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Identification of *ypqP* as a New *Bacillus subtilis* Biofilm Determinant That Mediates the Protection of *Staphylococcus aureus* against Antimicrobial Agents in Mixed-Species Communities

Pilar Sanchez-Vizueté,^{a,b} Dominique Le Coq,^{a,b,c} Arnaud Bridier,^d Jean-Marie Herry,^{a,b} Stéphane Aymerich,^{a,b} Romain Briandet^{a,b}
 INRA, UMR1319 MICALIS, Jouy-en-Josas, France^a; AgroParisTech, UMR1319 MICALIS, Jouy-en-Josas, France^b; CNRS, Jouy-en-Josas, France^c; IRSTEA, UR HBAN, Antony, France^d

In most habitats, microbial life is organized in biofilms, three-dimensional edifices sustained by extracellular polymeric substances that enable bacteria to resist harsh and changing environments. Under multispecies conditions, bacteria can benefit from the polymers produced by other species (“public goods”), thus improving their survival under toxic conditions. A recent study showed that a *Bacillus subtilis* hospital isolate (NDmed) was able to protect *Staphylococcus aureus* from biocide action in multispecies biofilms. In this work, we identified *ypqP*, a gene whose product is required in NDmed for thick-biofilm formation on submerged surfaces and for resistance to two biocides widely used in hospitals. NDmed and *S. aureus* formed mixed biofilms, and both their spatial arrangement and pathogen protection were mediated by YpqP. Functional *ypqP* is present in other natural *B. subtilis* biofilm-forming isolates. However, the gene is disrupted by the SP β prophage in the weak submerged-biofilm-forming strains NCIB3610 and 168, which are both less resistant than NDmed to the biocides tested. Furthermore, in a 168 laboratory strain cured of the SP β prophage, the reestablishment of a functional *ypqP* gene led to increased thickness and resistance to biocides of the associated biofilms. We therefore propose that YpqP is a new and important determinant of *B. subtilis* surface biofilm architecture, protection against exposure to toxic compounds, and social behavior in bacterial communities.

Bacillus subtilis is a nonpathogenic Gram-positive bacterium that can be found in its natural habitats as free cells or associated with surfaces in biofilms. In the soil, *B. subtilis* strains have been shown to form surface-associated communities on plant tissues that protect them from infection by pathogens (1–3). Because of its ability to resist stress through biofilm or spore formation, the bacterium has been isolated from extreme environments, such as sand deserts, clouds, and the digestive tracts of animals (4–6). As well as its ubiquitous presence in diverse habitats, *B. subtilis* has been used extensively in biotechnological applications, such as the production of natto, a traditional Japanese food made of fermented soybeans (7), and the production of industrial enzymes and pharmaceutical proteins (8, 9). As a generally recognized as safe (GRAS) organism, *B. subtilis* is also used as a biocontrol agent in agriculture and livestock buildings (10–14) and as a probiotic agent to improve human and animal health by preventing gastrointestinal infections (15, 16).

In fundamental research, *B. subtilis* has emerged as the model organism for deciphering the complex genetic regulation involved in the biofilm mode of life of Gram-positive bacteria. The domesticated strain *B. subtilis* 168 has been widely used to dissect metabolic and cellular processes. However, this strain is not able to form robust pellicles and complex colonies like those of its parental strain, NCIB3610, a descendant of the original Marburg strain that was deposited in 1951 (17). For easy use in the laboratory (rapid growth, efficient transformability, etc.), the domesticated strain 168 was selected from NCIB3610 by means of drastic sublethal UV and X-ray exposure, during which it lost some important genetic biofilm determinants (18, 19). Such determinants responsible for biofilm formation and its associated regulation have therefore mainly been characterized in strain NCIB3610, and this has shed light on the principal molecular players in the *B. subtilis* biofilm lifestyle. It has thus been shown that the switch from a motile planktonic to a sessile lifestyle is induced by specific exter-

nal stimuli, such as root exudates, impaired respiration, or the action of antimicrobials (1, 20, 21), which trigger phosphorylation of the regulator Spo0A (22). Phosphorylated Spo0A (Spo0A~P) then represses two important negative regulators of biofilm formation, AbrB and SinR, allowing expression of the genes involved in the synthesis of the biofilm matrix (the polysaccharides synthesized from the *epsA-O* operon (i.e., the operon comprising *epsA*, *epsO*, and the genetic material between those genes) and the amyloid-like fiber TasA encoded by the *tapA-sipW-tasA* operon) (23). The amphiphilic matrix protein BslA has been recently shown to be required for biofilm formation (24). The two principal biofilm models used for *B. subtilis* (i.e., colonies on agar and floating pellicles) are in direct contact with air (17). However, submerged biofilms are the most widely used model to study the formation of biofilms and their properties for pathogens such as *Pseudomonas aeruginosa* and *Staphylococcus aureus* (25, 26). While the submerged model represents environmental conditions confronted by *B. subtilis* in its natural habitats, it has only recently been applied to the species (22, 27), likely due to experimental

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Address correspondence to Romain Briandet, romain.briandet@jouy.inra.fr.

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TABLE 1 *B. subtilis* strains and plasmid used during the study

Strain or plasmid	Relevant genotype or isolation source ^a	Reference, source, or construction ^b
Strains		
NDmed	Isolated from endoscope washer-disinfector	29
NDmed GFP	NDmed <i>amyE::P_{hyperspank}-GFP (spec)</i>	30
GM3248	NDmed <i>ypqP::kan</i>	PCR product→NDmed (this work)
GM3248 GFP	NDmed <i>ypqP::kan amyE::P_{hyperspank}-GFP (spec)</i>	NDmed GFP→GM3248 (this work)
GM3326	NDmed <i>ypqP::kan amyE::ypqP (spec)</i>	pIC679→GM3248 (this work)
168	<i>trpC2 ypqP::SPβ</i>	Bacillus Genetic Stock Center
NCIB3610	Prototroph <i>ypqP::SPβ</i>	17
ATCC 6051	Prototroph <i>ypqP::SPβ</i>	American Type Culture Collection
PY79	SPβ cured	Bacillus Genetic Stock Center
NDfood	Isolated from a dairy product	27
BSn5	Isolated from a plant	3
BSP1	Isolated from poultry	6
Plasmid		
pIC679	pDR111 derivative containing <i>ypqP (spec)</i>	This work

^a *spec* and *kan* are genes coding for resistance to spectinomycin and kanamycin respectively.

