–13955.

Edited by: W. van Schaik and F. M. Commichau