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Transcriptional regulation of adhesive properties of *Bacillus subtilis* to extracellular matrix proteins through the fibronectin-binding protein YloA

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Summary

Bacterial adherence to extracellular matrix proteins (ECMp) plays important roles during host–pathogen interaction, however its genetic regulation remains poorly understood. *yloA* of the model bacterium *Bacillus subtilis* shows high homology to genes encoding fibronectin-binding proteins of Gram-positive pathogens. Here, we characterized the regulatory network of YloA-dependent adhesive properties of the probiotic *B. subtilis* natto (*Bsn*). YloA-proficient, but not YloA-deficient, *Bsn* specifically bound to ECMp in a concentration-dependent manner and were proficient in biofilm formation. *yloA* expression showed a continuous increase in activity during the growth phase and decreased during the stationary phase. The transcription factors AbrB and DegU downregulated *yloA* expression during the logarithmic and stationary growth phases respectively. Analysis of the *yloA* promoter region revealed the presence of AT-rich direct and inverted repeats previously reported to function as DegU-recognized binding sites. In *spo0A* cells, *yloA* expression was completely turned off because of upregulation of AbrB throughout growth. Accordingly, DNase I footprinting analysis confirmed that AbrB bound to the promoter region of *yloA*. Interestingly, *Bsn* bound fibronectin with higher affinity, lower *Kd*, than several bacterial pathogens and competitively excluded them from binding to immobilized-fibronectin, a finding that might be important for the anti-infective properties of *B. subtilis* and its relatives.

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Introduction

Adherence to and colonization of host tissues are bacterial abilities that facilitate the early steps and evolution of infections. In mammals, host mucosal surface represents an extensive area in contact with the external environment through which different pathogens can initiate infection (Joh *et al.*, 1999; Styriak *et al.*, 1999; Schwarz-Linek *et al.*, 2004; Pizarro-Cerda and Cossart, 2006). To date, a number of factors involved in the progression of the bacterial adhesive process have been identified including members of adhesion proteins MSCRAMM (microbial surface components recognizing adhesive matrix molecules) such as bacterial adhesins to extracellular matrix (ECM) components of the host mucosa (Joh *et al.*, 1999; Wann *et al.*, 2000; Styriak *et al.*, 2003; Schwarz-Linek *et al.*, 2004).

Fibronectin is a dimeric glycoprotein present in a soluble form in blood plasma and extracellular fluids, and in a fibrillar form of higher molecular weight present in the ECM (Romberger, 1997). Although fibronectin has critical roles in eukaryotic cellular processes, such as adhesion, migration and differentiation, it is also a common substrate for the attachment of bacteria (Proctor *et al.*, 1982; Singh *et al.*, 2015; Somarajan *et al.*, 2015). The fibronectin-binding proficiency displayed by several pathogens constitutes an advantage improving bacterial survival and virulence (Terao *et al.*, 2001; Singh *et al.*, 2015; Somarajan *et al.*, 2015). Several Gram-positive pathogenic cocci are proficient to bind fibronectin;

Staphylococcus aureus, an opportunistic pathogen producing superficial skin infections as well as invasive life threatening infections such as septic arthritis and endocarditis (Lindberg *et al*., 2004; O'Neill *et al*., 2008; Burke *et al*., 2010), binds to epithelial cells via fibronectin, facilitating internalization and systemic spread within the host (Bozzini *et al*., 1992; Joh *et al*., 1999; Styriak *et al*., 1999; Ingham *et al*., 2004; Loughman *et al*., 2008; Burke *et al*., 2011). *Streptococcus mutans* is routinely involved in coronal caries and dental root decay but it is also able to colonize heart valves and endocardium causing endocarditis when entering into the bloodstream. These processes are enhanced by the binding ability of this pathogen to ECM components of the host (Beg *et al*., 2002; Miller-Torbert *et al*., 2008). *Streptococcus pneumoniae* colonizes the nasopharynx in up to 40% of healthy subjects, and is a leading cause of middle ear infections (otitis media) in children, and meningitis and pneumonia in the elderly or immunocompromised. Accordingly, colonization of the nasopharynx by *S. pneumoniae* is a prerequisite for the development of pneumococcal disease and fibronectin-binding deficient mutants (*pavA* mutants) of encapsulated *S. pneumoniae* were approximately 10^4-fold attenuated in virulence in a mouse sepsis model (Holmes *et al*., 2001). Enterococci are among the first microbial colonizers of the infant gastrointestinal tract and they are part of the commensal intestinal microbiota in healthy adults. However, enterococci have also emerged as opportunistic pathogens and major causes of health care-associated infections, most notably urinary tract infections, bacteremia and endocarditis (Singh *et al*., 2015; Somarajan *et al*., 2015). *Enterococcus faecalis* strains deficient in fibronectin-binding proficiency (*efbA* mutants) were significantly attenuated in their virulence compared with wild-type cells in a rat endocarditis model (Singh *et al*., 2015). Not only Gram-positive cocci (staphylococci, streptococci and enterococci) are proficient to bind fibronectin. *Listeria monocytogenes* is a ubiquitous Gram-positive, food-borne bacillus responsible for intracellular life-threatening infections, causing sepsis and meningoencephalitis in pregnant women, fetuses and immunocompromised hosts. Adherence of *L. monocytogenes* to the host cell surface is a critical event during infection (Cossart, 2002; Dramsi *et al*., 2004; Osanai *et al*., 2013). This pathogen synthesizes a fibronectin-binding adhesin (FbpA) playing a key role on adherence and virulence as suggested by the reduced pathogenicity of Δ*fbpA L. monocytogenes* mutant strains in mouse model (Dramsi *et al*., 2004). Taken together, the fibronectin-binding ability displayed by several bacterial pathogens represents an important virulence factor for the development, progression and success of the infection.

In addition to antibiotic treatment, different alternative strategies have emerged to prevent or combat bacterial infections and multiresistance to antibiotics (Boucher *et al*., 2009; Brusselaers *et al*., 2011; Roldán *et al*., 2014; Karam *et al*., 2016). For example, inhibition of pathogen bacterial adhesion through vaccination with recombinant adhesins (Schennings *et al*., 1993; Kawabata *et al*., 2001) and competitive exclusion produced by probiotic bacteria (Kirjavainen *et al*., 1998; Ouwehand *et al*., 2001; Styriak *et al*., 2003; Buck *et al*., 2005, 2010) are suitable strategies for this purpose. Probiotics are live microorganisms producing beneficial effects on host health when administered or present on the host mucosa in adequate quantities (Tannock, 2005; Thomas and Versalovic, 2010; Mackowiak, 2013). The most common microorganisms used as probiotics are lactic acid bacteria, LAB, (Lee *et al*., 2011). In addition to LAB, some beneficial spore-forming bacteria of the genus *Bacillus* (i.e., *B. subtilis* and *B. coagulans*) have emerged as a new type of probiotic for human consumption (Senesi *et al*., 2004; Willians, 2007; Endres *et al*., 2009; Feldman *et al*., 2009; Cutting, 2011; Benson *et al*., 2012; Vidyalaxme *et al*., 2014; Donato *et al*., 2017). The difference between probiotic bacilli and LAB primarily relies on two beneficial properties: refrigeration is not required for maintaining bacilli viability, and these bacteria can be added to a wide range of foods and beverages in addition to dairy products (Duc *et al*., 2004; Barák *et al*., 2005; Cutting, 2011). Adhesion to intestinal surfaces is believed to be an important probiotic characteristic, as proposed by the FAO/WHO and several reports (Kirjavainen *et al*., 1998; Ouwehand *et al*., 2001; Araya *et al*., 2002; Lorca *et al*., 2002; Styriak *et al*., 2003; 2010; Buck *et al*., 2005; Perea Vélez *et al*., 2007).

Even though the abundant information related to the biochemistry of ECM-binding adhesins, very little is known about its genetic regulation. One attractive probiotic and genetically-tractable model-organism to investigate the genetic regulation of adherence to ECM components and competition with the adherence of bacterial pathogens is *B. subtilis* (Kearns *et al*., 2005, 2003; Willians, 2007; Hong *et al*., 2009; Vidyalaxme *et al*., 2014; Grau *et al*., 2015; Hanifi *et al*., 2015; Donato *et al*., 2017). In the present study, we characterize the adhesive properties of different wild strains of *B. subtilis* to components of the ECM and identify a novel gene encoding for a fibronectin-binding protein and its genetic regulation. In addition, we present experimental evidence supporting the suitability of *B. subtilis* to competitively exclude several fibronectin-binding proficient pathogens from adherence to ECM components.

Table 1. Strains used in this study.