^b Arrows indicate transformation with plasmid, chromosomal DNA, or PCR product.

limitations associated with the bacterium (17, 27). In a previous study, we proposed a new methodology to visualize and quantify *B. subtilis* submerged biofilms using a microplate-based model combined with confocal laser scanning microscopy (CLSM) (27, 28). This experimental system enabled the identification of a remarkable *B. subtilis* strain (NDmed), isolated from an endoscope washer-disinfector (29), capable of forming thick and protruding biofilms highly resistant to the biocides commonly used for endoscope disinfection in hospitals (30).

Although monospecies biofilms have been widely studied, bacteria mostly grow in complex multispecies communities where synergistic and antagonistic interactions modulate the spatial organization and biomass production. In most cases, interspecies relationships increase the fitness and the resistance to environmental stresses of the biofilm (30–35). Indeed, we previously demonstrated the ability of *B. subtilis* NDmed to protect the pathogen *S. aureus* from biocide action in dual-species biofilms (30).

The aim of the present study was to decipher the genetic determinants responsible for NDmed biocide resistance and pathogen protection. A comparative genomic analysis of the recently sequenced genome of the NDmed strain (36) showed that four genes described as defective in the domesticated strain 168 (*sfp*, *epsC*, *swrA*, and *degQ*) (18) were identical in NCIB3610 and NDmed (data not shown). One striking difference was the absence of the 134.4-kb SPβ prophage from the NDmed genome, whose insertion in NCIB3610 and 168 causes disruption of the *ypqP* gene. In this work, we showed that the inactivation of *ypqP* in NDmed led to changes in specific biofilm traits, such as thickness, high resistance to biocide, and pathogen protection capacities.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and mutant construction. The *B. subtilis* strains and plasmid used during this study are listed in Table 1. The *S. aureus* strain RN4220 with a plasmid allowing the constitutive expression of mCherry fluorescent protein (*ery*) (37) was used in the mixed-species biofilms. Bacterial stock cultures were kept at –20°C in tryptone soy broth (TSB) (bioMérieux, France) containing 20% (vol/vol) glycerol. Prior to each experiment, frozen cells were subcultured twice in TSB at 30°C. The final overnight culture was used as an inoculum for the growth of biofilms. Transformation of *B. subtilis* and *Escherichia coli* was

performed according to standard procedures, and the transformants were selected on Luria-Bertani (LB) (Sigma, France) plates supplemented with appropriate antibiotics at the following concentrations: ampicillin, 80 μg/ml; kanamycin, 8 μg/ml; and spectinomycin, 100 μg/ml. The primers used for genetic constructions are listed in Table 2. To construct the *ypqP* mutant (strain GM3248), a kanamycin resistance cassette was amplified from plasmid pDG780 (38) using the PSV013 and PSV014 primers and ligated by recombinant PCR to a 791-bp fragment corresponding to the region upstream of *ypqP* followed by the first 405 nucleotides of the gene amplified with PSV011 and PSV012 primers and to an 806-bp fragment corresponding to the last 605 nucleotides of *ypqP* and the region downstream amplified with the PSV015 and PSV016 primers. Transformation of the *B. subtilis* strain NDmed with the resulting fragment led to its integration into the genome via double-crossover recombination, selected by kanamycin resistance. To construct the *ypqP*-complemented strain (GM3326), a fragment containing the *ypqP* gene flanked by its promoter and terminator was amplified from NDmed chromosomal DNA with the PSV026 and PSV027 primers and substituted for the EcoRI-BamHI part of plasmid pDR111 (a kind gift from D. Z. Rudner, Harvard Medical School) to produce plasmid pIC679. This allowed insertion by double-crossover recombination of a functional complementing copy of the gene in the ectopic *amyE* site of the *ypqP* mutant strain GM3248.

Mono- and dual-species biofilm formation. Submerged biofilms were formed in polystyrene 96-well microtiter plates with a μclear base (Greiner Bio-one, France), enabling high-resolution fluorescence imag-

TABLE 2 Primers used in this work

Primer	Sequence
PSV011	GTTTTTGGAAACCTTCATTTGGTACCCTCCTC
PSV013	GTCATTAATATCAGTATAAACCAGCGAAC CATTGAGGTGATAGG
PSV014	TTGTGTTACAGGAGCAGAGTATGGACAGT TGCGGATGACTTCAG
PSV015	ACTGTCCATACTCTGCTCTGTGAACACAA TGGGTGCCACCAAG
PSV016	GCCGAAGCTGTGATTCTCATCTTGGCCAGC
PSV012	GTTTCGTTGGGTTTATCTGATATTAATGAC ATGCTGCACTCGGTGTG
PSV026	CGCGGATCCATACGAAAGCTATCTGCATCTC
PSV027	CCGGAATCTTTTACTACTAACAATACTATG

ing, as previously described (28). For single-species biofilms, 200 μ l of an overnight culture in TSB (adjusted to an optical density at 600 nm [OD₆₀₀] of 0.02) was added to the wells of a microtiter plate. For mixed-species biofilms, overnight cultures in TSB of *S. aureus* mCherry and the *B. subtilis* green fluorescent protein (GFP) strains were adjusted to an OD₆₀₀ of 0.02 in TSB and mixed at a ratio of 1:2. The microtiter plate was then kept at 30°C for 90 min to allow the bacteria to adhere to the bottom of the wells. After this adhesion step, the wells were rinsed with TSB to eliminate any nonadherent bacteria and then refilled with 200 μ l sterile TSB. The microtiter plate was then incubated for 48 h at 30°C to allow biofilm development. The medium was replaced with fresh medium after 24 h of development. When appropriate, the medium was supplemented with 200 μ M isopropyl- β -D-thiogalactopyranoside (IPTG) to induce GFP expression from the *P_{hyperspank}* promoter.

Confocal laser scanning microscopy. Forty eight-hour submerged biofilms were observed using a Leica SP8 AOBs inverter confocal laser scanning microscope (Leica Microsystems, Germany) at the INRA-MIMA2 platform (www6.jouy.inra.fr/mima2_eng/). For the observation of *B. subtilis* monospecies biofilms, cells were fluorescently stained in green with the nucleic acid marker SYTO9 (1:1,000 dilution in TSB from a SYTO9 stock solution at 5 mM in dimethyl sulfoxide [DMSO]; Invitrogen, France). After 20 min of incubation in the dark at 30°C to enable fluorescent labeling of the bacteria, the plate was mounted on the motorized stage of the confocal microscope. The microtiter plates were scanned using a 63 \times /1.2-numerical-aperture (NA) water immersion objective lens. Both single and mixed biofilms were scanned at excitation wavelengths of 488 nm (argon laser; 3% intensity) and 561 nm (helium-neon laser; 5% intensity), with emission wavelengths collected from 493 to 550 nm and from 590 to 690 nm for GFP or SYTO9 and mCherry fluorescence, respectively, using hybrid detectors (HyD Leica Microsystems, Germany). Three-dimensional (3D) projections of the biofilm structures were reconstructed using the Easy 3D function of the IMARIS software (Bitplane, Switzerland) from xyz image series.

