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Escudero JA, San Millan A, Gutierrez B, Hidalgo L, La Ragione RM, AbuOun M, Galimand M, Ferrándiz MJ, Domínguez L, de la Campa AG, Gonzalez-Zorn B.

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Fluoroquinolone efflux in *Streptococcus suis* is mediated by SatAB and not by SmrA

Jose Antonio ESCUDERO,[1, 2] Alvaro SAN MILLAN,[1, 2] Roberto LA RAGIONE,[3]

María José FERRANDIZ,[5] Lucas DOMINGUEZ,[2] Patrice COURVALIN,[4] Adela G. DE LA CAMPA,[5] and Bruno GONZÁLEZ-ZORN [1, 2,*]

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7 [1] *Departamento de Sanidad Animal, Facultad de Veterinaria, Universidad Complutense* 8 *de Madrid, Madrid, Spain*

9 [2] *Centro de Vigilancia Sanitaria Veterinaria (VISAVET), Universidad Complutense de* 10 *Madrid, Madrid, Spain*

11 [3] *Department of Food and Environmental Safety, Veterinary Laboratories Agency (VLA),*

12 *Weybridge, New Haw, Addlestone, Surrey KT15 3NB, UK.*

13 [4] *Institut Pasteur, Unité des Agents Antibactériens, Paris, France.*

14 [5] *Unidad de Genética Bacteriana, Centro Nacional de Microbiología, and CIBER*

15 *Enfermedades Respiratorias, Instituto de Salud Carlos III, 28220 Majadahonda,*

16 *Madrid, Spain.*

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20 *Correspondence: Departamento de Sanidad Animal, Facultad de Veterinaria,

21 Universidad Complutense de Madrid, 28040, Madrid, Spain. Tel.: +34 91 3943707. Fax:

22 +34 91 3943908. E-mail: bgzorn@vet.ucm.es

ABSTRACT

Streptococcus suis **is an emerging zoonotic pathogen. Lacking an effective vaccine, antibiotics remain the main tool to fight against infections caused by these bacteria. We have previously observed a reserpine-sensitive fluoroquinolone-efflux phenotype in this species. Here, SatAB and SmrA, two pumps belonging to the ATP Binding Cassette and the Major Facilitator Superfamily, respectively, have been analyzed in the fluoroquinolone-resistant clinical isolate BB1013. Genes encoding these pumps were overexpressed constitutively or in the presence of ciprofloxacin in this strain. However, these genes could not be cloned in plasmids in *E. coli* despite strong expression repression. Finally, site directed insertion of *smrA* and *satAB* in the *amy* locus of *Bacillus subtilis* chromosome using ligated PCR amplicons allowed for the functional expression and study of both pumps. Results showed that SatAB is a narrow spectrum fluoroquinolone exporter (norfloxacin and ciprofloxacin), susceptible to reserpine, whereas SmrA was not involved in fluoroquinolone extrusion. Direct chromosomal integration in *Bacillus* is a novel method for studying efflux pumps from Gram-positive bacteria, which allowed to demonstrate the implication of SatAB, but not of SmrA, in fluoroquinolone efflux in *S. suis*.**

INTRODUCTION

Streptococcus suis is a worldwide distributed zoonotic agent affecting pig and man (19). In industrialized countries it is mainly regarded as an important economic burden to the pig industry that only sporadically causes infections in humans. It is therefore defined as an occupational zoonosis where risk groups are people in close contact with pigs and pork. Nevertheless, cases of *S. suis* meningitis have also been reported in patients with no history of

contact with live pigs or their products (19, 24). In several developing countries in South East Asia, *S. suis* is one of the main causes of meningitis in humans, even though its prevalence is probably still under-estimated (18, 20, 36). Several outbreaks of *S. suis* have occurred in China (42). The largest outbreak took place during the summer of 2005 in Sichuan province, and affected 204 people causing 38 fatalities. Mortality was high as a result of the onset of a Toxic Shock

Syndrome (STSS) similar to the one produced by Group A streptococci (GAS) (37). Consequently, concern about this pathogen has risen in these areas, and measures to prevent or hinder its impact are already being taken (18).

Antimicrobial resistance is one of the most concerning phenomena in infectious diseases. In *S. suis*, resistance to neomycin, spiramycin, lincomycin, trimethoprimsulphamethoxazole, erythromycin and tetracycline antibiotics is a common feature (30, 33, 41). Interestingly, genome comparison of STSS-producing strains has led to the discovery of ICE*Ssu*$_{BM407}$2, a putative mobile Pathogenicity Island bearing tetracycline resistance determinants TetL and TetO, erythromycin ribosome methylase ErmB, a chloramphenicol acetyltransferase and a dihydrofolate reductase (17). Treatment of infections caused by *S. suis* remain, in general, effective by administration of beta-lactam antibiotics. Nevertheless, strains showing resistance to this family of drugs have been reported (14, 33). Fluoroquinolones are the main alternative to beta-lactam antibiotics for treatment of streptococcal infections (9). We have reported increasing fluoroquinolone (FQ) resistance among clinical *S. suis* isolates in Spain (8). Substitutions in the quinolone resistance determining regions (QRDR) of the genes encoding the antibiotic targets, *gyrA* and *parC*, were identified (8). Interestingly the FQ-resistance phenotype could be partially reduced by the efflux pump inhibitor reserpine, indicating that efflux may be involved in FQ-resistance in *S. suis*. Several efflux pumps conferring resistance to fluoroquinolones have been described in other Gram-positive bacteria. Mainly members of two families of transporters are involved: (i) proteins belonging to the Major Facilitator Superfamily (MFS), such as Bmr of *Bacillus subtilis* (27), NorA of *Staphylococcus aureus* (38), Lde from *Listeria monocytogenes* (13), and PmrA in

Streptococcus pneumoniae (12); and (ii) exporters belonging to the vast ATP Binding Cassette (ABC) family, such as PatA and PatB from *S. pneumoniae* (26) or LmrA from *Lactococcus lactis* (39). Efflux-mediated drug resistance is normally a consequence of overexpression of the genes encoding the pumps, either through mutations in regulators or in promoter regions thereof (28). Amino acid substitutions can also lead to resistance through a change in the substrate recognition of the pump (29, 31).

To date, no FQ efflux pumps have been described in *S. suis*. Here we report the implication of SatAB, an ABC transporter homologous to PatA and PatB, in resistance to ciprofloxacin and norfloxacin in *S. suis*. Further, intensive study of SmrA, a Major

Facilitator Superfamily transporter analogous to pneumococcal FQ-efflux pump PmrA, allows for neglecting its role in FQ-resistance. Study of these pumps was accomplished with a knock-in strategy, integrating the genes encoding the pumps in monocopy in the chromosome of *Bacillus subtilis* 168 under the control of an inducible promoter. This system has proven to be a promising tool for the study of efflux pumps in Gram-positive bacteria.