Strains	Relevant phenotype and/or genotype	Comments and/or source (reference)
JH642	Related to wild-type *B. subtilis* 168 strain	Laboratory collection (Arabolaza *et al.*, 2003)
NCIB3610	Related to wild-type *B. subtilis* Marburg strain	Laboratory collection (Grau *et al.*, 2015)
RG4365	Related to wild-type *B. subtilis* natto strain	Laboratory collection (Lombardía *et al.*, 2006)
RG4366	Δ*spo0A*::Ery^r (erythromycin resistance)	This work, SS955 (Méndez *et al.*, 2004) →RG4365 (DNA from donor strain → receptor strain)
RG4367	Δ*spoIIAC*::Kan^r (Kanamycin resistance) Deficient in Sigma F production	This work, RG19148 (Arabolaza *et al.*, 2003)→RG4365
RG4368	Δ*abrB*::Cm^r (Cloramphenicol resistance)	This work, RG12607 (Lombardía *et al.*, 2006)→RG4365
RG4369	Δ*sinR*::Spc^r (Spectinomycin resistance)	This work, RG432 (Gottig *et al.*, 2005)→RG4365
RG4370	Δ*spo0A*::Ery^r-Δ*abrB*::Cm^r	This work, RG4366→RG4368
RG4371	Δ*spo0A*::Ery^r-Δ*sinR*::Spc^r	This work, RG4366→RG4369
RG4372	Δ*sinR*::Spec^r-Δ*abrB*::Cm^r	This work, RG4369→RG4368
RG4373	Δ*spo0A*::Ery^r-Δ*sinR*::Spc^r-Δ*abrB*::Cm^r	This work, RG4366→RG4372
RG4374	*yloA*::Cm^r	This work, pJM103::Δ*yloA*→RG4365
RG4375	*amyE*::P*yloA-lacZ* Cm^r	This work, pJM116::P*yloA*→RG4365
RG4376	*yloA*::Spc^r	This work, pCm::Spc (Arabolaza *et al.*, 2003)→RG4374
RG4377	*yloA*::Spc^r-*amyE*::*yloA*^{wt} Cm^r	This work, pJM116::yloA^{wt}→RG4376
RG4378	Δ*abrB*::Spc^r	This work, pCm::Spc (Arabolaza *et al.*, 2003)→RG4368
RG4379	Δ*abrB*::Spc^r-*amyE*::P*yloA-lacZ* Cm^r	This work, RG4375→RG4378
RG4380	Δ*spo0A*::Ery^r-*amyE*::P*yloA-lacZ* Cm^r	This work, RG4366→RG4375
RG4381	Δ*abrB*::Spc^r-Δ*spo0A*::Ery^r-*amyE*::P*yloA-lacZ* Cm^r	This work, RG4366→RG4379
RG4382	Δ*roK*::Kan^r-*amyE*::P*yloA-lacZ* Cm^r	This work, 168 Δ*roK*::Kan^r (Kovács and Kuipers, 2011)→RG4375
RG4383	Δ*luxS*::Spc^r-*amyE*::P*yloA-lacZ* Cm^r	This work, RG1340 (Lombardía *et al.*, 2006)→RG4375
RG4384	Δ*sinR*::Spc^r-*amyE*::P*yloA-lacZ* Cm^r	This work, RG432 (Gottig *et al.*, 2005)→RG4375
RG4385	Δ*comA*::Kan^r-*amyE*::P*yloA-lacZ* Cm^r	This work, BD1777 (Kovács and Kuipers, 2011)→RG4375
RG4386	Δ*degSU*::Kan^r-*amyE*::P*yloA-lacZ* Cm^r	This work, QB4238 (Kovács and Kuipers, 2011)→RG4375
RG4387	Δ*TnrA*::Spc^r-*amyE*::P*yloA-lacZ* Cm^r	This work, JH19190 (Wang *et al.*, 1997)→RG4375
RG4388	Δ*codY*::Spc^r-*amyE*::P*yloA-lacZ* Cm^r	This work, JH19199 (Wang *et al.*, 1997)→RG4375
RG4389	Δ*sigB*::Ery^r-*amyE*::P*yloA-lacZ* Cm^r	This work, MR744 (Méndez *et al.*, 2004)→RG4375
RG4390	Δ*spo0A*::Ery^r- Δ*degSU*::Kan^r-*amyE*::P*yloA-lacZ* Cm^r	This work, RG4366→RG4386
RG4391	Δ*abrB*::Spc^r-Δ*degSU*::Kan^r-*amyE*::P*yloA-lacZ* Cm^r	This work, RG4378→RG4386
RG4392	Δ*abrB*::Spc^r-Δ*spo0A*::Ery^r-Δ*degSU*:: Kan^r-*amyE*::P*yloA-lacZ* Cm^r	This work, RG4366→RG4391
DH5α	*E. coli* strain used for DNA constructions	Laboratory collection (Gottig *et al.*, 2005)
ATCC12600	*S. aureus*	Laboratory collection (Roldán *et al.*, 2014)
ATCC7644	*L. monocytogenes*	Laboratory collection (Roldán *et al.*, 2014)
ATCC29212	*E. faecalis*	Laboratory collection (Roldán *et al.*, 2014)

Results

Adhesive properties of B. subtilis *to ECM components and its regulation*

Human-beneficial gut bacteria should have the ability to adhere to components of the ECM to grow as a biofilm and to produce beneficial effects in the gastrointestinal tract (Donato *et al.*, 2017). To test whether this property was present in *B. subtilis*, we initially utilized the crystal violet (CV) technique to analyze three wild-type *B. subtilis* strains (Table 1) of different ancestry for their proficiency to bind immobilized human fibronectin. Figure 1 shows the amount of crystal violet (Ab_{570} nm) bound to fibronectin- and BSA-adhered *B. subtilis* cells. All the tested *B. subtilis* strains (JH642, NCIB3610, RG4365) showed a concentration-dependent, saturable and specific binding to fibronectin and low affinity for bovine serum albumin (BSA) used as a negative control of binding specificity (Fig. 1). Because the natto-related strain RG4365 showed the highest whole-cell fibronectin-binding proficiency, we selected this strain to unravel the genetic control of *B. subtilis* adherence to ECM components.

Surface adherence would represent a prerequisite for *B. subtilis* to display different collective behaviors such as sessile biofilm formation and surface-associated swarming and sliding motilities (Kearns *et al.*, 2003; 2005; Stanley and Lazazzera, 2004; Grau *et al.*, 2015; Vlamakis *et al.*, 2013). These social behaviors are under the control of several master transcription factors such as AbrB, SinR and the active form of Spo0A (phosphorylated Spo0A, Spo0A∼P) (Grau *et al.*, 2015). We hypothesize that initial commitment with a surface constitutes a property that should be genetically controlled (Shapiro, 1998; Ben Jacob *et al.*, 2004; Parsek and Greenberg, 2005). Therefore, we were intrigued to know the identity of the regulatory proteins affecting the binding ability of *B. subtilis* to ECM components. For this purpose, we analyzed the ability of fluorescein isothiocyanate (FITC)-labeled RG4365 *B. subtilis* cells and isogenic mutant derivatives (Table 1) to adhere to immobilized fibronectin and

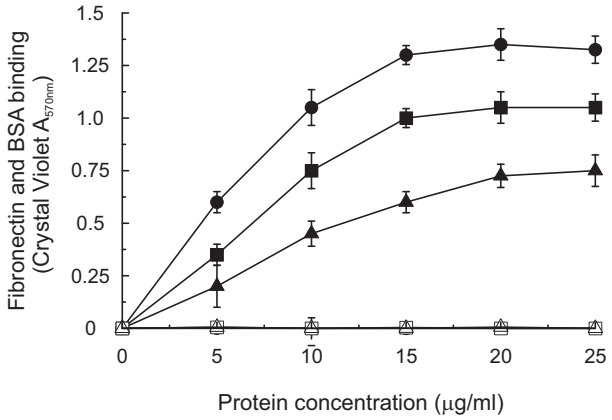

Fig. 1. *Bacillus subtilis* is proficient in specific binding to fibronectin.
The fibronectin-binding proficiency of different wild-type *B. subtilis* strains (natto-related RG4365, Marburg-relate NCIB3610, domesticated JH642) was assayed in microtiter wells coated with a fixed amount of bacteria and increasing amounts of fibronectin (black symbols) or BSA (empty symbols). After incubation, with gentle shaking, samples were treated to remove unbound bacteria as indicated in Experimental Procedures. Fibronectin- and BSA-bound bacteria were stained with CV, washed and the stain bound to bacteria was removed and quantified (A_{570}). Data are the average values of three independent experiments performed by quadruplicate. The specificity of fibronectin-binding was tested using BSA as immobilized control protein. The mean value obtained from bacteria bound to BSA was subtracted from data points shown in the figure. The assayed *B. subtilis* strains are: RG4365 (circle), NCIB3610 (square) and JH642 (triangle). Filled and empty symbols indicate the mean binding ± S.E.M. to fibronectin and BSA respectively.

collagen as representative components of the ECM and using BSA as control of specificity. As shown in Fig. 2, FITC-labeled wild-type *B. subtilis* was able to adhere specifically to fibronectin and collagen but not to BSA (Fig. 2A and H). The specific binding to fibronectin and collagen was Spo0A∼P-dependent but independent of sporulation proteins as observed by the deficiency (Fig. 2B and H) and proficiency (Fig. 2C and H) of the *spo0A* and *sigF* (encoding for Sigma F, the first sporulation-specific sigma factor) cells to bind ECM components respectively. These results indicate that, in addition to the surface-associated phenomena of biofilm formation and social motility, Spo0A∼P controlled the adhesive properties of *B. subtilis*.

Insufficient levels of Spo0A∼P to trigger sporulation are sufficient to upregulate exoenzyme and antimicrobial compounds production through downregulation of the transition state regulators AbrB and SinR (Strauch *et al*., 1990; Arabolaza *et al*., 2003; Hamon *et al*. 2004; Gottig *et al*., 2005; Chu *et al*., 2006). Low levels of Spo0A∼P repress *abrB* expression (Strauch *et al*., 1990; Grau *et al*., 2015) and intermediate levels of Spo0A∼P induce *sinI*, which encodes for an inhibitor of SinR activity (SinI protein) (Shafikhani *et al*., 2000; Gottig *et al*., 2005). By contrast, in the absence of Spo0A activity, AbrB and SinR are upregulated (Strauch *et al*., 1990; Shafikhani *et al*., 2000; Gottig *et al*., 2005; Chu *et al*., 2006). Therefore, the cell ability to specifically bind fibronectin and collagen may be under the direct control of Spo0A∼P or regulated indirectly through Spo0A∼P effects on AbrB and/or SinR. Inactivation of the genes encoding for the master regulators AbrB (Δ*abrB*, Fig. 2D) and SinR (Δ*sinR*, Fig. 2E) did not abolish but increased, in the case of Δ*abrB* cells, the *B. subtilis* proficiency to bind the selected ECMp (Fig. 2H). However, surface binding of the Δ*sinR* strain became unspecific since these mutant cells also bound significant amounts of BSA (Fig. 2E and H). The high and nonspecific adherence of SinR-deficient *B. subtilis* cells to other surfaces (i.e., solid LB and DSM media) has been previously observed (Gottig *et al*., 2005 and unpublished results). As shown in Fig. 2F and H, the deficiency in specific adherence to fibronectin and collagen of Spo0A-deficient cells was completely restored after the inactivation of *abrB*. The inactivation of *sinR* in a *spo0A* minus background was also able to restore the surface binding ability but it was nonspecific since this double mutant strain also showed a strong adherence to BSA (Fig. 2G and H). In addition, the adherence proficiency of Δ*abrB*Δ*sinR*, Δ*spo0A*Δ*sinR*Δ*abrB* and wild-type spore cells to fibronectin and collagen was high but nonspecific (data not shown).

To confirm the previous results, we measured the binding of the different *B. subtilis* strains to fibronectin and collagen using the crystal violet technique. As shown in Fig. 3, the requirement of Spo0A∼P activity to block the AbrB-mediated inhibition of fibronectin and collagen binding was confirmed. Therefore, the overall results (Figs 1–3) uncover a novel negative role for AbrB on the regulation of the specific and high adherence of *B. subtilis* to proteins of the ECM.