Quantification of biofilm biovolume and maximum thickness. The maximum thickness (in μ m) was measured directly from xyz stacks. Biofilm biovolume is widely used to represent the overall volume (in μ m³) of bacteria associated with the surface. To calculate this biovolume, images were analyzed with a homemade java script executed by ICY, an open-community image analysis platform (39). The images were first binarized using the K-means function. The biovolume was then defined as the number of foreground pixels in an image stack multiplied by the voxel volume, which is the product of the squared pixel size and the scanning step size (40).

Biocide treatments. After 48 h of biofilm development in the microtiter plates, 100 μ l of peracetic acid (PAA) or ortho-phthalaldehyde (OPA) (Sigma-Aldrich, France) was added at a final concentration of 3.5 g/liter or 10 g/liter, respectively. After 5 min of contact, the wells' contents (including nonadherent cells and biocide solution) were gently removed, and the wells were refilled with 300 μ l of quenching solution (3 g/liter L- α -phosphatidylcholine, 30 g/liter Tween 80, 5 g/liter sodium thiosulfate, 1 g/liter L-histidine, 30 g/liter saponin) and left for 5 min to halt the action of the biocide. The bottoms of the wells were then scraped with tips, and the suspension was aspirated and expelled several times to detach and recover any surviving cells from the biofilms. The suspensions were serially diluted in 150 mM NaCl and plated in duplicate on tryptone soy agar (TSA) (bioMérieux, France) for total counts or on TSA supplemented with 3 μ g/ml erythromycin for selective counts of *S. aureus* cells before being incubated at 30°C for 24 to 48 h. Survivors were enumerated, and the log₁₀ reduction was calculated from the initial population. The sensitivity of planktonic cells to the action of OPA and PAA was also analyzed. Cell suspensions of the three NDmed strains (NDmed wild type [WT], GM3248, and GM3326) were harvested from a 24-h culture in TSB at 30°C by centrifugation (7,000 \times g; 10 min; 20°C) and washed by resuspending them in 150 mM NaCl and vortexing for 30 s. The suspensions were adjusted to 10⁸ CFU/ml, and OPA and PAA activities were then evaluated according to the standard European NF EN 1040 procedure

(41) at final concentrations of 150 mg/liter and 5 mg/liter, respectively. The suspensions were serially diluted in 150 mM NaCl, plated on TSA, and incubated at 30°C for 24 to 48 h. Survivors were enumerated, and the log₁₀ reduction was calculated from the initial population.

Growth of bacterial colonies. In order to analyze complex colony architecture, 3 μ l of an overnight culture in TSB was spotted on 1.5% TSA. The plates were then incubated at 25°C for 72 h. Digital images of the colonies on the plates were taken using a Nikon Coolpix P100 digital camera.

Statistical analysis. One-way analysis of variance (ANOVA) was performed using Statgraphics (Manugistics, Rockville, MD, USA) v16.1 software. Significance was defined as a *P* value associated with a Fisher test value lower than 0.05.

RESULTS

The *ypqP* gene is disrupted by the SP β prophage in several *B. subtilis* laboratory strains, but not in the NDmed strain and other natural isolates. The role of the candidate gene *ypqP* in biofilm-associated phenotypes of *B. subtilis* NDmed has been investigated. This gene, coding for a 341-amino-acid (aa) protein in the NDmed strain, is disrupted by the insertion of the SP β prophage in both strains 168 and NCIB3610, which results in an early stop codon, potentially leading to a shorter protein of 141 aa (Fig. 1). Most of the natural isolates of *B. subtilis* whose genomes have been sequenced, including BSn5, BSP1, RO-NN-1, PS216, and AUS198, also have an undamaged *ypqP* gene (Fig. 1A). Likewise, the laboratory strain PY79 (a strain derived from 168 but cured of the SP β prophage); other *B. subtilis* subspecies, such as *spizizenii* or *natto*; and some other strains in related *Bacillus* species, such as *Bacillus amyloliquefaciens*, *Bacillus pumilus*, or *Bacillus stratosphericus*, also have a nondisrupted *ypqP*.

Eight *B. subtilis* strains with different origins (Table 1) containing either an uninterrupted or a disrupted *ypqP* gene were therefore analyzed for submerged-biofilm formation. These biofilms were grown in 96-well plates and examined after 48 h of development using CLSM. As shown in Fig. 2A, all the *B. subtilis* strains tested containing a nondisrupted *ypqP* gene (NDmed, NDfood, PY79, BSn5, and BSP1) formed denser biofilms with more protruding structures than those formed by the strains whose *ypqP* gene is disrupted by the SP β prophage (168, NCIB3610, and ATCC 6051). The maximum thickness of the biofilms formed by the latter group of strains was less than 85 μ m (only 50 μ m for the laboratory strain 168), whereas it could reach more than 160 μ m for strains with a nondisrupted *ypqP* gene (Fig. 2B). The laboratory strain PY79, cured of the SP β prophage, also showed greater ability than strain 168 to grow on submerged surfaces. Although other genetic differences between strains are probably involved, these observations have pinpointed the fact that the product of the candidate gene *ypqP* could be involved in the traits of *B. subtilis* submerged biofilms.

***ypqP* is required for the spatial organization of *B. subtilis* NDmed biofilms.** An NDmed *ypqP* mutant strain (GM3248) was constructed by inserting a resistance cassette in the *ypqP* gene immediately upstream of the prophage attachment site. Both the growth rate and cellular morphology of the mutant were identical to those of the parental strain (data not shown). Observation of complex colonies formed by the NDmed WT strain and the *ypqP* mutant derivative revealed that *ypqP* disruption markedly affected their structure; whereas the colonies formed by the NDmed WT strain were small and highly wrinkled, those formed by the *ypqP* mutant were flat and more widely spread over the surface (Fig. 3A). In order to confirm that the *ypqP* mutation alone was respon-