MATERIALS AND METHODS

Bacterial strains, culture conditions and susceptibility testing

The characteristics of the strains and plasmids used in this study are listed in Table 1. *B. subtilis* 168 and pDR67 were kindly donated by Tarek Msadek from *Institut Pasteur* in Paris.

S. suis and *S. pneumoniae* strains were grown on Columbia sheep blood agar plates, Todd-Hewitt broth and a casein hydrolysate-based medium with 0.3% sucrose (AGCH) as energy source. *B. subtilis* 168 was grown on LB. Chloramphenicol was added to a final concentration of 5 μg/ml and 2.5 μg/ml for *B.* subtilis and *S. pneumoniae* transformants respectively. Growth media were obtained from Oxoid (Oxoid Ltd., Basingstoke, United Kingdom) and Biomérieux (BioMérieux, France). Chemicals, additives and antibiotics were supplied by Merck (Merck KGaA, Darmstadt, Germany) and Sigma-Aldrich (Sigma Chemical Co. St Louis, Mo). IPTG was purchased from Invitrogen (Invitrogen, Carlsbad, CA).

Susceptibility tests were carried out following CLSI guidelines (6). Efflux phenotype visualization was performed by adding 40 μg of ciprofloxacin, enrofloxacin and levofloxacin, and 100 μg of norfloxacin, in 10 μl volume, to non-impregnated discs that were used for diffusion antibiograms on standard media with and without 10 μg/ml of reserpine dissolved in acetone. Non-impregnated and antibiotic discs were obtained from Biomerieux (BioMérieux, France) and

Oxoid (Oxoid Ltd., Basingstoke, United Kingdom). For sugar assays, API 50CH were obtained from Biomérieux and used following manufacturer's instructions.

DNA analysis and manipulation

Primers used in this study were supplied by Roche (Roche, Germany), and SigmaGenosys (Sigma Chemical Co. St Louis, Mo). A list of the primers used can be found in Table 2. PCRs were performed using *Taq* Polymerase from Biotools (B&M Labs, Spain). Phusion polymerase (Finnzymes, Woburn, MA) was used for the obtention of long amplicons. pCR2.1 and pBAD plasmids and *E. coli* IncF' (Top10) strain were purchased from Invitrogen (Invitrogen, Carlsbad, CA). Plasmid DNA extraction and purification of

PCR fragments were performed using Plasmid Midi, QIAprep Spin Miniprep, Qiagen PCR purification or Gel extraction Kits (Qiagen, Inc., Chatworth, California, USA). For plasmid extraction from *S. pneumoniae* and *S. suis* a modified first step with sodium deoxycholate was used as previously described (35). Automated sequencing was carried out at Secugen S. L. (Madrid, Spain). Sequence analysis was performed using DNA Strider 1.4f13 (CEA, France), 4Peaks 1.6 (Mek&Tosj, Netherlands), CLC DNA Workbench Software (Denmark), ClustalW, NEBCutter (40) and NIH online analysis tools (http://www.ncbi.nlm.nih.gov). Promoter sequence analysis was performed with Bprom (Softberry Inc. Mount Kisco, NY). Protein modeling was done using Phyre server (22), and illustrated using PyMol Molecular Graphics System, (Version 1.3, Schrödinger, LLC.).

RT-PCR

RNA from all strains was obtained from liquid cultures in early exponential phase (DO_{600}=0.3). When ciprofloxacin was added to the growth media, concentrations used were ¼ of the MIC of each strain. RNA extractions were performed twice from each strain and condition using RNA-easy Kit (Qiagen, Inc., Chatworth, California, USA).

Digestions with DNase were performed following manufacturers instructions. Absence of DNA was checked through PCR. Several digestions were performed until PCR results obtained were negative. cDNA was obtained using Superscript III retrotranscriptase (Invitrogen, Carlsbad, CA) and 6bp random primers. Presence of cDNA was confirmed with conventional PCR. RT-PCR primers (Table 2) were designed using Allele ID 7.7 software (Premier Biosoft, Palo Alto, CA). Q-PCR was carried out in triplicates using MyiQ thermal cycler (Bio-Rad Laboratories Inc. Spain). Every condition and strain was tested three times. No primer dimer was obtained in any of the PCRs. Efficiency of all assays was 100% ± 5%. Results on the expression of *smrA* and *satAB* were normalized using *rpoB.* Primers rpoB428 and rpoB468R, targeting the *rpoB* gen of *S. pneumoniae*, have been previously described (4).

Cloning of *smrA* and *satAB* in *B. subtilis* chromosome

In order to clone *smrA* and *satAB* in pDR67, XbaI and BglII restriction sites were used. Amplification of the ORFs including their putative RBSs was carried out using primers designed to bear both restriction sites (Table 2). Plasmid and inserts were digested using XbaI and BglII (Takara Bio, Inc., Japan) and ligated using T4 DNA ligase (New England Biolabs, Beverley, MA). Ligation product was directly transformed in *B. subtilis* using a two-step transformation procedure as previously described (15). Colonies were phenotypically tested for pDR67 integration in the chromosome. Briefly, *amyE* disruption leads to the loss of alpha amylase activity. This can be evidenced by growth on 1% potato starch and staining with iodine pearls. Clones not showing a light halo around the colonies were selected, for its amylase activity was lost, presumably because of pDR67 integration (Figure 5). These observations were confirmed by PCR and sequencing.

Mobilization of the expression system bearing *smrA* to pJS3

Streptococcal plasmid pJS3 was amplified by PCR with primers bearing EcoRI and BamHI restriction sites located close together and facing outwards (Table 2). The product was phosphorilated using polinucleotide kinase, ligated using T4 DNA ligase and transformed in *S. pneumoniae* R6 as previously described (23). The resulting plasmid was called pJS3EB and bore both restriction sites plus a new unique NdeI site in between. P_{spac}-*lacI* and P_{spac}-*smrA-lacI* from strains BB1066 and BB1067 respectively, were amplified using primers with EcoRI and BamHI restriction sites (Table 2). Both inserts and pJS3EB were digested using EcoRI and BamHI and ligated with T4 DNA ligase. All enzymes were purchased from New England Biolabs (New England Biolabs, Beverley, MA).

Influence of pH in the activity of SmrA

LB was used as growth media and pH was adjusted to 5, 6, 7 and 8 adding HCl or NaOH after autoclaving. Inocula were adjusted to 0.5 MacFarland and diluted 1:200 in the growth medium. IPTG was added to a final concentration of 1mM. Ciprofloxacin and norfloxacin were tested covering a wide range of concentrations from subinhibitory to inhibitory (0.02 to 0.3 μ g/ml for ciprofloxacin and from 0.06 to 0.8 μ g/ml for norfloxacin). Growth curves were performed in a Tecan Infinite 200 apparatus (Tecan Group Ltd. Männendorf Switzerland). 24-well plates were incubated at 37ºC and OD_{620} measurements were taken every 10 minutes after 15 seconds shaking with 2.5 mm amplitude.