The B. subtilis yloA *gene encodes for a fibronectin-binding protein homolog present in pathogenic enterococci, streptococci and bacilli*

After a search in the genomic data bank available for *B. subtilis* (www.genobd.pasteur.fr), we found a long 1716 bp open reading frame (ORF) encoding for a putative fibronectin-binding protein (*yloA* accession number BG13383) which is flanked by *yloB* (encoding a putative calcium-transporter ATPase) and the *cysH* operon (involved in L-cysteine synthesis) divergently transcribed (Fig. 4A). The putative promoter region of *yloA* contains a consensus housekeeping (Sigma A) RNA-dependent polymerase −10 and −35 promoter sequences (Nicolas *et al*., 2012) and a typical *B. subtilis* ribosomal binding

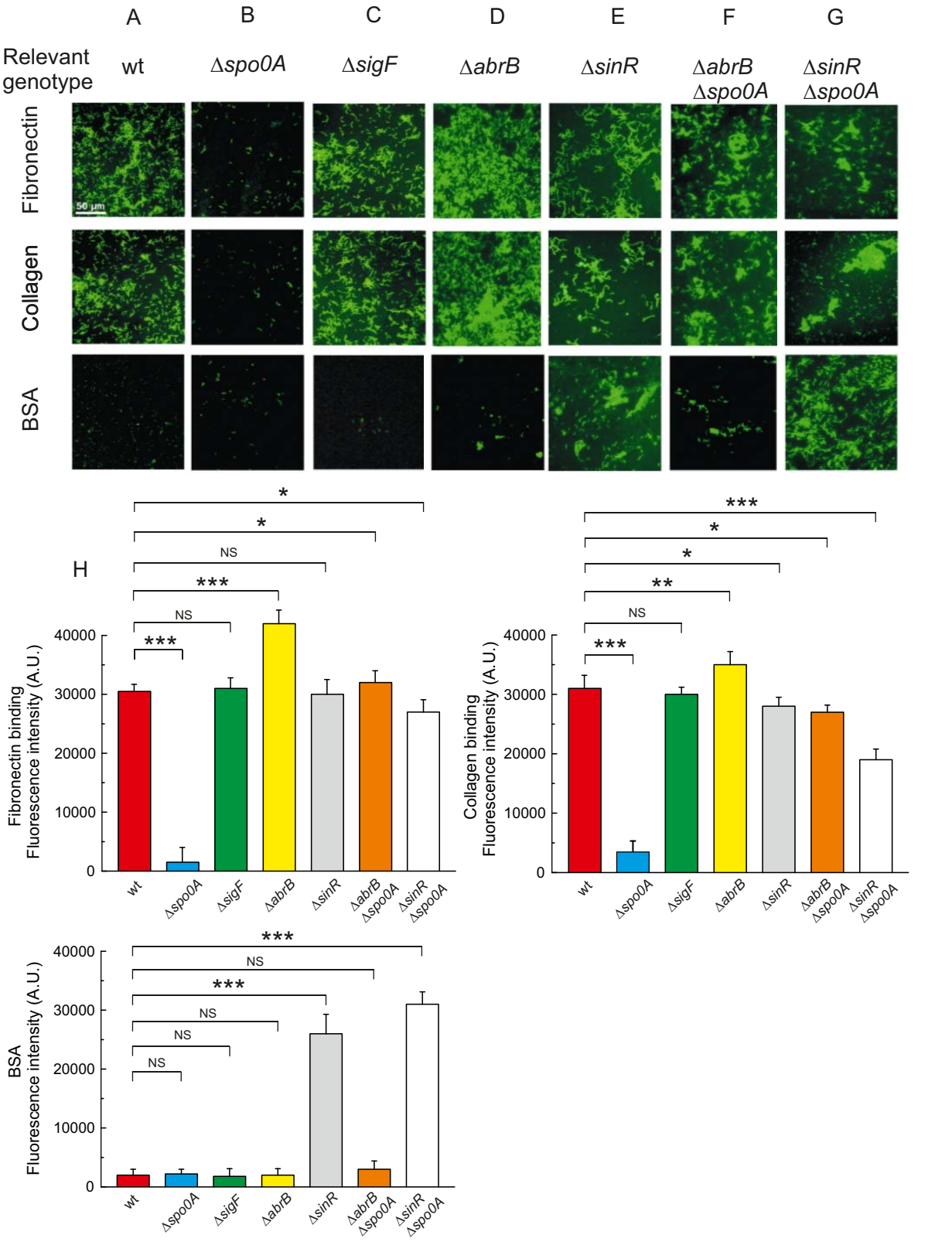
A
B
C
D
E
F
G
Relevant genotype
wt
ΔspoOA
ΔsigF
ΔabrB
ΔsinR
ΔabrB ΔspoOA
ΔsinR ΔspoOA
Fibronectin
Collagen
BSA
50 μm
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Fig. 2. AbrB plays a key role on the specific adhesive properties of *B. subtilis* to ECM components.
A–G. The photographs show FITC-labeled wild-type RG4365 cells and isogenic derivatives (Table 1) adhered to immobilized-fibronectin (top panel) and immobilized-collagen (middle panel) as representative proteins of the ECM. The bottom panel shows the adherence level of the FITC-labeled cells to BSA (negative control). The absence of specific binding of *spo0A* deficient-cells (panel B, strain RG4366) to both ECM proteins was restored after the inactivation of *abrB* (panel F, strain RG4368). Inactivation of *sinR* (strain RG4369) in any genetic background leads to an unspecific protein-binding (panels E and G, strains RG4368 and RG4371). A representative result of three experiments performed by separate is shown. Size bar represents 50 μm and strains are listed in Table 1. (H) Fluorescence intensity of FITC-labeled cells to fibronectin, collagen and BSA. NS: no significant difference, $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$ (ANOVA with Bonferroni test).

site (RBS) located 6 bp upstream of the start TTG codon (Fig. 4A, top segment). The putative YloA protein consists of 572 amino acids and contains an N-terminal region homologue of the N-terminus domain of well-characterized atypical fibronectin-binding proteins (amino acids 4 to 431), followed by a conserved domain of unknown function (Duf814; amino acids 451 to 535) which is also conserved in atypical fibronectin-binding proteins (Fig. 4A, bottom expanded segment).

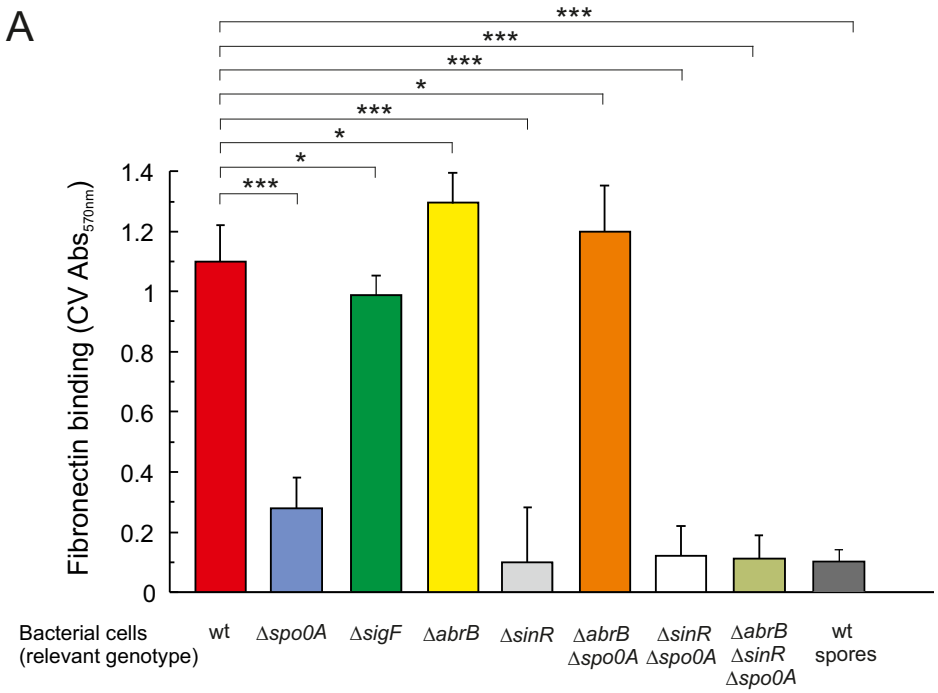
A neighbor-joining phylogenetic tree of *yloA* was constructed based upon the sequence of Gram-positive genes encoding for staphylococci-related FnBPs and atypical fibronectin-binding proteins of enterococci and streptococci. As expected, *B. subtilis yloA*. exhibits strong homologies (approximately 70% similarity and 45% identity over its full length) to the fibronectin-binding proteins present in streptococci, enterococci and other Gram-positive bacilli such as *B. anthracis* (accession number Q81WG3) and *B. cereus* (accession number A0A158RSA1) (Fig. 4B and Supporting Information Fig. S1). These are atypical fibronectin-binding proteins in the sense that do not contain the conventional secretion signal and anchorage motif present in staphylococci-related fibronectin-binding proteins (FnBPA and FnBPB) (O'Neill *et al*., 2008; Burke *et al*., 2010, 2011). Despite this, it was shown that the final localization of atypical fibronectin-binding proteins is in the cell surface (Holmes *et al*., 2001; Dramsi *et al*., 2004).

To confirm that *B. subtilis* YloA contributes to fibronectin adherence, we constructed a *yloA* deletion mutant (strain RG4376, Table 1) which completely lost its fibronectin binding proficiency ($p < 0.001$) and a derivative strain (RG4377, Table 1) in which a functional copy of *yloA* was ectopically introduced at the nonessential *amyE* locus, in which this property was completely restored (Fig. 5). The physiological parameters of both strains were similar to that of the parental strain RG4365 (data not shown). Similarly, the Δ*yloA B. subtilis* strain was unable to bind collagen, a proficiency that was recovered after the transcomplementation with a wild-type copy of Δ*yloA* (Fig. 5B). Interestingly, the EfbA fibronectin binding protein of *E. faecalis* (highly homolog to YloA of *B. subtilis*, see Fig. 4B) was shown to be important for the biofilm formation proficiency in pathogenic enterococci (Singh *et al*., 2015). As shown in Fig. 5C, the density and amount of the biofilm produced by the Δ*yloA B. subtilis* strain was reduced compared with the wild-type ($p < 0.001$). As expected, restoration of full biofilm formation proficiency was observed in the YloA-proficient trans-complemented strain RG4377 (Table 1). The overall results confirmed that *B. subtilis yloA* encodes for a functional fibronectin-binding protein (Fig. 5) homologous to the atypical fibronectin-binding proteins present in pathogenic cocci and bacilli (Fig. 4 and Supporting Information Fig. S1).