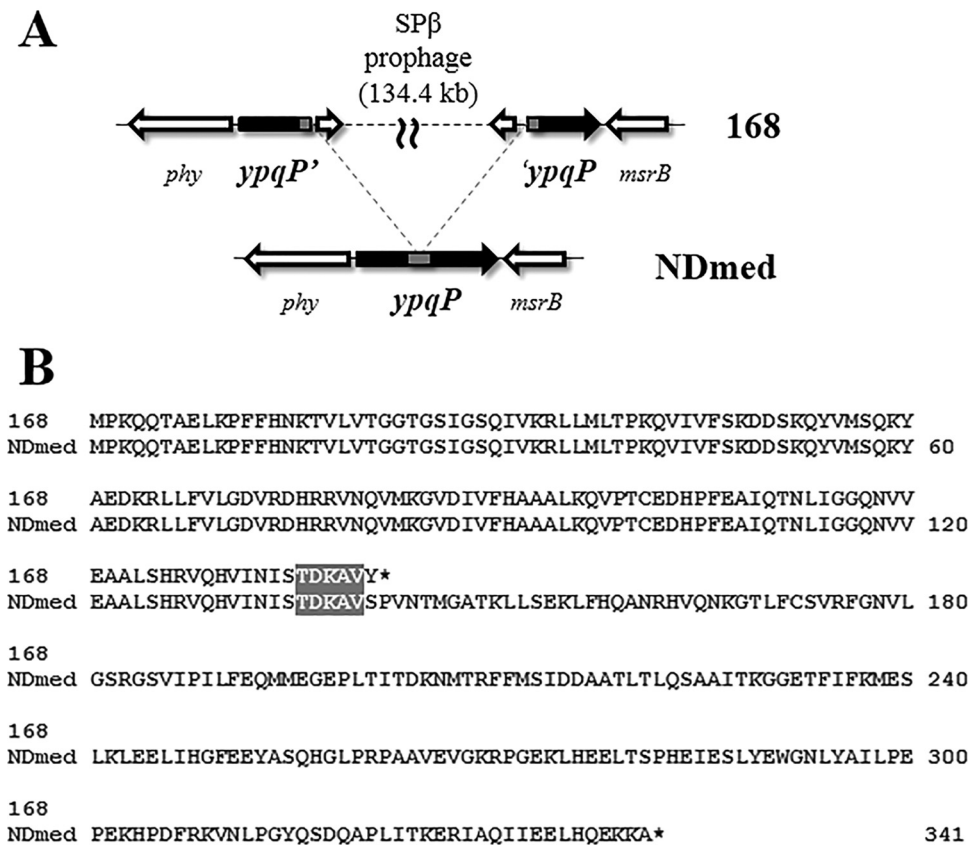


FIG 1 YpqP is disrupted in the laboratory strain 168 but not in the nondomesticated strain NDmed. (A) Schematic representation of the *B. subtilis* *ypqP* gene and its genetic environment in natural strains, such as NDmed, and the disruption of *ypqP* by the SP β prophage in the domesticated strain 168. (B) Protein sequence of YpqP in NDmed (341 aa) and its alignment with the truncated protein in 168 (141 aa). The phage attachment site (A) and the corresponding protein sequence (B) are shaded in gray; asterisks symbolize stop codons.

sible for the phenotype observed, a complemented strain was also constructed. To achieve this, a copy of the *ypqP* WT gene under the control of its own promoter was introduced into the *ypqP* mutant at the ectopic *amyE* locus (strain GM3326). Complete restoration of the WT phenotype was observed in colonies of this *ypqP*-complemented strain (Fig. 3A). The abilities of NDmed WT, *ypqP* mutant, and *ypqP*-complemented strains to form submerged biofilms in 96-well plates were also investigated. Observation of the architecture of biofilms using CLSM after 48 h of development showed that the biofilms formed by the NDmed *ypqP* mutant differed significantly from those formed by the WT strain (Fig. 3C). The *ypqP* mutant biofilms were thinner ($P < 0.05$), with a maximum thickness of 60 μm (compared to 160 μm for the WT strain), and had a biovolume of $256 \times 10^3 \mu\text{m}^3$ compared to $525 \times 10^3 \mu\text{m}^3$ for the WT (Fig. 4). By zooming in specifically on the layer of cells attached to the surface, it could be seen that the *ypqP* mutant cells were grouped in compact rafts while the WT cells were randomly dispersed over the surface (Fig. 3B). The complemented strain presented a phenotype identical to that of the WT, thus confirming the key role of *ypqP* in complex colony architecture and submerged biofilm formation in the NDmed strain.

Disruption of *ypqP* dramatically decreases the biocide resistance of *B. subtilis* NDmed biofilms. The marked resistance of *B. subtilis* NDmed biofilms to PAA has previously been demonstrated (30). Here, we investigated the resistance to this oxidizing

agent, as well as to an aldehyde that is also widely used in hospitals (namely, OPA), of biofilms formed by several *B. subtilis* strains endowed or not with a functional *ypqP* gene. Biofilms grown for 48 h were subjected to 10.0 g/liter OPA or 3.5 g/liter PAA for 5 min. The results are presented for each strain as \log_{10} reductions of CFU per well (log red) in Fig. 5A for OPA and in Fig. 5B for PAA. Treatment with OPA triggered only 2.61 ± 0.18 log red in NDmed WT biofilms, which thus appeared to be far more resistant than NCIB3610 biofilms (4.96 ± 0.21 log red) ($P < 0.05$). Remarkably, *ypqP* disruption in the *ypqP* mutant strain led to a drastic decrease in the resistance of biofilm cells to the biocide (4.73 ± 0.18 log red), with values similar to those of the laboratory strain 168 and with both being significantly different from that of NDmed ($P < 0.05$). The complementation of *ypqP* disruption (strain GM3326) led to restoration of the resistance observed in the WT strain. It should also be noted that the strain PY79, a laboratory strain with an undisrupted *ypqP* gene, also showed marked tolerance for OPA that was similar to those of both the NDmed WT and *ypqP*-complemented strains. The results obtained following the disinfection of NDmed WT and 168 biofilms using 3.5 g/liter PAA were similar to those found previously under similar conditions (30). The NDmed WT strain exhibited only 3.91 ± 0.30 log red, while no survivors were detected for strain 168, corresponding to 7.00 ± 0.42 log red. Once again, the reference strain NCIB3610 exhibited less resistance (5.03 ± 0.28 log red) than NDmed WT ($P < 0.05$).