BIOLOG

Biolog phenomic assays were performed at the Veterinary Laboratories Agency, Weybridge, UK. Strains were grown on agar plates under selective pressure as described above. Cell suspensions were prepared as previously described (2) to directly obtain an 81% transmittance (T). Inoculating fluids were available from Biolog. Inocula were prepared following manufacturer's instructions and IPTG was added to a final concentration of 1 mM for *B. subtilis* and 0.5 mM for *S. pneumoniae*. All assays were performed at least twice. Colorimetric readings were performed during incubation at 37ºC for 48h with OmniLog reader. Kinetic data were analyzed with OmniLog PM software

(Biolog). Microarrays used in both *B. subtilis* and *S. pneumoniae* constructions included PM1 and 2A for carbon pathways, and PM11A to 20B for sensitivity to 240 chemicals at 4 different concentrations. Furthermore *S. pneumoniae* constructions were also tested against panels PM9 and 10 for pH and ion/osmotic effects.

Nucleotide sequence accession numbers

The nucleotide sequences of this study have been deposited in GenBank under the following accession numbers: *smrA*, including the promoter region, in BB1001, JF416696; in BB1002, JF416694; and in BB1013, JF416698; and *satRAB* in BB1001, JF416697; in BB1002, JF416695; and in BB1013, JF416699.

RESULTS

Efflux is involved in FQ resistance in *S. suis*

In our previous work *S. suis* isolates of animal origin showing high level FQ resistance were studied (8). These isolates carried resistance mutations in the quinolone resistance determining regions (QRDR) of both *gyrA* and *parC*, genes encoding the antibiotic targets. In order to assess the involvement of drug efflux in the FQ-resistance phenotype, ciprofloxacin MIC were determined with and without the efflux pump inhibitor reserpine. Briefly, in all but one resistant isolates, resistance levels were 2 to 4fold lower in the presence of reserpine, indicating that efflux was involved in FQresistance in these strains. No decrease in the MIC was observed in any of the FQsusceptible strains in presence of reserpine. To unequivocally prove these results, a diffusion antibiogram method that enabled a clear visualization of the efflux phenotype was developed. Custom made antibiogram discs bearing high concentrations of various fluoroquinolones were used on standard media with and without reserpine against BB1013, an isolate with ciprofloxacin MICs of 64 and 16 μ g/ml in the absence and presence of reserpine, respectively, known to also carry ParC Ser79Tyr and GyrA Ser81Lys changes involved in FQ-resistance (8). Clear growth-inhibition zones were obtained to ciprofloxacin and norfloxacin only when reserpine was added, showing the existence of reserpine-sensitive FQ-efflux. However, resistance to enrofloxacin and levofloxacin was unaffected by reserpine (Figure 1).

Identification of *smrA* and *satAB*

Three FQ efflux pumps have been widely studied in the close member of the genus *S. pneumoniae*. PmrA, first described as a norfloxacin extruder (12), is an MFS transporter, whereas PatA and PatB are ABC transporters involved in ciprofloxacin and norfloxacin efflux among other

compounds (26). We searched for homologues of these pumps in the available *S. suis* genomes, in order to study their possible implication in FQ resistance in this species.

smrA: an ORF encoding a predicted protein with a 58% identity with PmrA was found in the genome of *S. suis* and named *smrA*. The genetic environment of *smrA* was analogous to that of *pmrA*. Protein modeling of the 401 amino acid-long product, SmrA, revealed a 12 transmembrane segments structure found in most MFS transporters (Figure 3). The complete sequence of the gene with the corresponding promoter region was obtained from resistant strain BB1013 and from the two susceptible, genetically unrelated, clinical isolates BB1001, and BB1002 (8). SmrA of BB1013 presented two residue substitutions (Thr107Ala and Ile126Val) when compared to the susceptible alleles, which were identical between each other (Figure 3). The promoter region of *smrA* presented a canonical -35 box (TTGACAA) and Ribosome Binding Site (RBS) (GGAGG) at positions -66 and -14 (Figure 4). No -10 box was identified. Interestingly, between the -35 box and the region where the -10 box should be located, a 7-bp inverted repeat, separated by 5 bp, was found. When any 2 of the 5 bp are erased *in silico* a sequence resembling a -10 box is found immediately after the 3' repeat, suggesting *smrA* could be regulated the way *bmr* is (16). A mutation in the 3' inverted repeat was found in the sequence of the resistant strain suggesting a possible deregulation of *smrA* in BB1013. Further experiments were focused on elucidating whether overexpression of *smrA* or/and the amino acidic substitutions found in $SmrA^{BB1013}$ could be involved in fluoroquinolone resistance in BB1013.

satA and satB: two homologues of pneumococcal FQ-efflux pumps PatA and PatB, showing 66% and 67% identity, were identified in *S. suis* genomes and named SatA and SatB. Protein modeling revealed an N-terminal transmembrane domain and a Cterminal nucleotide binding domain structure characteristic of ABC transporters (Figure 3). Interestingly, in contrast to the pneumococcal organization of the ORFs, in wich these genes seem to be independently cotranscribed, *satA* and *satB* showed in *S. suis* a classical operon conformation, with both genes immediately contiguous on the same strand and reading frame. Furthermore, upstream *satA* and partly overlapping with it, an ORF coding for a regulator of the MarR family was found and named *satR*. To determine if *satA* and *satB* were cotranscribed on the same mRNA a PCR from one gene to the other, using primers SatAIntF and SatBIntR (Table 2) was performed on cDNA from BB1001 and BB1013. A 2 kb fragment was amplified from the cDNA and the control DNA of the two strains, proving the suspected operon conformation of both ORFs (data not shown). This organization points to the function of SatA and SatB being intimately related and is in accordance to other authors suggestion of a heterodimeric SatAB transporter (11). Primers satAF and satBR, amplifying the whole operon and its regulator, were designed using the available *S. suis* genomes in GenBank.

The sequences of *satR* and *satAB* of BB1001, BB1002 and BB1013 were obtained by primer walking and analyzed. Several amino acid differences, mainly located in the transmembrane segments, were found among the three alleles of both genes (Figure 4). Interestingly, sequence analysis of the putative regulator SatR revealed in BB1013 a C238T mutation leading to a premature stop codon (CAA80TAA) compared to BB1001, BB1002 and GenBank sequences.

Expression analysis

Efflux pumps can lead to antimicrobial resistance through overexpression. In order to assess if *smrA* and *satAB* where overexpressed in BB1013 levels of mRNA of these pumps were measured by means of RT-Q-PCR using BB1001 as a control (Figure 2).

Expression of *smrA* was not significantly different between BB1013 and the control strain in the absence of fluoroquinolones. When subinhibitory concentrations of ciprofloxacin were added to the growth culture, to a final concentration of ¼ of the MIC (0,12 μg/ml for BB1001 and 16 μg/ml for BB1013), BB1013 increased the levels of expression of *smrA*, leading to a statistically significant four-fold difference between both strains.

Expression of *satAB* was found to be about fifteen times higher in BB1013 than in the control strain in the absence of ciprofloxacin. Interestingly, expression was not enhanced, but mildly repressed by the presence of ciprofloxacin in the growth media.