Transcriptional regulation of yloA *expression*

The biochemical properties of bacterial fibronectin-binding proteins have been extensively studied, however little is known about their genetic regulation. Our results (Figs 2 and 3) suggest an inhibitory role for the transcription factor AbrB on the adhesive properties of *B. subtilis*, possibly due to an effect on *yloA* transcription. Indeed, when we monitored an *yloA-lacZ* transcriptional fusion expression in wild-type and Δ*abrB B. subtilis* strains we found that expression was up-regulated in the absence of AbrB. The low level of expression observed in the Δ*spo0A* strain was likely due to derepression of AbrB, as shown previously (Strauch *et al*., 1990), since expression was restored in the double Δ*spo0A*Δ*abrB* mutant strain (Fig. 6A).

The downregulation of *yloA* during the stationary phase of growth in wild-type and Δ*abrB* strains suggested that other transcriptional factors would be affecting *yloA* expression. Therefore, we tested other well-characterized *B. subtilis* transcriptional regulators in their proficiency to affect *yloA* expression. As shown in Fig. 6B *yloA* expression was not affected by the regulatory proteins SinR (Gottig *et al*., 2005), ComA (Kovács and Kuipers, 2011), LuxS (Lombardía *et al*., 2006), Rok (Kovács and Kuipers, 2011), SigB (Méndez *et al*., 2004), TnrA (Wang *et al*., 1997) and CodY (Sonenshein, 2005). The absence of *yloA* regulation by SinR confirmed our previous observation of the high but unspecific binding of Δ*sinR* cells to fibronectin and collagen (see Fig. 2). Interestingly *yloA* expression was upregulated during the stationary phase of growth in the



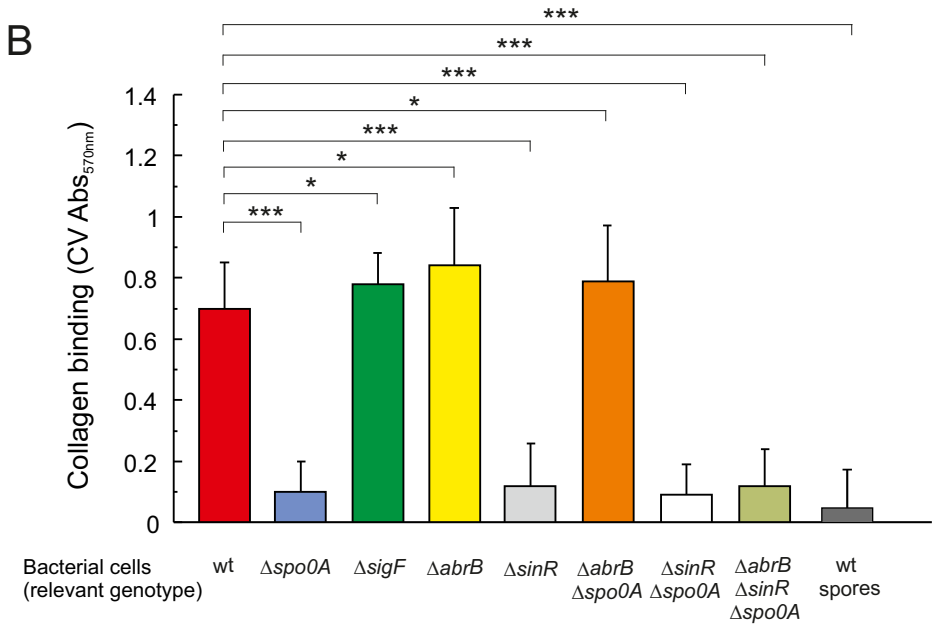
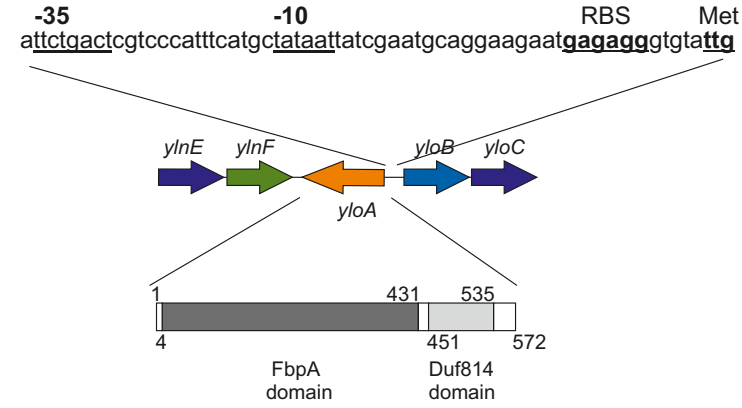


Fig. 3. Contribution of different regulatory genes to the adhesiveness of *B. subtilis* to ECM components. Immobilized fibronectin (A), collagen (B) or BSA were incubated with *B. subtilis* suspensions from different RG4365-derived isogenic strains for 2h at 37°C. Unbound bacteria were removed, and the adhered bacteria to the wells were fixed and stained with CV as indicated in Experimental procedures. After processing, the CV stain bound to bacteria was released and quantified (A_{570}). The BSA-coated wells were included as negative control, and the mean value obtained from bacteria of each strain bound to BSA was subtracted from all other data points. Each data point contains the mean ± S.E.M. of a representative experiment performed by quadruplicate. NS: no significant difference, $*p < 0.05$ and $***p < 0.001$ (ANOVA with Bonferroni test).

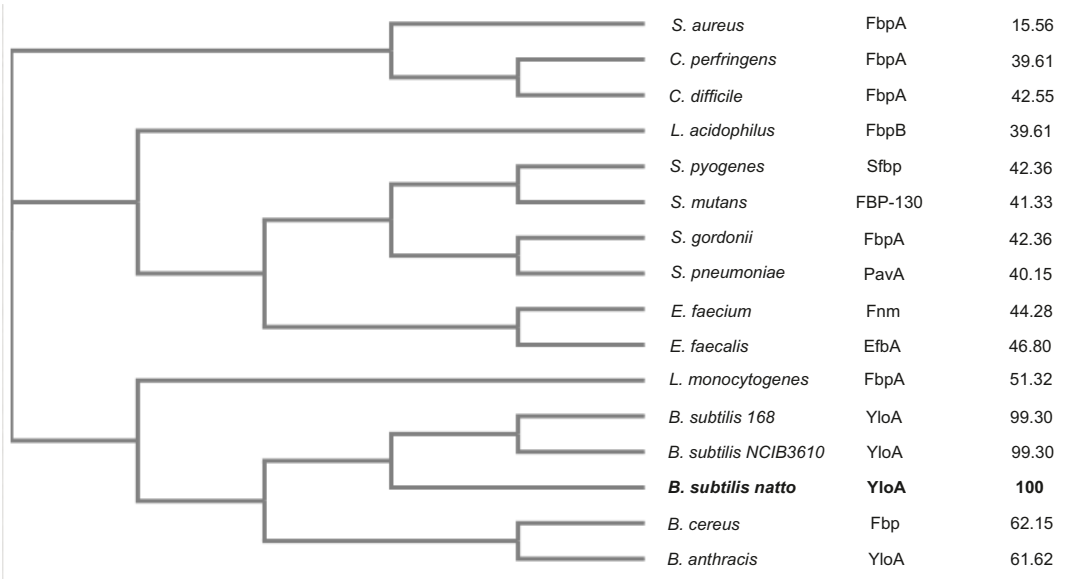
absence of activity of the response regulator DegU (Δ*degSU*). The DegU-dependent downregulation of *yloA* expression was independent of AbrB and Spo0A (Fig. 6C) suggesting that both regulators (AbrB and DegU) produced a synergistic negative effect on *yloA* expression. Accordingly, the fibronectin-binding proficiency of Δ*degSU* mutant cells was higher than in wild-type cells and maximal fibronectin-binding proficiency was observed in Δ*degSU*Δ*abrB* cells, Fig. 6D. These overall results point to AbrB and DegU as the key regulators of *yloA* expression (Fig. 6A–C).

The DegU- and AbrB-dependent downregulation of *yloA* expression could be due to a direct effect (binding) of the transcription factors to *yloA* or an indirect effect mediated by the product of other regulatory gene under DegU or AbrB control (Strauch *et al.*, 1989; Qian *et al.*, 2002; Hamon *et al.*, 2004; Tsukahara and Ogura, 2008a,b). For AbrB a consensus DNA binding sequence

A



B



	S.a.	L.a.	L.m.	BS168	BS3610	BSN	B.c.	B.a.	S.p.	S.m.	S.g.	S.py.	E.fc.	E.fl.	C.p.	C.d.
S. aureus	**100.00**	13.79	16.73	15.56	15.50	15.56	18.16	18.36	17.67	15.20	16.57	15.74	18.15	15.41	16.63	14.84
L. acidophylus	13.79	**100.00**	38.39	39.43	39.43	39.61	38.24	38.78	38.97	40.15	41.91	40.81	42.99	43.47	33.82	35.91
L. monocytogenes	16.73	38.39	**100.00**	51.68	51.68	51.32	50.44	50.09	43.67	42.94	45.32	44.40	46.38	45.47	38.75	40.35
BS 168	15.56	39.43	51.68	**100.00**	100.00	99.30	62.32	61.80	41.99	41.14	42.54	40.33	44.46	46.98	37.86	42.91
BS NCIB3610	15.50	39.43	51.68	100.00	**100.00**	99.30	62.32	61.80	41.83	41.14	42.54	40.33	44.46	46.98	37.86	42.91
BSN	15.56	39.61	51.32	99.30	99.30	**100.00**	62.15	61.62	42.36	41.33	42.36	40.15	44.28	46.80	37.68	42.55
B. cereus	18.16	38.24	50.44	62.32	62.32	62.15	**100.00**	94.73	41.33	41.51	42.80	40.96	42.99	45.36	39.46	40.85
B. anthracis	18.36	38.78	50.09	61.80	61.80	61.62	94.73	**100.00**	42.25	41.70	42.44	41.88	42.99	45.89	38.93	41.21
S. pyogenes	17.67	38.97	43.67	41.99	41.83	42.36	41.33	42.25	**100.00**	74.32	73.27	71.09	50.56	49.82	36.87	37.20
S. mutans	15.20	40.15	42.94	41.14	41.14	41.33	41.51	41.70	74.32	**100.00**	74.50	70.86	49.91	50.28	36.50	38.01
S. gordonii	16.57	41.91	45.32	42.54	42.54	42.36	42.80	42.44	73.27	74.50	**100.00**	79.09	49.63	50.73	37.06	36.46
S. pneumonia	15.74	40.81	44.40	40.33	40.33	40.15	40.96	41.88	71.09	70.86	79.09	**100.00**	51.58	50.92	37.73	35.85
E. faecium	18.15	42.99	46.38	44.46	44.46	44.28	42.99	42.99	50.56	49.91	49.63	51.58	**100.00**	69.73	39.34	39.02
E. faecalis	15.41	43.47	45.47	46.98	46.98	46.80	45.36	45.89	49.82	50.28	50.73	50.92	69.73	**100.00**	37.97	38.15
C. perfringens	16.63	33.82	38.75	37.86	37.86	37.68	39.46	38.93	36.87	36.50	37.06	37.73	39.34	37.97	**100.00**	42.88
C. difficile	14.84	35.91	40.35	42.91	42.91	42.55	40.85	41.21	37.20	38.01	36.46	35.85	39.02	38.15	42.88	**100.00**