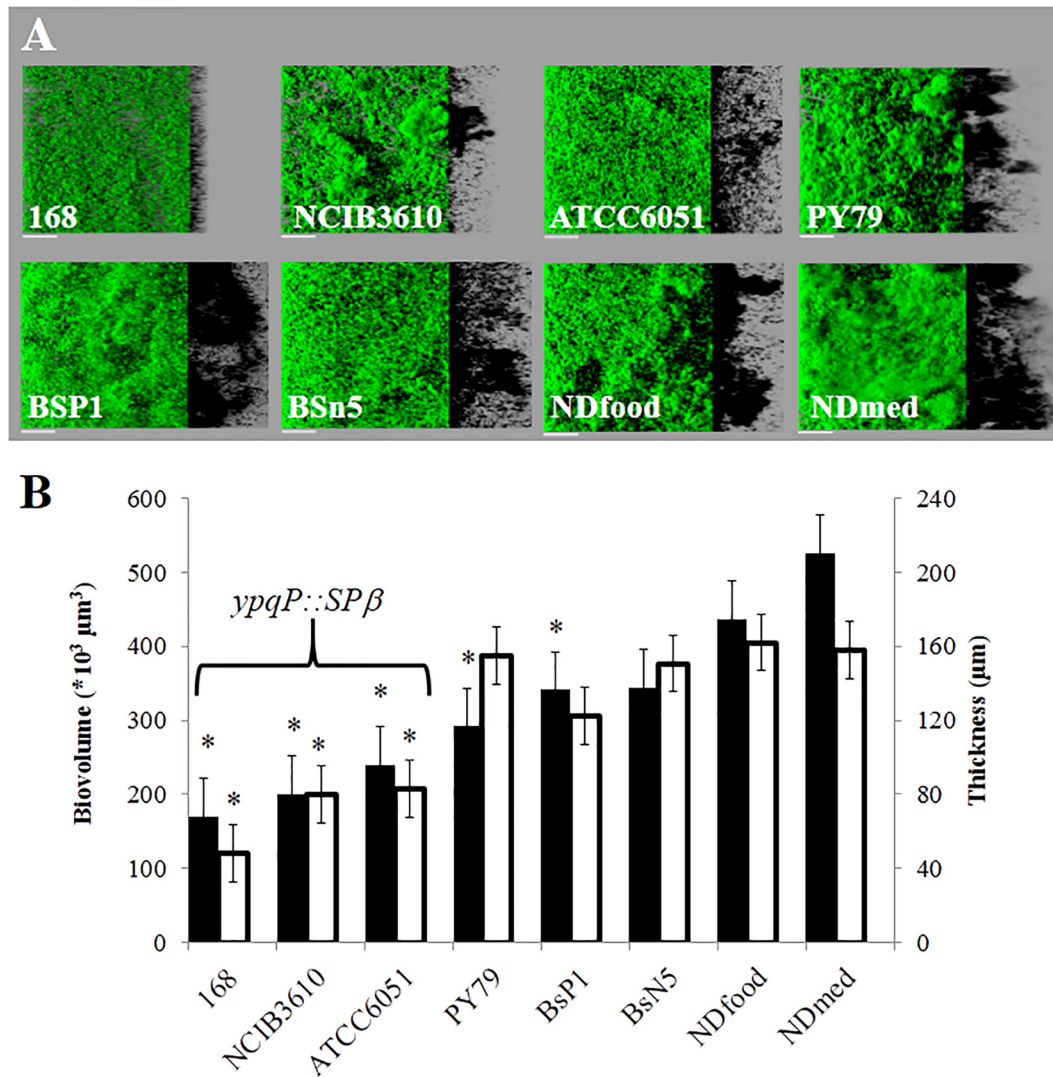


FIG 2 Variability in the architecture of submerged biofilms formed by natural and domesticated *B. subtilis* strains. (A) Submerged biofilms formed by eight *B. subtilis* strains in 96-well plates for 48 h were stained with SYTO9, and their architecture was analyzed by CLSM. A representative image obtained from confocal series reconstruction using IMARIS software with the virtual shadow projection on the right is presented for each strain. The scale bars represent 50 μm . (B) Biovolumes (black bars) and maximum thicknesses (white bars) calculated from six series of images. The error bars indicate the standard errors, and statistically significant differences from the NDmed strain ($P < 0.05$) observed are indicated by asterisks.

The inactivation of *ypqP* led to a dramatic decrease in tolerance for PAA, with 6.30 ± 0.48 log red, and once again, the tolerance for the biocide shown by the NDmed WT strain was completely restored in the complemented strain GM3326. Strain PY79 exhibited 5.93 ± 0.42 log red, which was significantly more resistant than the laboratory strain 168 ($P < 0.05$). In contrast, *ypqP* disruption had no significant effect on NDmed planktonic-cell resistance to both biocides ($P > 0.05$) (see Table S1 in the supplemental material). These findings, therefore, highlighted the involvement of the *ypqP* gene in NDmed biofilm resistance to the biocides tested.

***ypqP* is involved in the protection of *S. aureus* against biocides in mixed-species biofilms.** Dual-species biofilms formed by the *B. subtilis* NDmed derivative strains expressing GFP (WT or *ypqP* mutant) and an *S. aureus* mCherry strain were grown under conditions similar to those applied to monospecies biofilms. Cell counts of the NDmed monospecies biofilms after 48 h of develop-

ment were lower for the *ypqP* mutant than for the WT strain (7.04 ± 0.15 and 7.25 ± 0.11 log CFU/well, respectively; $P < 0.05$). When grown together with *S. aureus*, the *B. subtilis* cell counts rose to similar values for both the WT and the *ypqP* mutant strains (7.52 ± 0.23 and 7.45 ± 0.30 log CFU/well, respectively; $P > 0.05$). In contrast, *S. aureus* cell counts were higher when the pathogen was grown with the *ypqP* mutant than with the WT strain (8.42 ± 0.18 and 7.52 ± 0.23 log CFU/well, respectively; $P < 0.05$), findings that agreed with confocal-microscopy observations (Fig. 6). *S. aureus* formed a flat and regular biofilm with a maximum thickness of 40 μm (30). When the pathogen was grown with NDmed WT, both strains formed a well-mixed biofilm in which the pathogen cells occupied spaces left free by NDmed both on the surface and also inside the protruding structures formed by the strain, in line with previous results (30). In contrast, in the biofilm formed with *S. aureus*, the *B. subtilis* *ypqP*