Expression analysis showed that both pumps were overexpressed in BB1013, either under induction by ciprofloxacin, as is the case of SmrA, or constitutively as for SatAB. Moreover, these data suggest that *satR* acts as a repressor of *satAB* in *S. suis*.

Cloning and expression of *smrA* and *satAB* in *B. subtilis*

Cloning of *satAB* and *smrA* in *E. coli*, as performed successfully with other pumps such as NorA or Bmr (27, 38), was investigated. All assays were negative when trying to clone the genes encoding the putative pumps in pCR2.1 plasmid and *E. coli* IncF' strain. Cloning under the control of pBAD promoter was tested. Despite enhanced repression by addition of G-6-P (glucose-6-phosphate) and fucose, cloning of *S. suis* efflux pumps was unsuccessful in all cases, suggesting a high toxicity of the insert in a Gram-negative environment. Thus, cloning in *B. subtilis* was explored as an alternative strategy.

pDR67 (21) is a plasmid capable of replicating in *E. coli* and integrating in *Bacillus subtilis* chromosome. It is designed for cloning inserts under the control of the IPTG-inducible promoter P_{spac}. Expression is tightly repressed by the presence, downstream the insert, of the promoter's operator, *lacI*. Furthermore, repression can be strengthened using $lacI^q$ *E. coli* strains (such as XL1-Blue). pDR67 bears two homology regions with the non-essential *amyE* chromosomal gene of *B. subtilis*, as well as a chloramphenicol marker suitable for selection in this species. It can therefore be used for mobilizing constructions obtained in *E. coli* to a Gram-positive environment as a single copy in the chromosome. IPTG induction is also feasible in this bacterium.

smrA and *satAB* were cloned with their corresponding RBS in pDR67 and transformed in XL1-Blue *E. coli*. Despite the high levels of repression of the insert, transformation efficiency was low (data not shown). Furthermore, growth of transformants was evidently impaired and, when streaking these colonies in new plates, various morphologies were recognizable. These clones did not seem to be suitable and were not used for further study. Interestingly, for each pump, one colony was found to be positive to the insert by PCR but did not display toxicity signs. Sequencing revealed premature stop codons in both inserts suggesting the non-functionality of the pumps as the reason for the absence of toxicity (*satA* CAG□TAG in the case of *satAB* operon, and a deletion of A1008 in *smrA* leading to a frame shift altering the protein sequence of the following 15 amino acids and a CTA□TAG premature stop codon). These strains were used as controls when needed. Altogether, our data suggest that a cloning procedure including a passage of *smrA* and *satAB* through *E. coli* is unfeasible. Thus, the ligation products of *satAB* from the three strains (BB1001, BB1002 and BB1013) and *smrA* from BB1013 with pDR67 were directly transformed into *B. subtilis* 168. Strains BB1068, B1069 and BB1070 bearing the three different alleles of *satAB* and BB1067 bearing *smrA* from BB1013, were obtained (Table 1). Sequencing confirmed that the inserts bore no mutations affecting the protein sequence. A transformant with the complete cloning system but no insert, namely BB1066, was obtained and conserved as a control. Last, plasmid pDR67 bearing a non-functional copy of $satAB^{BB1002}$ obtained in *E. coli* was also transformed in *B. subtilis*, giving rise to BB1071, a strain bearing the whole operon but with an interrupted *satA* gene.

satAB confers resistance to fluoroquinolones. _smrA_ does not _smrA:_

Antimicrobial susceptibility testing to a wide variety of compounds was performed with strain BB1067, bearing $smrA^{BB1013}$. Strain BB1066 was used as a control. No differences were found for any of the antibiotics and toxics tested. Primer walking confirmed the correct sequence of the insert. RT-PCR was performed proving the induction of the system in this strain (data not shown). As MFS transporters obtain their energy from the proton gradient between the periplasm and the cytoplasm, we investigated whether pH of the medium could play a role in the activity of SmrA against fluoroquinolones. Growth curves on LB medium at pH 5, 6, 7 and 8 were performed.

Several norfloxacin and ciprofloxacin concentrations, ranging from subinhibitory to inhibitory, were tested in all pHs in presence of 1mM of IPTG. No significant difference could be observed when comparing BB1066 and BB1067 in any condition (data not shown). Therefore, our data suggest that *smrA* is not involved in fluoroquinolone resistance in *S. suis*. MFS transporters have also been described as sugar transporters (25). To test whether *smrA* could be involved in carbohydrate import or export API 50CH strips were inoculated with BB1067 with and without induction and compared to

BB1066. No differences were observed among strains nor conditions, suggesting that SmrA is not a carbohydrate pump either. In order to determine the natural substrate of *smrA*, our strains were tested using BIOLOG, a phenomic array technology allowing to easily test a wide variety of compounds. A panel of 240 antibiotics and toxic molecules at four different concentrations were tested on BB1067 under induction conditions, using BB1066 as the control strain. Strikingly, of all the set of molecules, BB1067 only showed a slight improvement of respiration in one of the dilutions of minocycline, a drug belonging to the family of tetracyclines (data not shown). Interestingly minocycline is generally not considered to be a substrate of MFS pumps except for the case of TetB (7). BIOLOG® panel of carbon pathways was also tested confirming previous results on sugar usage and adding other compounds to SmrA's list of non-substrate molecules.

In order to rule out the possibility of SmrA not being active in *B. subtilis* the construction, from P_{spac} to *lacI* (see Figure 5), was mobilized to *Streptococcus pneumoniae* R6, a close member of the genus. pJS3, a plasmid derived from streptococcal native plasmid pMV158 (3), was modified to bear EcoRI and BamHI restriction sites and named pJS3EB. The constructions in the chromosome of *B. subtilis* were amplified by PCR and cloned in pJS3EB using the new restriction sites, giving rise to plasmids pB1011, containing the empty system (P_{spac}-*lacI*) as in BB1066, and pB1012 with the system and $smrA^{BB1013}$ (P_{spac}-*smrA-lacI*), as in BB1067. Strains BB1072, and BB1073 (Table 1) were obtained by transformation of pB1011 and pB1012 respectively into *S. pneumoniae* R6 (see materials and methods). Sequence of the insert was checked. RTPCR was performed in order to confirm that P_{spac} was inducible by IPTG in *S. pneumoniae*. Strains BB1072 and BB1073 were confronted to BIOLOG® panel of antibiotics, carbohydrates and ion/osmotic effects, and confirmed previous observations on minocycline, proving *B. subtilis* to be a suitable model for the study of streptococcal

MFS pumps. The natural substrate of this pump remains, though, elusive.

satAB:

Antimicrobial susceptibility testing to a wide variety of antimicrobials of strains bearing the three alleles of *satAB* was performed (data not shown). When IPTG was added to the growth media to a final concentration of 1mM, strains bearing a functional copy of *satAB* showed a

decrease in susceptibility to norfloxacin and ciprofloxacin but not to levofloxacin, enrofloxacin, moxifloxacin, or nalidixic acid (Table 3). In order to quantitate the effect of SatAB, MICs to ciprofloxacin and norfloxacin were determined with and without induction with IPTG. All alleles conferred at least a four-fold decrease in susceptibility to both antibiotics. Interestingly the allele originated from BB1002 conferred an extra doubling-dilution increase in resistance (up to eight-fold) against norfloxacin, and the allele from BB1013 did so to both norfloxacin and ciprofloxacin.