Fig. 4. Schematic representation and phylogenetic conservation of *yloA*.
A. Genetic organization of the *yloA* region present in *B. subtilis* and its relatives. YloA harbors two Pfam domains, one of which (FbpA domain) is predicted to be involved in fibronectin binding and the other (Duf814 domain) is systematically associated with the FbpA domain. The putative −35 and −10 promoter sequences, the ribosome binding site (RBS) and the start codon for *yloA* are shown.
B. Phylogenetic relationship of the fibronectin-binding coding gene *yloA* of *B. subtilis*. Genes encoding atypical fibronectin-binding proteins were used to generate a comparative matrix and a neighbor-joining tree using Clustal Omega (www.ebi.ac.uk/tools/msa/clustalo). The phylogenetic distance with a gene encoding for typical fibronectin-binding proteins (*fnbA* gene from *S. aureus*) is also shown. The identity % refers to identical amino acid present in the protein sequence. Identity is the amount of amino acids which match exactly between two different sequences.

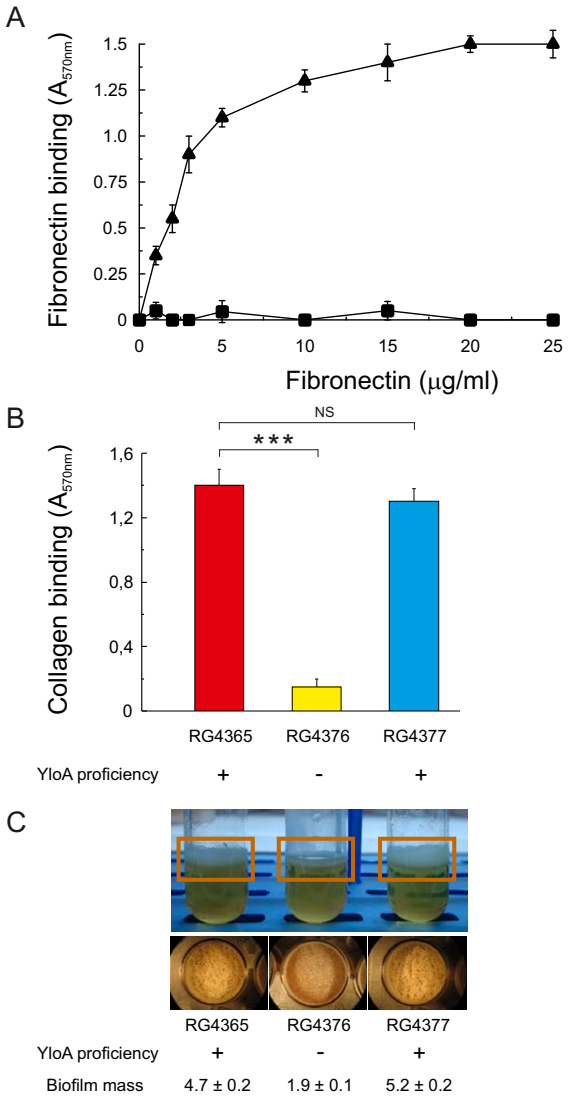


Fig. 5. *yloA* encodes for a functional fibronectin-binding protein of *B. subtilis* required for full biofilm formation proficiency.
A and B. The specific fibronectin- and collagen-binding proficiencies (A and B, respectively) of YloA-deficient and ectopically *yloA*$^{+}$-complemented cells (strains RG4376 and RG4377, respectively, Table 1) was assayed in microtiter wells coated with a fixed amount of each bacteria and increasing amounts of fibronectin (A) or collagen (B). After incubation, fibronectin- and collagen-bound bacteria were stained with CV and staining was quantified (A_{570}) as indicated in Experimental Procedures. Squares and triangles symbols are for YloA-deficient and YloA-proficient strains RG4376 (*yloA*::Spc^{r}) and RG4377 (*yloA*::Spc^{r}-*amyE*::*yloA*^{wt}Cm^{r}) respectively. In panel A data ± S.E.M. are the average values of three independent experiments performed by quadruplicate. In panel B ANOVA statistical analysis was used to compare adhesion of each type of bacteria, NS: no significant difference, ***$p < 0.001$.
C. Biofilm development (floating pellicles) of standing cultures of YloA-proficient and YloA-deficient cells grown in LBY broth (Pedrido *et al.*, 2013). Biofilms were developed in glass tubes (side views) and microtitre wells (top-down images) at 30°C, and pictures were taken after 36 h of incubation. Biofilm mass was quantified as indicated in Experimental Procedures. Similar results were observed in several independent experiments.

(AbrB box) in the promoter genes under its direct control does not exist. It has been hypothesized that AbrB recognizes an unknown three-dimensional arrange of the DNA promoters under its control (Strauch, 1995; Strauch *et al.*, 2007). Therefore, to test if the AbrB-dependent downregulation of *yloA* expression was due to a direct binding of AbrB to *yloA*, we performed a DNase I footprinting analysis of purified AbrB protein to the promoter region of *yloA*. As shown in Fig. 7A, a region of DNase protection from DNase I cleavage was visible between the −35 and −10 regions to the RBS of *yloA* with increasing concentrations of AbrB. Consistent with this binding pattern (Fig. 7A) it has been previously reported that AbrB downregulated extracellular alkaline protease activity (AprE or Subtilisin activity) by direct binding to the promoter region of *aprE* between the position −40 to the start site of transcription (Strauch, 1995). Therefore, this result strongly suggests that the AbrB-dependent downregulation of *yloA* is due to a direct binding of the repressor protein (Fig. 7A). The regulatory protein DegU belongs to the two-component system DegS-DegU controlling more than one hundred and twenty genes related to many cellular processes, including exoprotease production, motility, DNA competence and biofilm development (Ogura *et al.*, 2001; Mäder *et al.*, 2002; Verhamme *et al.*, 2007; Murray *et al.*, 2009; Marlow *et al.*, 2014). Unphosphorylated DegU activates genetic competence while low and high levels of DegU∼P activates and inhibits complex colony formation respectively (Verhamme *et al.*, 2007). Several studies showed that phosphorylated and unphosphorylated forms of DegU bind to DNA promoter regions containing AT-rich direct- and inverted-repeats (DR and IR, respectively) with or without nucleotide spacing (Shimane and Ogura, 2004; Verhamme *et al.*, 2007; Tsukahara and Ogura, 2008a, 2008b; Ohsawa *et al.*, 2009). These, completed or uncompleted, DR and IR have been shown to work as DegU binding sites (DegU boxes). Accordingly, a bioinformatic analysis of the *yloA* promoter region revealed the presence of putative DegU-recognized DR (5′-GAATG-N_6-GAATG-3′) and IR (5′-ATTTCA/TATCGT-3′ and 5′-TTCATGCTATAA/ACGTAAGCTATT-3′) sequences located on the −10 and RBS region of *yloA* that are partially superposed to the AbrB binding region (Fig. 7B). The presence of these DR and IR sequences functioning as DegU-binding sites suggests that the DegU-dependent downregulation of *yloA* expression could be due to a direct binding of the response regulator to the *yloA* promoter region (Fig. 7B). The validation of this hypothesis as well as the level of DegU∼P required to downregulate *yloA* and the possibility of a DegU–AbrB interaction to regulate *yloA*, is the subject of future investigations.