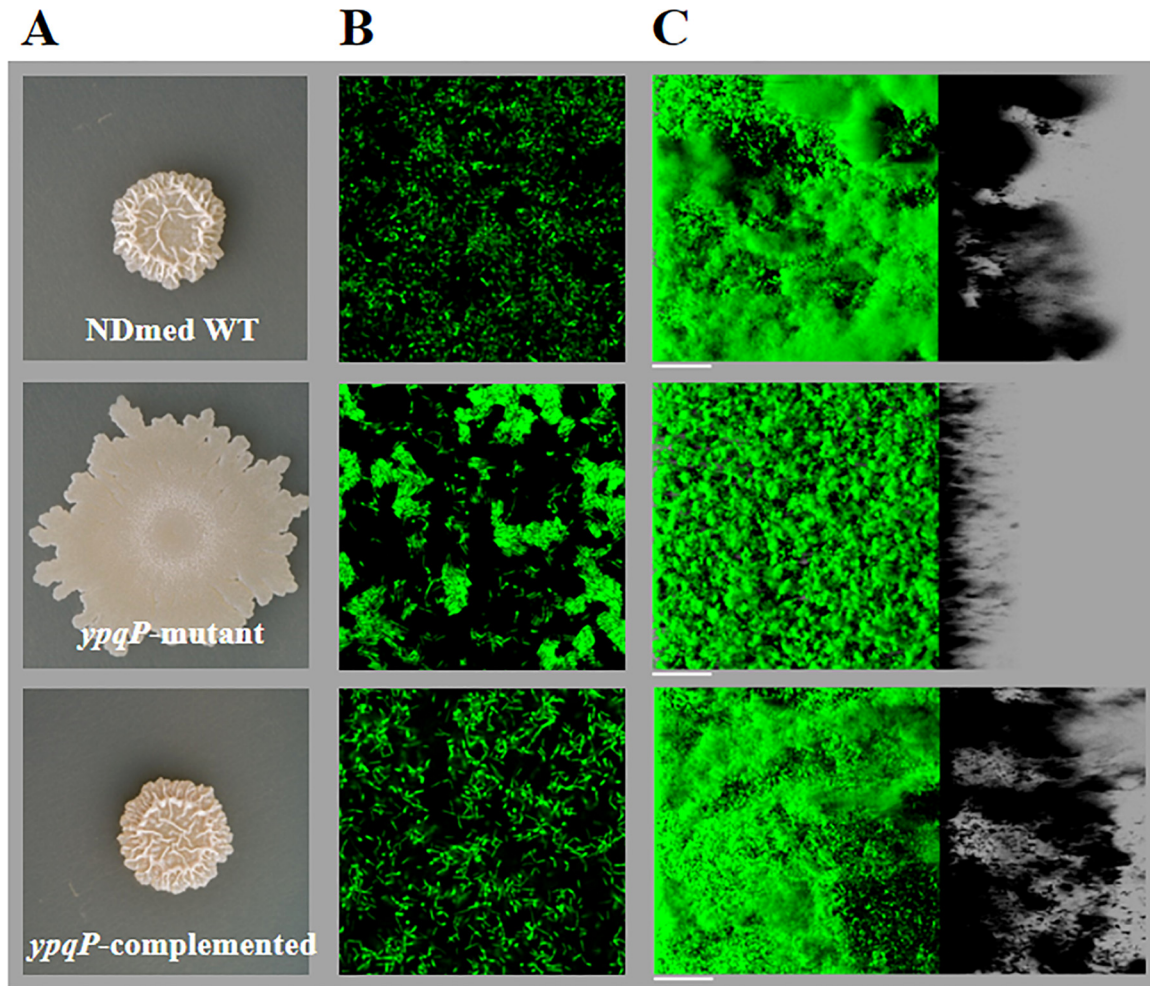


FIG 3 Visualization of the effect of *ypqP* disruption on submerged-biofilm structure and complex colony morphology in *B. subtilis* NDmed WT and mutant strains. (A) Colonies of the NDmed WT, *ypqP* mutant (GM3248), and *ypqP*-complemented (GM3326) strains were grown in TSB agar for 3 days. (B and C) Biofilms of the three strains were grown for 48 h and stained with SYTO9. For each strain, representative images of the adherent cells in contact with the surface (B) and the 3D reconstruction using IMARIS software (C) are presented. The scale bars represent 50 μm .

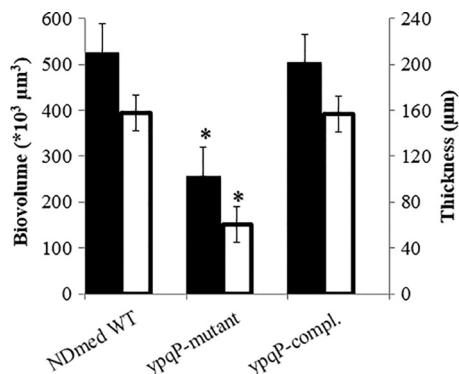


FIG 4 Quantification of biovolumes and thicknesses of submerged biofilms of *B. subtilis* NDmed WT and mutant strains. Biovolumes (black bars) and maximum thicknesses (white bars) were calculated from six series of images for each strain (NDmed WT, *ypqP* mutant, and *ypqP* complemented [compl.]). The error bars indicate the standard errors, and statistically significant differences from the NDmed strain ($P < 0.05$) observed are indicated by asterisks.

mutant formed rafts of cells on the surface (similar to those formed in monospecies biofilms) and the *S. aureus* cells remained separated from them, colonizing the void spaces on the surface and above the *B. subtilis* cells. In order to determine whether *ypqP* was also involved in *S. aureus* biocide protection, dual-species biofilms were subjected to the same biocide treatments as *B. subtilis* monospecies biofilms. When *S. aureus* monospecies biofilms were exposed to OPA or PAA, no survivors could be counted with either biocide, which corresponded to 8.85 ± 0.14 log red. In contrast, tolerance of the pathogen for both biocides was increased when cocultured with the *B. subtilis* NDmed WT strain: log red values of only 3.44 ± 0.14 and 3.33 ± 0.36 were observed with OPA and PAA, respectively (Fig. 7A and B). These results are consistent with those previously obtained with PAA (30). The *ypqP* mutant and the complemented strain were also cocultured with *S. aureus* and subjected to the actions of these antimicrobials. When grown with the *ypqP* mutant, the tolerance of *S. aureus* for OPA or PAA decreased markedly (5.95 ± 0.14 and 7.23 ± 0.41 log red, respectively) compared with that observed in the coculture with the WT strain, whereas similar tolerance was observed when it was cocul-

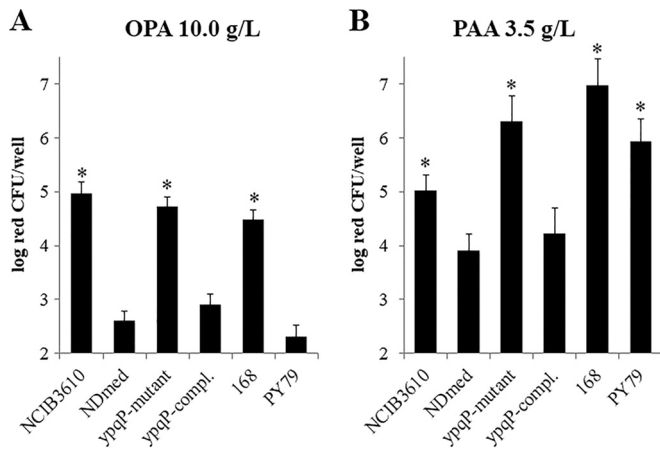


FIG 5 Antimicrobial resistance of submerged biofilms formed by *B. subtilis* strains. Biofilms formed by the strains with *ypqP* disrupted (NCIB3610, 168, and *ypqP* mutant) or *ypqP* functional (NDmed WT, *ypqP* complemented, and PY79) were subjected to 10-g/liter OPA (A) or 3.5-g/liter PAA (B) treatment for 5 min. Survivors were enumerated, and the log₁₀ reduction from the initial population was calculated. The results presented are the means of at least 8 experiments; the error bars indicate the standard errors, and statistically significant differences from the NDmed WT strain ($P < 0.05$) are indicated by asterisks.

tured with the *ypqP*-complemented *B. subtilis* strain, GM3326 ($P < 0.05$). These results therefore confirmed that the protective role of YpqP is also effective under multispecies conditions.