This points to the diversity in amino acid composition of the three alleles of SatAB as a possible factor in the levels of resistance through modifications in the affinity for the drug. The addition of reserpine to the medium reverted the resistance phenotype. No differences were observed between strain BB1066 bearing the expression system but no insert, and BB1071, bearing $\Delta\, satAB^{BB1002}$ confirming the non-functionality of the mutated insert. Fluoroquinolone efflux was only detected in presence of IPTG proving the system's tight regulation (Table 2). No other fluoroquinolone tested was a substrate of *satAB*, nor were any of the antibiotics belonging to a variety of other families that were tested (data not shown). SatAB is therefore a narrow spectrum pump of norfloxacin and ciprofloxacin.

DISCUSSION

Antimicrobial resistance is a major threat in the treatment of infectious diseases. Efflux is a worrying mechanism of resistance as efflux pumps are genes involved in many basic life functions, such as osmoregulation, and are hence ubiquitous in bacteria. Furthermore, efflux pumps often confer resistance to a wide array of unrelated compounds, allowing for a one-step selection of multi-resistant clones as well as for an undesirable co-selection phenomenon.

Here we present SatAB, an efflux pump involved in fluoroquinolone resistance in *S. suis*. We also assessed the involvement of SmrA, an MFS transporter homologous to FQ-efflux pump PmrA, in this phenotype. Our data show that SatAB but not SmrA is involved in fluoroquinolone efflux in *S. suis*.

Expression analysis of both pumps has been performed in clinical isolate BB1013. In the case of *satAB*, overexpression was constitutive, probably as a consequence of the loss of function of SatR due to a premature stop codon in its coding sequence. The expression of *smrA* was not significantly different from that of susceptible strain BB1001 in the absence of antibiotic. Nevertheless, when sub-inhibitory concentrations of ciprofloxacin were added to the media, *smrA* was significantly overexpressed in BB1013 as compared to BB1001, while *satAB* levels of expression remained stably high. Overexpression of efflux pumps in the presence of ciprofloxacin has already been observed for transporters not related to the efflux of this drug (26). Therefore, the link between expression and resistance is not concluding. Genetic approaches, such as knockout or knock-in mutants, are needed to confirm the implication of a pump in a given resistance phenotype. Cloning in *E. coli* is an easy and convenient approach. Interestingly some MFS pumps from Gram-positive bacteria, such as NorA or Bmr, have been cloned and are functional in *E. coli* (27, 38), while others, such as PmrA, have, to our knowledge, never been cloned in a Gram-negative species, despite the interest this pump raises. Altogether our data suggest that *smrA* and *satAB* are extremely toxic for *E. coli*. We hypothesize that a cell wall destabilization due to structural differences between *E. coli* and *S. suis*, is at the basis of this phenomenon. It would be interesting to elucidate the features that make some Gram-positive MFS pumps, and not others, toxic in Gramnegative bacteria even though they all share the same basic 12-transmembrane segments structure. When cloning toxicity is an impediment, knock-out mutants are the main strategy to study and characterize pumps. Nevertheless, tools to perform these assays have not been developed for all species. Even when available, wild type strains are often difficult to manipulate, and modifiable laboratory strains might not show the desired efflux phenotype. In *S. suis*, although some genetic tools are available (34), DNA transformation in BB1013 was found to be a bottleneck for further experiments. SmrA and SatAB were found to be very toxic in *E. coli* rendering the cloning and further study unviable in this species. A system

enabling the introduction of SmrA and SatAB pumps, while avoiding toxicity, was needed. pDR67 and *B. subtilis* 168 were used to effectively obtain knock-in mutants that were suitable for studying efflux pumps. This system has proven to be a fast, easy and reliable tool in the study of MFS and ABC transporters of *S. suis*, and will surely be helpful for the study of other pumps from different Gram-positive species.

PmrA is an MFS efflux pump from *S. pneumoniae* that has been, at least for some time, considered to be the main norfloxacin exporter in this bacterium. Since its first description (12) PmrA has generally failed to be further related to FQ resistance in either wild type strains or laboratory mutants (1, 5, 26, 32). Its role in fluoroquinolone resistance remains therefore unclear and is now questioned. The function of SmrA, the homolog of pneumococcal PmrA in *S. suis*, was intensively studied. Our results point to

SmrA as not being involved in fluoroquinolone resistance in BB1013. Among more than 450 compounds tested, only minocycline, an antibiotic from the family of tetracyclines, seemed to be affected by SmrA. These differences were subtle and further investigations are needed to define the possible clinical impact of SmrA in minocycline resistance. Nevertheless they where consistent in genetic constructions both in *B. subtilis* and *S. pneumoniae* bearing an inducible copy of the pump, allowing the inference that the whole expression system works not only for ABC, but for MFS transporters too. Also, *S. pneumoniae* seems a suitable species for the use of P_{spac}-*lacI* expression system.

Expression levels of *smrA* where measured in BB1013. Interestingly, expression of *smrA* increased in this strain in the presence of ciprofloxacin. As mentioned above, similar results have also been observed in *S. pneumoniae* for other transporters that are not involved in FQ extrusion (26). *S. pneumoniae*'s chromosome has been shown to be organized in topology-reacting gene clusters. DNA relaxation by a gyrase inhibitor modified global gene expression, wich affected both *pmrA* (downregulation) and *patApatB* (up-regulation) in opposite ways (10). It would be interesting, from both a clinical and a basic research perspective, to elucidate the reasons why a subset of unrelated transporters are overexpressed when in presence of antibiotics that are not a substrate thereof. Unraveling whether it is a general response to stress or an indirect consequence of the effect of fluoroquinolones in the coiling and topology of the chromosome (10) would be of great interest.

Our results prove that SatAB can extrude the fluoroquinolones norfloxacin and ciprofloxacin. Interestingly the alleles from both susceptible and resistant strains increased the levels of resistance to fluoroquinolones when cloned in *B. subtilis*. It is unlikely that the natural function of SatAB is FQ efflux as these antibiotics are fully synthetic. Therefore, other functions of this transporter are yet to be unveiled. Interestingly, levels of resistance conferred by SatAB differed depending on the allele. A possible influence of the amino acid differences found among

alleles cannot be ruled out. Still, SatAB overexpression seems to be the main requisite to obtain fluoroquinolone efflux. As it is the case for other pumps, of all fluoroquinolones tested, only ciprofloxacin and norfloxacin were affected by SatAB. This is interesting from both a drugdevelopment and a structural point of view as the substrate ciprofloxacin and the nonsubstrate enrofloxacin only differ in a methyl group.