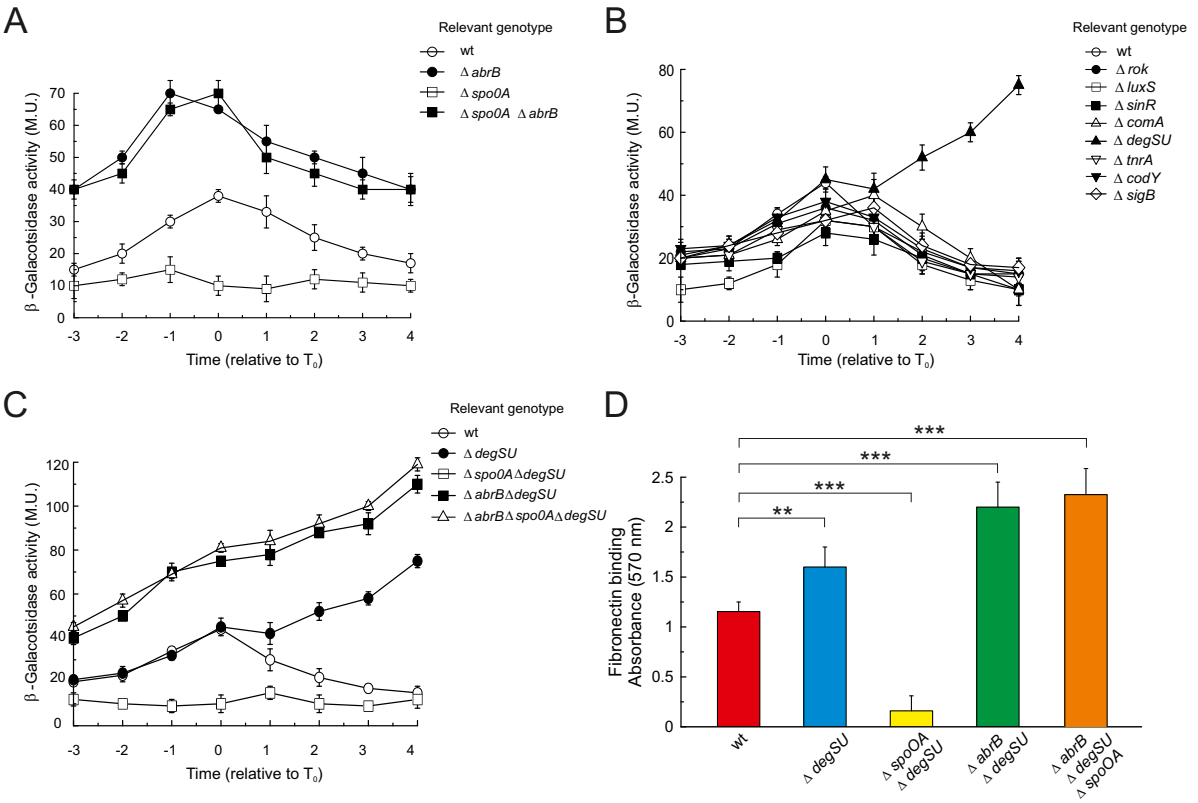


Fig. 6. AbrB and DegU downregulate *yloA* expression.
A–C. β-galactosidase expression of transcriptional *lacZ*-reporter fusions of the *yloA* promoter in different genetic backgrounds (Table 1). Cultures were developed in SM broth at 37°C with shaking at 150 rpm and samples were taken at the indicated times referred to T_0 (end of exponential growth). Each data point is the average M.U. values ± S.E.M. of three independent experiments performed by duplicate. Symbols for $P_{yloA\text{-}lacZ}$ carrying strains are indicated in the figures.
D. Immobilized fibronectin was incubated with *B. subtilis* suspensions of the indicated genetic backgrounds (Table 1) for 2h at 37°C. Unbound bacteria were removed, and the adhered bacteria to the wells were fixed and stained with CV as indicated in Experimental Procedures. After processing, the CV stain bound to bacteria was released and quantified (A_{570}). The BSA-coated wells were included as negative control, and the mean value obtained from bacteria of each strain bound to BSA was subtracted from all other data points. Each data point contains the mean ± S.E.M. of a representative experiment made by triplicate. $^{**}p < 0.01$ and $^{***}p < 0.001$ (ANOVA with Bonferroni test).

YloA-proficient B. subtilis *competes and interferes with the adherence of bacterial pathogens to fibronectin*

Numerous bacterial pathogens possess the ability to bind to fibronectin and this binding is important for virulence. Thus, if a strategy could be developed to inhibit the early binding of bacterial pathogens to the host cells, it would be possible to prevent or combat gastrointestinal colonization and disease at early steps of infection (competitive exclusion of pathogens). In this regard, it has been reported that *S. aureus* is able to colonize the gut of healthy infants, perhaps due to poor competition from other gut bacteria (Lindberg *et al.*, 2004). We hypothesize that the probiotic *B. subtilis* RG4365 would be able to inhibit the binding ability of diverse bacterial pathogens to ECM components (Cho and Blazer, 2012; Clemente *et al.*, 2012). To experimentally investigate this hypothesis, we analyzed the kinetics of fibronectin-binding to different bacterial pathogens by ELISA and compared these values with the fibronectin-binding proficiency of *B. subtilis* strains proficient and deficient in YloA production. As shown in Fig. 8, the binding to increasing amounts of fibronectin of a constant amount of *S. aureus*, *L. monocytogenes* and *E. faecalis* cells was specific and saturable. Interestingly, the highest and lowest fibronectin-binding proficiencies were obtained with wild-type and Δ*yloA B. subtilis* cells, respectively (Fig. 8. The fibronectin-binding proficiencies (measured as the apparent dissociation constant, Kd) of the three bacterial pathogens were similar to the previously reported values (Proctor *et al.*, 1982; Ingham *et al.*, 2004; O'Neill *et al.*, 2008) but significantly lower than the fibronectin-binding proficiency of wild-type *B. subtilis* cells (Fig. 8). As expected (see Fig. 1), the fibronectin-binding proficiency of the strain RG4365 was higher (lower Kd) than the ones observed for the *B. subtilis* strains NCIB3610 and JH642, Kd values for each strain of (1.45 ± 0.31) nM and (1.77 ± 0.33) nM were reproducibly obtained respectively (data not shown).

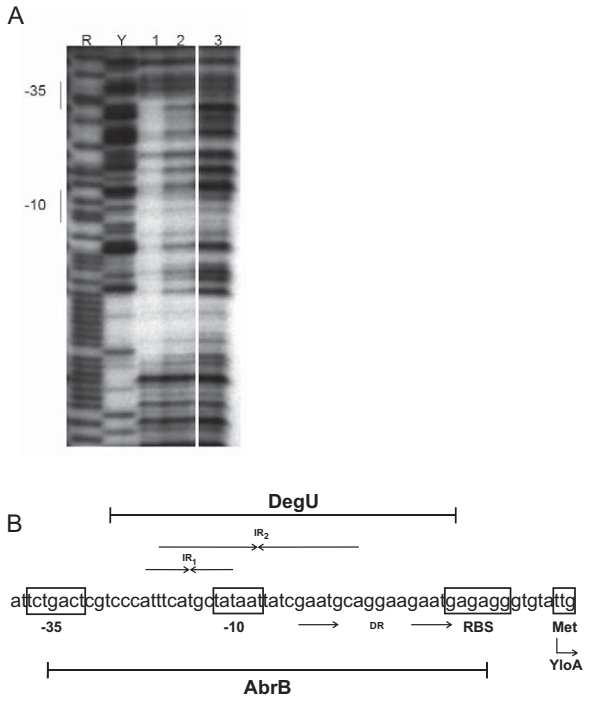


Fig. 7. AbrB- and DegU-recognized promoter regions of *yloA*.
A. DNAase I footprinting analysis of AbrB binding to a DNA fragment of the strain RG4365 containing the *yloA* promoter region. Shown is the non-template strand labeled at its 3′ end. Lanes: 1, 30 μM AbrB; 2, 9 μM AbrB; 3, no AbrB. Maxam-Gilbert purine (R) and pyrimidine (Y) sequencing ladders are shown for positioning reference. The putative −10 and −35 regions of the promoter are indicated by the vertical bars on the left. All binding reactions were performed at pH 8.
B. Sequence of the *yloA* promoter region showing extends of AbrB-afforded protection from DNAase cleavage and the putative DegU-recognized binding sites (see text for details).

The results presented in Fig. 8 suggest the possibility to use probiotic *B. subtilis* as an anti-infective competitor bacterium against the adherence of bacterial pathogens to ECM components of the host. To test this hypothesis, we modified our original FITC-labeled bacterial assay (shown in Fig. 2) to measure the *in vitro* pathogen adherence to immobilized-fibronectin under conditions of *B. subtilis* adherence competence. As shown in Fig. 9, YloA-proficient *Bs*n cells were very efficient in interfering with the adherence of the three assayed bacterial pathogens (*S. aureus*, *L. monocytogenes* and *E. faecalis*), to immobilized-fibronectin. For instance, at a cellular ratio of 1:1 between both bacteria (wild type *B. subtilis: S. aureus*) an inhibition of the pathogen adherence of 75% was observed (Fig. 9A). At higher cellular ratios (4:1), the ability of the pathogenic bacteria to compete with *B. subtilis* and to adhere to fibronectin was insignificant. In contrast, YloA-deficient *B. subtilis* cells were completely unable, even at a high cellular ratio (10:1), to interfere with the adherence of *S. aureus* to fibronectin. Similar behaviors were obtained when compared the fibronectin binding proficiency of *L. monocytogenes* and *E. faecalis* in presence of wild-type and Δ*yloA B. subtilis* cells (Fig. 9B and C). These results pointed out the potential suitability of the probiotic RG4365 *Bs*n strain to interfere with bacterial pathogen adhesion to ECM components of the host.

Discussion

The key of bacterial adaptability and survival relies on the individual or collective capacity to initiate the correct response at the appropriate time under a specific circumstance (Shapiro, 1998; Ben Jacob *et al*., 2004; Parsek and Greenberg, 2005; Grau *et al*., 2015). Examples of physiological fluctuations that bacteria face during growth include changes in nutrient concentration, temperature, osmolarity, pH and cellular density (Ben Jacob *et al*., 2004; Gottig *et al*., 2005; Parsek and Greenberg, 2005). Significant changes in transcriptional regulation occur upon sensing environment fluctuations, and transition state regulatory proteins play a pivotal role in these phenomena (Kearns *et al*., 2003; 2005; Méndez *et al*., 2004; Vlamakis *et al*., 2013). Like many other bacteria, *B. subtilis* responds to a multitude of environmental stimuli by using transcription factors to orchestrate gene expression patterns (Kearns *et al*., 2005; Pedrido *et al*., 2013; Grau *et al*., 2015). Accordingly, when *B. subtilis* is posed on a surface, it must take the decision to remain in that place or move away (i.e., forming a structured biofilm or activating surface-associated motility

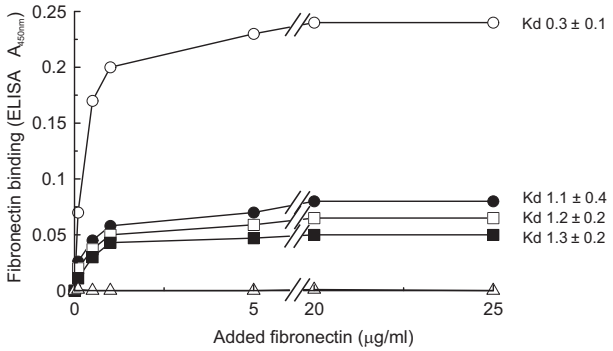


Fig. 8. Kinetics and saturability of the binding of *B. subtilis* and Gram-positive pathogens to fibronectin. Microtitre plates were coated with the corresponding bacterial strains and incubated with increasing amounts of fibronectin for 2 h at 37°C. Bound protein was detected with anti-fibronectin monoclonal antibody and mouse anti-IgG peroxidase-conjugated and quantified with TMB one-step substrate system. The results are representative of three experiments performed by triplicate. The apparent dissociation constants (Kd) ± S.E.M. (indicated on the figure) were calculated (in nM) using the computer program Grafit 4 (see Experimental procedures for details). Symbols: (○) RG4365 (wild-type *B. subtilis*); (•) BBB (*S. aureus*); (□) BBB (*E. faecalis*); (■) ATCC7644 (*L. monocytogenes*); (Δ) RG4376 (*yloA B. subtilis*).