DISCUSSION

In a previous study, we showed that the thick and spatially structured biofilms formed by the *B. subtilis* NDmed strain were highly resistant to biocide action and enhanced the survival of the hospital pathogen *S. aureus* in multispecies communities (27, 30). In this work, we highlighted the involvement of the *B. subtilis* gene *ypqP* in these biofilm-associated phenotypes. Disruption of *ypqP* resulted in the loss of the typical protruding structures observed in

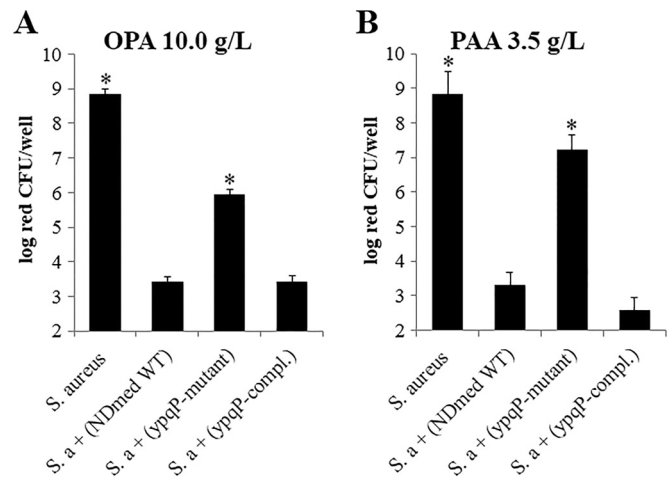


FIG 7 Antimicrobial resistance of *S. aureus* (*S. a*) cells in mono- and dual-species biofilms with *B. subtilis* NDmed. *S. aureus* mono- and dual-species biofilms were subjected to 10 g/liter OPA or 3.5 g/liter PAA. The log₁₀ reductions of *S. aureus* populations, alone or in a mixed biofilm with *B. subtilis* (NDmed WT, *ypqP* mutant [GM3248], and *ypqP* complemented [GM3326]) are presented for OPA (A) and PAA (B). The results presented are the means of at least 8 experiments; the error bars indicate the standard errors, and statistically significant differences from *S. aureus* in dual-species biofilms with NDmed WT ($P < 0.05$) are indicated by asterisks.

NDmed WT submerged biofilms and in the decrease of the resistance to two biocides widely used for decontamination in the medical setting, an oxidizing agent (PAA) and an aldehyde (OPA). NDmed was far more resistant to these two biocides than the reference strains NCIB3610 and 168, in which *ypqP* is naturally disrupted by insertion of the SPβ prophage. The participation of a functional *ypqP* gene in the hyperresistance of NDmed to biocides was also demonstrated in multispecies biofilms. The disruption of *ypqP* in NDmed led to reduced protection of *S. aureus* when subjected to biocides. The visualization of mixed-species biofilms using CLSM revealed that the spatial distribution of both species was

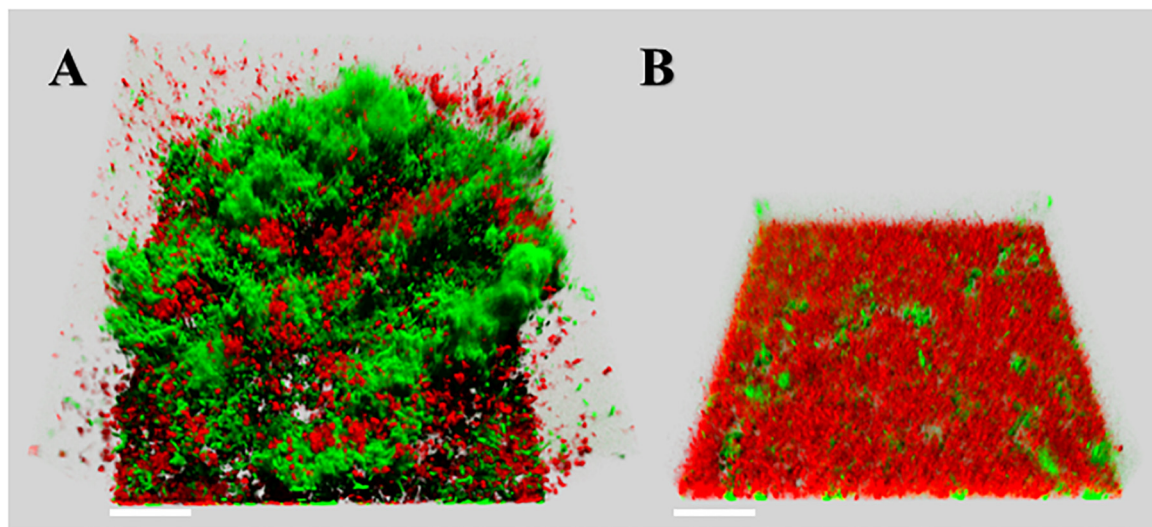


FIG 6 Three-dimensional organization of *B. subtilis* NDmed and *S. aureus* mixed biofilms. Mixed biofilms of *S. aureus* mCherry (red) and *B. subtilis* GFP (green) strains were grown for 48 h as described in Materials and Methods. Representative 3D reconstruction images of *S. aureus* and *B. subtilis* NDmed WT (A) or *ypqP* mutant (B) mixed biofilms are presented. The scale bars represent 50 μm.

markedly affected when *ypqP* was disrupted. Disruption of *ypqP* did not alter planktonic-cell resistance to biocides, and no spores were detected in biofilms formed by NDmed WT and mutant strains (data not shown). However, the absence of YpqP lessens both the three-dimensional structure and the resistance to antimicrobial action, suggesting, in accordance with *in silico* analysis, its involvement in the production of extracellular polymeric substances (EPS).