Altogether our data on SmrA allows neglecting the possibility of it being a fluoroquinolone efflux pump of clinical relevance. Therefore, in order to further investigate *S. suis* mechanisms of FQ-resistance efforts should be directed to characterize in depth SatAB and the discovery of other possible transporters. Experiments to elucidate the implication of SatAB in other resistant clinical isolates showing efflux are under way.

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FIGURE LEGENDS

Figure 1

Diffusion antibiograms of BB1013 in media with (A) and without (B) 10 μg/ml of reserpine. CIP, ciprofloxacin; NOR, norfloxacin; LEV, Levofloxacin; ENR, enrofloxacin. Numbers indicate the micrograms of antibiotic in the disc.

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Levels of expression of *smrA* and *satAB* in BB1013, compared to BB1001. Striped bars represent growth on subinhibitory concentrations of ciprofloxacin (1/4 of the MIC of each strain). Pump expression was normalized against *rpoB* expression levels.

Figure 3.

Protein modeling of SmrA (A and B), SatA (C) and SatB (D). **A.** Lateral view of SmrA showing a 12 transmembrane segments structure typical of Major Facilitator Superfamily of transporters. Arrows show the amino acid substitutions present in BB1013 compared to BB1001 and BB1002. Red and yellow stained residues are T107A and I126V substitutions respectively. **B.** view down the channel of SmrA. **C** and **D**, modeling of SatA and B. A six transmembrane segments structure followed by a nucleotide-binding domain is observed in each protein.

Figure 4

Figure 4

A. Promoter region of *smrA* from BB1001, BB1002 and BB1013. Canonical -35 box and RBS are highlighted with black boxes. Two 7 bp inverted repeats (arrows) were found downstream the -35 box, separated by a 5 bp spacing region. *in silico* studies using SoftBerry Bprom software and the 133 bp immediately upstream *smrA* showed that the loss of

two base pairs in the spacer region led to the recognition of a previously undetected -10 box immediately downstream the 3' repeat (grey box). BB1013 bore three mutations when compared to FQ-susceptible strains. G-43A is located in the 3' inverted repeat and could interfere with regulation of *smrA* expression. **B.**

Alignment of the amino acid sequence of SatA and SatB from the three strains. Conserved residues are represented in black over a white background. Light grey and dark grey backgrounds represent one and two differences among sequences respectively.

Figure 5

A. Schematic representation of *smrA* and *satAB* integration in *B. subtilis*. Open reading frames are shown as arrows, with the direction of transcription indicated by the arrowhead. pDR67 bears regions of homology to the nonessential *amyE locus* of *B. subtilis*. These gene fragments are shown as broken arrows. Homologous recombination thereof with the chromosome allows for pDR67 integration. *smrA* and *satAB* amplicons were ligated to pDR67 and transformed directly in *B. subtilis*. **B**. Phenotypic screening of pDR67 integration in the chromosome. Bacteria are grown on LB containing 1% potato starch. Upper lane: *B. subtilis* 168 with a functional copy of *amyE*. Degradation of starch appears as a light unstained halo. Lower lane: BB1066, with pDR67 disrupting *amyE*, has no amylase activity. Starch is stained and no light zone is visible.

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TABLE 1. Strains and plasmids used in this study.

Strains	Relevant genotype	Description	Source or reference
S. suis			
BB1001	Wild type	Fluoroquinolone susceptible	Escudero *et al*. (8)
BB1002	Wild type	Fluoroquinolone susceptible	Escudero *et al*. (8)
BB1013	Wild type	Fluoroquinolone resistant. Efflux	Escudero *et al*. (8)
B. subtilis			
BS168	Reference strain. Used as recipient strain.	Parental strain	Courtesy Msadek, T.
BB1066	*amyE*::P_{spac}*lacI* Δ *lacZcat*	Control strain	This work
BB1067	*amyE*::P_{spac}*smrA*^{BB1013}*lacI* Δ *lacZcat*		This work
BB1068	*amyE*::P_{spac}*satAB*^{BB1001}*lacI* Δ *lacZcat*	Inducible resistance to NOR and CIP	This work
BB1069	*amyE*::P_{spac}*satAB*^{BB1002}*lacI* Δ *lacZcat*	Inducible resistance to NOR and CIP	This work
BB1070	*amyE*::P_{spac}*satAB*^{BB1013}*lacI* Δ *lacZcat*	Inducible resistance to NOR and CIP	This work
BB1071	*amyE*::P_{spac} Δ *satAB*^{BB1002}*lacI* Δ *lacZcat*		This work
S. pneumoniae R6	Reference strain		Rockefeller Inst.
BB1072	R6/pB1011	Control strain	This work
BB1073	R6/pB1012	Strain bearing *smrA*^{BB1013}	This work
Plasmids			
pJS3	*cat*	Replicative in *Streptococcus* spp.	Ballester *et al*. (3)

pJS3EB	-	EcoRI and BamHI restriction sites	This work
pB1011	pJS3EB:: P_{spac}*lacI*	Control plasmid	This work
pB1012	pJS3EB:: P_{spac}*smrA*^{BB1013}*lacI*	Plasmid bearing *smrA*^{BB1013}	This work

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Table 2. List of primers used in this study.

Name	Sequence 5□□3□	Reference
Amplification and Sequencing satAF	GTTGAGAACTTGTCCTAGGG	This work
satBR	TGACCAGTTCGAATCCACGG	This work
smrAF	ATGGCTGCTCAGCTTTCTTT	This work
smrAR	AAACTAAAAGACTGTATTTTG	This work
Operon determination		
satAIntF	CTGGTTTTGACAGAGAAGGG	This work
satBIntR	CCGCAATGGCATTTCCAAGG	This work
Cloning in pDR67 and insert checking		
satAXbaIRBS	GCGCTCTAGAGGTTAAACAGGTGGGCAATC	This work
satBBglIIStop	GCGCAGATCTACTTTATTCAAACACAAACT	This work
smrAXbaIRBS	GCTCTAGATTTTGGAGGAATTAAAGGAT	This work
smrABglIIR	GCAGATCTTCACTACACATCCCTTACTT	This work
pDR67F	ACATCCAGAACAACCTCTGC	This work
pDR67R	CTCGTTTCCACCGAATTAGC	This work
Mobilisation to pJS3 and insert checking		
pJS3EcoRI	GAACCGAATTCTCCTTTTTCGCTTC	This work
pJS3BamHI	ATATGGATCCGGAGCTGTAATATAAAAAC	This work

pDR67EcoRI	ATGAGAATTCTACACAGCCC	This work
pDR67BamHI	CAGTGCAGGGATCCTAACTC	This work
pJS3InsF	AATGTCACTAACCTGCCCCG	This work
pJS3InsR	TGCCAAAAAGCTTCTGTAGG	This work
RT-Q-PCR smrARTF		
	AAGCAGAATTTGAAGGTG	This work
smrARTR	AAGGGCATTAACAGATACCG	This work
satABRTF	AATCCAGAACCTTGTCAT	This work
satABRTR	AATAATCATCCACCAGAGT	This work
rpoBsuisF	AACTGGCGAGATCAAGAC	This work
rpoBsuisR	AACGATGATACGCTCTGC	This work
rpoBBSF	GGATGGCTACAACTATGAG	This work
rpoBBSR	GCTTCTGATTCGTATTCTTC	This work
rpoB428	CGGTTGGTGAATTGCTTGCCAACC	4
rpoB468R	ACTGCAGCTGTTACAGGACGG	4