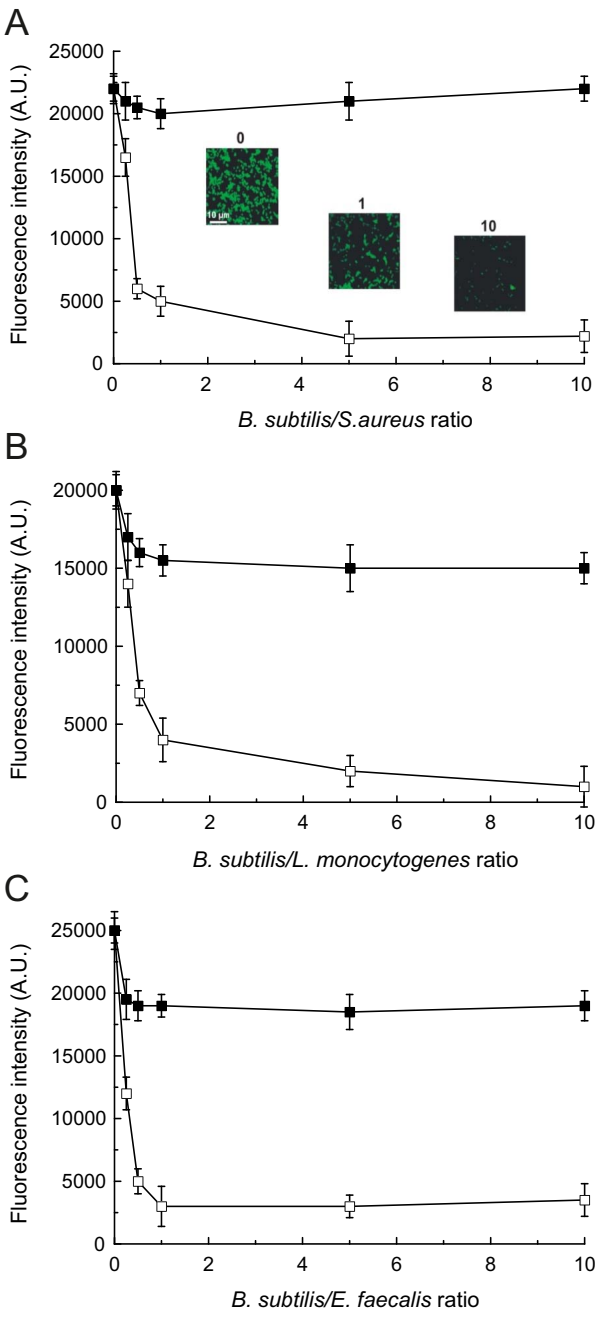


Fig. 9. YloA-proficient *B. subtilis* cells interfere and inhibit the adherence of pathogenic bacteria to immobilized-fibronectin. FITC-labeled bacterial pathogen suspensions; (A) *S. aureus* (ATCC12600), (B) *L. monocytogenes* (ATCC7644) and (C) *E. faecalis* (ATCC29212) were incubated with increasing amounts of YloA-proficient *B. subtilis* (strain RG4365, □) or YloA-deficient *B. subtilis* (strain RG4374, ■) cells and applied to fibronectin-coated plates. After incubation, unbound bacteria were removed and fluorescence intensity of remaining fixed pathogen bacteria was measured as indicated in Experimental Procedures. Data are indicated as arbitrary fluorescence units ± S.E.M. as previously reported. In addition, plates were observed in an inverted fluorescence microscope (Axiovert 25 Zeiss) and photographed to register the image. Data, from a representative experiment performed by triplicate, ± S.E.M. are shown. The insert-photographs in (A) show the fibronectin-adhered FITC-labeled pathogen incubated in the presence of increasing amounts of YloA-proficient *B. subtilis* cells as example. Ratios are (*B. subtilis*:pathogen) 0:1; 1:1 and 10:1 for each inserted photograph in panel A respectively.

mechanisms; respectively), (Grau *et al.*, 2015). Therefore, it is conceivable that expression of adhesive molecules for specific surface adherence would represent a valuable property in which *B. subtilis* is proficient (Fig. 1). The observed specific binding to ECMp (Figs 1–3) was mediated by the product of *yloA* (Fig. 5), a gene sharing strong homology with genes encoding for atypical fibronectin binding proteins present in several Gram-positive pathogens (Fig. 4).

In *B. subtilis*, several transition state regulators (i.e., Spo0A, AbrB, DegU, CodY, SinR) play essential roles in cellular adaptation and survival. Our binding assays to ECM components pointed to AbrB and DegU as the key negative regulators for specific binding to fibronectin and collagen (Figs (2 and 3) and 6). AbrB is a pleiotropic protein that inhibits the expression of genes for stationary-phase functions during exponential growth (Strauch *et al.*, 1990, 2007; Strauch, 1995; Cosby and Zuber, 1997; Qian *et al.*, 2002; Hamon *et al.*, 2004; Gonzalez-Pastor, 2011). Effectively, the *abrB* transcript was detectable only at the onset of exponential growth, and AbrB protein levels diminished continuously towards the end of exponential phase because increasing amounts of Spo0A∼P repress its transcription. Thus, AbrB-mediated regulation is progressively attenuated towards the transition phase (Strauch *et al.*, 1989, 1990, 2007; Strauch, 1995; Grau *et al.*, 2015). Our β-galactosidase assays in different *B. subtilis* genetic backgrounds strongly suggested an AbrB-regulated *yloA* expression pattern and showed that the adherence deficiency of Spo0A∼P-deficient cells was due to overexpression of the global regulator AbrB (Fig. 6). AbrB is also a negative regulator of *B. subtilis* biofilm formation in part because of its repressive effect on *bslA*, a gene encoding for a flotillin-like protein essential for biofilm formation (Grau *et al.*, 2015). Interestingly, we showed that YloA expression is also required for full biofilm formation proficiency in *B. subtilis* (Fig. 5C), a property previously reported for YloA homologs in pathogenic cocci (Singh *et al.*, 2015). Therefore, AbrB is a negative regulator of *B. subtilis* biofilm formation because it downregulates *bslA* and *yloA* expression (Fig. 5C).

AbrB binds DNA specifically, but does not recognize a single consensus sequence. The examination of many AbrB-regulated chromosomal genes has failed to

uncover a consensus base sequence having substantive predictive value explaining AbrB-mediated target selection and affinity (Strauch, 1995; Bobay *et al*., 2005, 2006). It has been hypothesized that AbrB achieves binding specificity by recognizing three-dimensional DNA architectures shared by a finite subset of base consensus (Strauch, 1995; Bobay *et al*., 2005, 2006). One consequence of the particular kinetics of the DNA-binding ability of AbrB is the complete absence of a DNA consensus sequence (i.e., absence of a typical "AbrB-box") and the requirement of *in vitro* methodologies to test the AbrB proficiency to bind a particular gene. Our footprinting analysis confirmed that AbrB bound to *yloA* and located the region of AbrB-binding in the – 35 and – 10-promoter region of *yloA* (Fig. 7A). This protected DNA region is coincident with previous works reporting the DNA region protected by direct AbrB-binding to diverse genes (Strauch, 1995; Bobay *et al*., 2005, 2006), suggesting a direct negative effect of AbrB on *yloA* expression. For the response regulator DegU (the other negative regulator of *yloA*, see Fig. 6) it has been reported that different amounts of its phosphorylated and unphosphorylated forms were able to functional control (either activating or repressing) the expression of many genes (Verhamme *et al*., 2007). The amount of phosphorylated DegU (DegU∼P) increases during the stationary phase of growth (Murray *et al*., 2009) and because *yloA* expression was under DegU control only during this phase of growth, we hypothesize that DegU∼P might be the active form of the *yloA* repressor. In contrast to AbrB, other works showed that tandem direct- and inverted-repeats (DR and IR, respectively) consisting of AT-rich sequences functioned as DegU/DegU∼P binding sites (Shimane and Ogura, 2004; Verhamme *et al*., 2007; Tsukahara and Ogura, 2008a,b; Ohsawa *et al*., 2009). Precisely, these types of DR and IR are present in the promoter region of *yloA* superposed to the AbrB binding region revealed by the footprinting analysis (Fig. 7B). Future studies will test the hypothesis of the direct binding of DegU∼P to the DR and IR motifs found in the *yloA* promoter region.

Bacterial adherence to host tissues and ECMp is a critical step in the process of infection since it establishes the initial contact with the host (Beg *et al*., 2002; Cossart, 2002; Ingham *et al*., 2004; Pizarro-Cerda and Cossart, 2006). These interactions can facilitate translocation across the mucosal barrier and internalization into subcellular compartments eventually leading to bacterial spread within eukaryotic cells (Schwarz-Linek *et al*., 2004; Pizarro-Cerda and Cossart, 2006; O'Neill *et al*., 2008; Osanai *et al*., 2013). The ability to bind fibronectin is mediated by fibronectin-binding MSCRAMM molecules exhibiting typical structural features of cell wall anchored proteins, as well as anchorless but surface-located adhesins and invasins (Patti *et al*., 1994; Joh *et al*., 1999; Wann *et al*., 2000; Holmes *et al*., 2001; Terao *et al*., 2001; Miller-Torbert *et al*., 2008; Singh *et al*., 2015). The rising incidence of multiantibiotic-resistant nosocomial infections caused by Gram-positive cocci has led to the inclusion of these organisms in the list of "no ESKAPE" (*Enterococcus*, *Staphylococcus*, *Klebsiella*, *Acinetobacter*, *Pseudomonas* and Enterobacteriaceae) pathogens that constitute a challenge to clinicians and threaten patient safety (Boucher *et al*., 2009). It has been shown that vaccination of rats with purified recombinant EfbA, the fibronectin binding determinant of *E. faecalis*, protected against enterococcal endocarditis development (Singh *et al*., 2015). Similar protective results were obtained in animals immunized with fibronectin-binding proteins of S*treptococcus pyogenes* and *S. aureus* (Schennings *et al*., 1993; Kawabata *et al*., 2001). The specific and high adherence ability of *Bs*n to fibronectin (Fig. 8) suggested that this property would be of importance for the enhancement of the beneficial properties of this probiotic strain in the host gastrointestinal tract (Duc *et al*., 2004; Hosoi and Kiuchi, 2004; Hong *et al*., 2009; Mackowiak, 2013; Vidyalaxme *et al*., 2014; Donato *et al*., 2017). Supporting this hypothesis, our *in vitro* experimental evidences suggested the suitability of probiotic RG4365 *B. subtilis* cells to efficiently interfere with the fibronectin-binding proficiency of different Gram-positive pathogens (Fig. 9). It will be of the interest of future studies to determine the *in vivo* (in animal model) anti-infective properties of *Bs*n and if the higher fibronectin-binding proficiency of *Bs*n in comparison with the ECM-adhesiveness of other bacteria is due to a higher synthesis of YloA per cell and/or a higher affinity of YloA for fibronectin.