In several reports, the resistance of biofilm cells to antimicrobials has been linked to the production of EPS, which are also required to build the three-dimensional edifice (42, 43). The biofilm matrix has been involved in bacterial resistance to biocides through direct interference with diffusion and/or reaction of antimicrobials or through physiological heterogeneities driven by the three-dimensional organization (44, 45). Our previous results suggested direct interference between the biocidal molecules and the matrix components, as a matrix extract from NDmed biofilms, but not from 168 biofilms, could protect planktonic cells of *S. aureus* from the action of PAA (30). A direct-interference mechanism preventing water and organic-solvent penetration has been demonstrated for the BslA protein, which forms a hydrophobic raincoat on the inner layer of *B. subtilis* colonies (46–49), as well as curli fibers in *E. coli* colonies or extracellular DNA in *P. aeruginosa* submerged biofilms (50, 51). Biocide molecules can be delayed by the biofilm polymer matrix (52) or diffuse easily into the biofilm but strongly react with organic material, such as the oxidizing agent PAA (53, 54). Aldehydes, such as OPA, act as cross-linking agents reacting against proteins, DNA, and RNA (55). Hence, many of the biofilm polymeric substances produced by *B. subtilis* cells have the potential to hamper biocide reactivity. In addition to the amyloid protein TasA, the BslA protein, and the polysaccharides synthesized from the *epsA-O* operon, other components that are not essential for biofilm formation but are produced in large quantities under specific conditions (such the anionic polymer poly- γ -glutamate [PGA]) may be responsible for the hyperresistance observed in NDmed biofilms (56–58). Nevertheless, no differences were found between the sequences of the genes involved in the production of these polymers in the NDmed and NCIB3610 strains (data not shown), reinforcing our hypothesis regarding the important role played by the product of *ypqP*. In NDmed, as well as in the other *B. subtilis* strains used in this work, the gene *ypqP* is placed between the *phy* and *msrB* genes (encoding phytase and peptide methionine R-sulfoxide reductase, respectively), two genes in opposite orientations not described as being involved in biofilm formation and not expressed under those conditions (59). An identical gene organization can be found in the closely related *B. amyloliquefaciens*. In more distantly related species (*Bacillus licheniformis*, *Bacillus cereus*, or *S. aureus*), the nearest homolog of *ypqP* (named *capD* or *capE*) apparently belongs to an operon or to a cluster annotated as being involved in capsular-polysaccharide synthesis. According to the *in silico* analysis, *ypqP* codes for a UDP-*N*-acetylglucosamine 4,6-dehydratase, an enzyme likely involved in polysaccharide synthesis. In addition, a global study of the transcriptome of *B. subtilis* subjected to various conditions revealed that the *ypqP* promoter was highly active in biofilm cells (59). Two homologous proteins, FlaA1 and PseB (46% and 49% similarity to the *B. subtilis* NDmed YpqP protein, respectively) (see Fig. S1 in the supplemental material), have been characterized biochemically as UDP-*N*-acetylglucosamine 5-inverting 4,6-dehydratases, which catalyze the first step in the biosynthesis of

pseudaminic acid in *Helicobacter pylori* and *Campylobacter jejuni* (60, 61). Complementary analytical analysis would be needed to determine if *B. subtilis* can produce pseudaminic acid. Alternatively YpqP could be involved in the biosynthesis of a different polymer. The presumed substrate of this enzyme, UDP-*N*-acetylglucosamine (GlcNAc), is one of the most abundant building blocks for polymer synthesis in bacteria (62). Particularly in *Staphylococcus* spp. and *E. coli*, poly-*N*-acetylglucosamine is an important matrix component essential for biofilm formation and protection against host defenses and antimicrobial peptides (63–65). To our knowledge, none of the *B. subtilis* matrix polymers described is composed of GlcNAc. Interestingly, a recent study demonstrated that the protein responsible for the interconversion of UDP-*N*-acetylglucosamine and UDP-*N*-acetylgalactosamine in *B. subtilis*, GalE, is required for biofilm formation, since its product is used for exopolysaccharide production (66). The products of the *epsA-O* operon are responsible for the synthesis of the principal polysaccharide in *B. subtilis* biofilms, but its composition remains unknown. YpqP could also have a regulatory effect on the production of other polymers, as recently demonstrated with the *epsA-O* operon (67). The role of YpqP in either the synthesis of a new polysaccharide or the modification of known polysaccharides requires further investigation.

During this study, we were able to demonstrate the important function of YpqP in the properties of *B. subtilis* submerged biofilms and the formation of wrinkled colonies on agar, a widely used model associated with abundant matrix production (17). In contrast, we did not observe any significant effect of *ypqP* disruption on the macroscopic aspect of floating pellicles, the principal model used to study the multicellular behavior of *B. subtilis* (data not shown). Other studies have highlighted specific determinants for multicellular development in these two liquid-air and solid-liquid interfaces. For example, the PGA polymer is required for submerged-biofilm formation but not for floating pellicles (68); the signal peptidase SipW, required for TasA maturation in complex colonies, plays a regulatory role only in biofilm formation on submerged surfaces (69). The roles of the biofilm determinants identified using the pellicle model (e.g., the *tapA-sipW-tasA* operon, the *epsA-O* operon, and *bslA*) have to be further investigated at the solid-liquid interface. In natural and human-made environments, cells are mostly associated with surfaces or interfaces, likely alternating contact with liquid and air. Hence, the interest of using a submerged model as a complementary and relevant system to study the genetic regulation of *B. subtilis* biofilms formed under other specific ecological conditions is highlighted by this work. We propose YpqP as a major determinant for surface-associated communities and the resistance to antimicrobials of the ubiquitous bacterium *B. subtilis*. Functional *ypqP* found in other strains isolated from very diverse environments (i.e., plant tissues, desert sand, animal gut, dairy products, or medical devices) suggests that its involvement in biofilm architecture and physiology may constitute an ecological advantage for *B. subtilis* in some of its natural habitats. In contrast, disruption of the gene by insertion of the SP β prophage in other strains might be a regulatory mechanism or enable adaptation to certain specific conditions.

In conclusion, in this work, we identified a new biofilm determinant required for protection of a sensitive *S. aureus* strain from the action of disinfectants by the hyperresistant *B. subtilis* strain NDmed. YpqP is likely involved in the production of “public

goods" that can protect biofilm inhabitants against the actions of biocides (70, 71). These findings underline the importance of studying the interactions between pathogens and resident microflora, such as the ubiquitous bacterium *B. subtilis*, to develop efficient control strategies against infectious microorganisms.

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