Restriction sites are underlined

3

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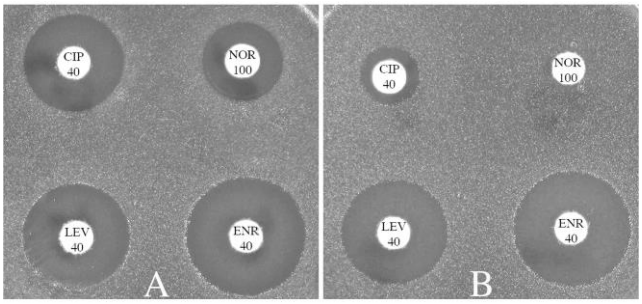
Table 3. MIC in µg/ml of strains from this study against several fluoroquinolones.

Strain	Relevant Genotype	ofloxacin			Norfloxacin			Enrofloxacin			Levofloxacin		
		-	I	IR	-	I	IR	-	I	IR	-	I	IR
BB1066	No insert	0,06	0,06	0,06	0,25	0,25	0,12	0,06	0,06	0,06	0,12	0,12	0,12
BB1067	*smrA* BB1013	0,06	0,06	0,06	0,25	0,25	0,12	0,06	0,06	0,06	0,12	0,12	0,12
BB1068	*satAB*$_{BB1001}$	0,06	0,25	0,12	0,25	1	0,5	0,06	0,06	0,06	0,12	0,12	0,12
BB1069	*satAB*$_{BB1002}$	0,06	0,25	0,12	0,5	2	0,5	0,06	0,06	0,06	0,12	0,12	0,12
BB1070	*satAB*$_{BB1013}$	0,06	0,5	0,12	0,25	2	0,5	0,06	0,06	0,06	0,12	0,12	0,12
BB1071	Δ *satAB*$_{BB1002}$	0,06	0,06	0,06	0,25	0,25	0,12	0,06	0,06	0,06	0,12	0,12	0,12

-, standard media. I, 1mM IPTG added; IR, 1mM IPTG and 10 µg/ml of reserpine added.



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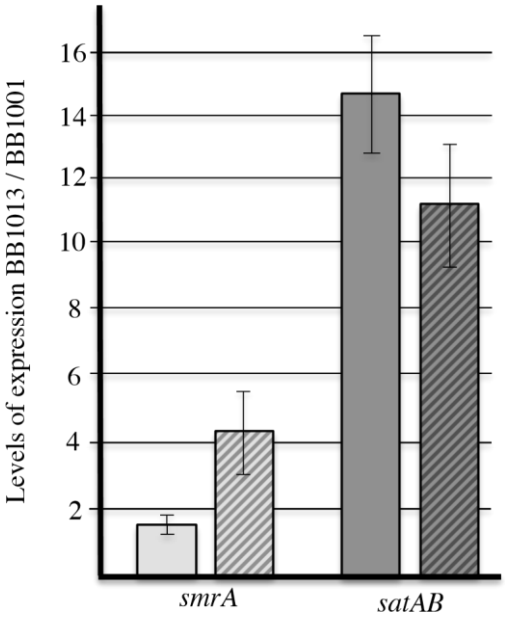
Diffusion antibiograms of BB1013 in media with (A) and without (B) 10 μg/ml of reserpine. CIP, ciprofloxacin; NOR, norfloxacin; LEV, Levofloxacin; ENR, enrofloxacin. Numbers indicate the micrograms of antibiotic in the disc.



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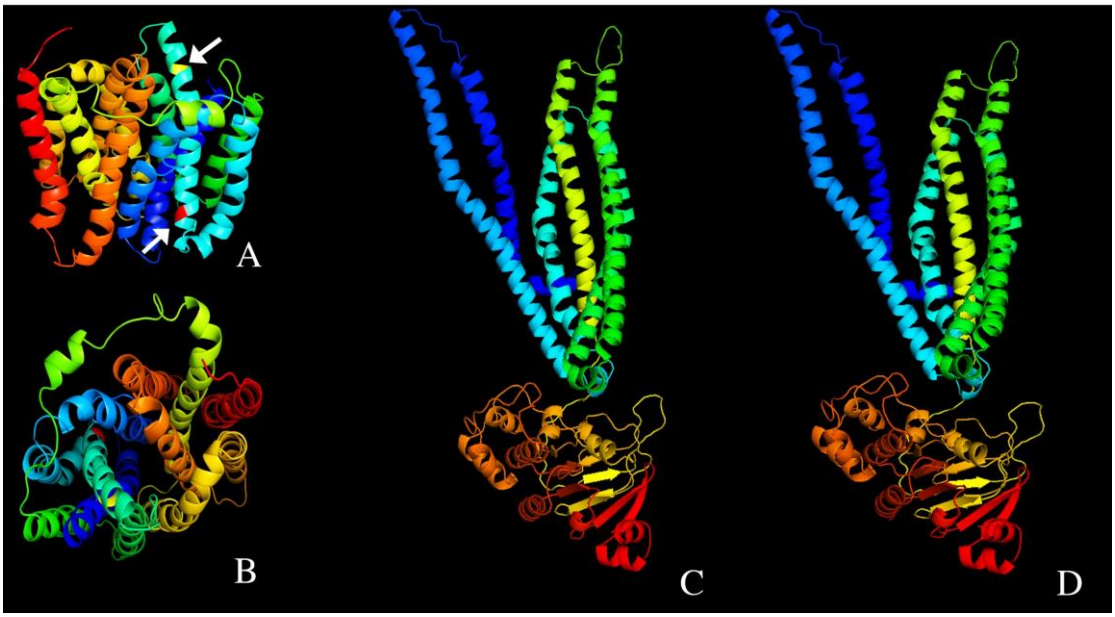

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Protein modeling of SmrA (A and B), SatA (C) and SatB (D). **A.** Lateral view of SmrA showing a 12 transmembrane segments structure typical of Major Facilitator

Superfamily of transporters. Arrows show the amino acid substitutions present in BB1013 compared to BB1001 and BB1002. Red and yellow stained residues are T107A and I126V substitutions respectively. **B.** view down the channel of SmrA. **C** and **D**, modeling of SatA and B. A six transmembrane segments structure followed by a nucleotide-binding domain is observed in each protein.

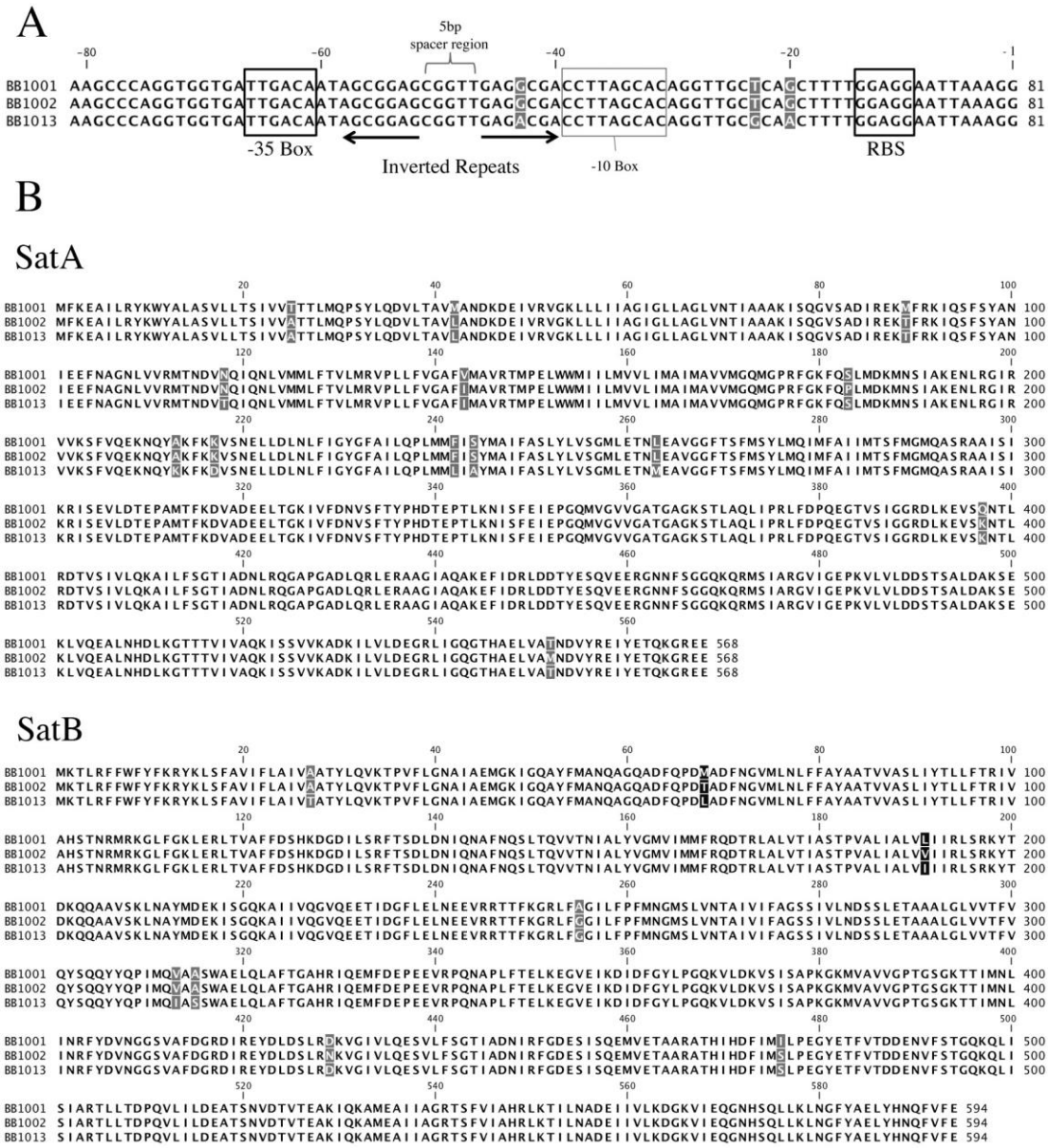

Figure 4

A. Promoter region of *smrA* from BB1001, BB1002 and BB1013. Canonical 35 box and RBS are highlighted with black boxes. Two 7 bp inverted repeats (arrows) were found downstream the -35 box, separated by a 5 bp spacing region. *in silico* studies using SoftBerry Bprom software and the 133 bp immediately upstream *smrA* showed that the loss of two base pairs in the spacer region led to the recognition of a previously undetected -10 box immediately downstream the 3' repeat (grey box). BB1013 bore three mutations when compared to FQ-

susceptible strains. G-43A is located in the 3' inverted repeat and could interfere with regulation of *smrA* expression. **B.** Alignment of the amino acid sequence of SatA and SatB from the three strains. Conserved residues are represented in black over a white background. Light grey and dark grey backgrounds represent one and two differences among sequences respectively.

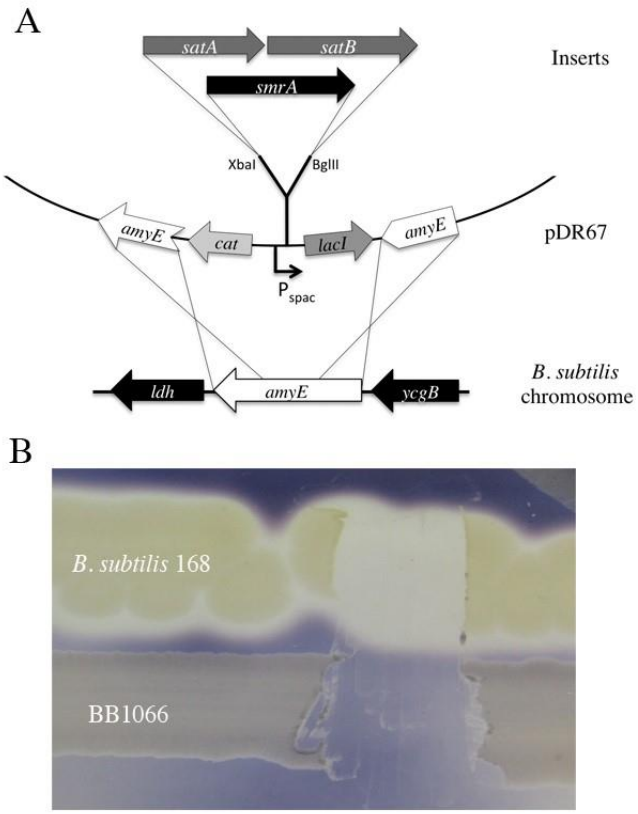

Figure 5

A. Schematic representation of *smrA* and *satAB* integration in *B. subtilis*. Open reading frames are shown as arrows, with the direction of transcription indicated by the arrowhead. pDR67 bears regions of homology to the nonessential *amyE locus* of *B. subtilis*. These gene fragments are shown as broken arrows. Homologous recombination with the chromosome allows for pDR67 integration. *smrA* and *satAB* amplicons were ligated to pDR67 and transformed directly in *B. subtilis*. **B.** Phenotypic screening of pDR67 integration in the chromosome. Bacteria are grown on LB containing 1% potato starch. Upper lane: *B. subtilis* 168 with a functional copy of *amyE*. Degradation of starch appears as a light unstained halo. Lower lane: BB1066, with pDR67 disrupting *amyE*, has no amylase activity. Starch is stained and no light zone is visible.