Experimental procedures

Strains and growth media

The wild-type *B. subtilis* natto strain RG4365 and their isogenic derivatives, as well as other bacteria, used in this study are listed in Table 1. Bacterial cultures were maintained on Luria-Bertani (LB) broth, for *B. subtilis*; trypticase soy broth (TSB), for *S. aureus*; and brain heart infusion (BHI) broth for *E faecalis* and *L. monocytogenes*. For *B. subtilis*, the growth medium (LB broth) was supplemented with 0.5% glucose and 1 mM glutamine to avoid spore formation (Gottig *et al*., 2005). Efficiency of spore formation was measured by counting viable vegetative and spore cells before and after heat treatment at 80°C as previously described (Gottig *et al*., 2005). When spores were required, *B. subtilis* cultures were developed on Schaeffer broth (DS medium) as previously described (Gottig *et al*., 2005).

When appropriate, antibiotics were included at the following final concentrations: 1 μg/ml erythromycin (Ery), 5 μg/ml kanamycin (Kan), 5 μg/ml chloramphenicol (Cm) and 50 μg/ml spectinomycin (Spc). Transformation of *B. subtilis*, to obtain isogenic derivatives of the parental strains, was carried out as previously described (Arabolaza *et al.*, 2003; Gottig *et al.*, 2005).

Adhesion assays of B. subtilis *to ECM proteins*

For the binding assays, microtiter plates were coated with 100 μl of each individual protein solution: human purified fibronectin, collagen and bovine serum albumin, BSA, (10 μg/ml in bicarbonate buffer pH 9.6); and subsequently incubated overnight at 4°C. Protein solutions were removed and plates were washed three times with phosphate buffered saline pH 8 (PBS). After blocking with 0.5% non-fat milk (in PBS), to prevent non-specific bacterial binding, wells were washed again twice with PBS. Finally, bacterial suspensions (100 μl; $1 \times s10^8$ CFU/ml) were added and the plates incubated on an orbital shaker for 2 h at 37°C. Unbound bacteria were removed by washing the wells five times with PBS. Adhered bacteria to the wells were fixed at 60°C for 20 min and stained with 100 μl/well of CV (0.3% in methanol – H_2O 30% v/v) for 45 min. Wells were subsequently washed extensively with PBS to remove the unbound stain. After adding 100 μl of citrate buffer pH 4.3 to each well and 45 min incubation at room temperature (to release the stain bound to bacteria) the absorbance values (A_{570}) were determined in a Multiscan enzyme-linked immunosorbent assay reader (O'Neill *et al.*, 2008). Average values of three independent experiments performed in quadruplicate were calculated. The specificity of binding was tested using BSA as immobilized protein. Data are expressed as the mean absorbance value (A_{570}), with non-specific binding corrected by subtraction. ANOVA statistical analysis was used to compare adhesion of bacterial suspensions and significance was inferred at $p < 0.05$.

FITC labeling and observation of B. subtilis *cells bound to ECM proteins*

Labeling of bacterial cells for fluorescence microscopy was performed as previously described (Styriak *et al.*, 1999). Briefly, 1 ml of the bacterial culture was adjusted to a density of 1×10^9 CFU/ml, washed once with pre-warmed PBS at 37°C and re-suspended in 0.8 ml of pre-warmed PBS containing 0.2 ml of freshly prepared fluorescein isothiocyanate (FITC) solution (1 mg/ml in PBS). After incubation for 45 min at 37°C bacteria were washed three times with pre-warmed PBS. The pellet was re-suspended in 1 ml of pre-warmed PBS and used to test bacterial adhesion to fibronectin and collagen (Sigma-Aldrich Co) immobilized on glass slide; immobilized-BSA was used as control of binding specificity (each protein was used at a final concentration of 1 mg/ml). Glass slides coated with each particular immobilized protein to be tested were incubated with 100 μl of FITC-labeled bacterial suspension (1×10^8 CFU/ml) for 1 h at room temperature. Unbound cells were removed by gently washing with pre-warmed PBS. Bacterial binding to each immobilized protein was visualized by fluorescence microscopy. Fluorescence microscopy was performed using an IMT-2 inverted (Olympus, Lake Success, NY). Images were acquired with a digital Olympus camera.

Apparent dissociation constant (Kd) values for bacteria adhered to Fn

The Kd values were calculated following the procedures previously described (Bozzini *et al.*, 1992; Ingham *et al.*, 2004). Briefly, apparent Kd values were determined by ELISA. Microtiter plates were coated with 100 μl/well of bacterial suspension. Bacteria from cultures grown until late log phase were centrifuged, washed two times with PBS, and adjusted to an optical density of 5.0 (OD_{650}) in PBS. For coating, 0.3 ml of this suspension was diluted to a final volume of 10 ml with a carbonate-bicarbonate buffer (pH 9.6). Costar polyvinyl strips were coated overnight at 4°C with 100 μl of this suspension and washed three times with PBS. After blocking with 100 μl of non-fat milk 5% in PBS, wells were incubated with the indicated concentrations of fibronectin (0–500 nM) for 2 h at 37°C on an orbital shaker. Then, the wells were washed and incubated 1 h at room temperature with anti-Fn monoclonal antibody (Sigma-Aldrich Co). After washing with PBS, wells were again incubated for 1 h at room temperature with mouse anti-IgG peroxidase-conjugated (1/1000; Sigma-Aldrich Co). After washing, binding was quantified with TMB one-step substrate system (DAKO) and read on a Wiener microplate reader at 450 nm (Wiener-Group Corporation). The experimental design consisted in three independent experiments made by triplicate. The resulting concentration-dependent increases in absorbance were fit to the "built in" equations of the computer program Grafit 4 which derived the apparent dissociation constant values (Kd) and maximal binding.

Competitive inhibition assays

Suspensions of each bacterial pathogen from late log phase cultures were FITC-labeled as described above, mixed with different concentrations of competitor (wild type and *yloA B. subtilis* cells) grown until late log phase and applied to Fn-coated plates. After 1 h of incubation on an orbital shaker at room temperature, non-bound bacteria were removed by extensive washing with PBS and fluorescence intensity of remaining fixed bacteria was measured in a Beckman Coulter Multimode Detector (DTX 880). In addition, plates were observed in an inverted fluorescence microscope (Axiovert 25 Zeiss), photographed and registered with an image capture system.

Strain constructions

Strain RG4375 is an isogenic derivate of the wild-type strain RG4365 carrying a transcriptional reporter fusion to the *yloA* promoter region (Table 1). To obtain the promoter region of *yloA*, we performed a PCR amplification of chromosomal DNA from strain RG4365 with oligonucleotides 5′CTAGTAAATCTGTT**G**GT**A**CCATTTCATGAAAC3′ and 5′GTGATATGCA**TG**CGTGAATAGCTCGGATG3′ (the introduced restriction sites for *Kpn*I and *Sph*I are underlined; boldface indicates substitutions used to create the restriction site).

The PCR product (342 bp) containing the promoter region of *yloA* was cloned into the *mcs* of the vector PJM116 to make a transcriptional fusion to the *lacZ* gene of *Escherichia coli* (Lombardía *et al*., 2006). The resulting plasmid was linearized and integrated into the chromosome of wild-type RG4365 competent cells via double crossover at the *amyE* locus by transformation and selection for resistance to chloramphenicol (Arabolaza *et al*., 2003). For the construction of *B. subtilis yloA*-negative mutant strain (RG 4374), we amplified a 488 bp internal fragment of *yloA* using chromosomal DNA from strain RG4365 as a template and oligonucleotides 5′TGTTTACATAC**T**G**CAG**GACACACG 3′ and 5′CTGAGCAGCGGA**TC**CTTATAATCTTGGCCG3′ (the introduced restriction sites for *Pst*I and *Bam*HI are underlined; boldface indicates substitutions used to create the restriction site). The amplified DNA fragment was cloned into the *mcs* of the suicide vector pJM103 (Lombardía *et al*., 2006). This plasmid was used to transform competent cells of RG4365 and to select, by a single crossover event, chloramphenicol resistant colonies that were screened by Southern blotting and PCR (data not shown) to corroborate the integration of the disruptive vector into the *yloA* locus.

β-Galactosidase assay

β-Galactosidase activity was assayed in *B. subtilis* cells harboring *lacZ* fusions. All strains were grown on solid LB at 37°C overnight with the appropriate antibiotics. Then a single colony was picked, streaked out onto the same medium, and incubated at 37°C overnight. Broth media were inoculated to an initial absorbance at 595 nm (A_{595}) of about 0.17. Cultures were grown in flasks at 37°C with shaking at approximately 130 rpm. When the culture A_{595} reached about 0.25, collection of 1.0-ml samples was started and continued at 30-min intervals for assays of β-galactosidase activity (Arabolaza *et al*., 2003; Méndez *et al*., 2004). The samples were centrifuged for 5 min in a microcentrifuge. The cell pellets were stored at −20°C until they were analyzed for β-galactosidase activity. The specific activity was expressed in Miller Units (M.U.). The β-galactosidase experiments described in the figures were independently repeated three times, and a representative set of results is shown in each figure.

Biofilm experiments

For biofilm (pellicle) formation, overnight *B. subtilis* cultures were grown in LB medium until they reached the stationary phase. Then, 50 μl of each culture was diluted in 2 ml of fresh LBY medium (LB broth supplemented with 6.0% yeast extract, Pedrido *et al*., 2013). All the tubes (or microplates when indicated) were statically incubated at 30°C for 36 h. For quantification of formed biofilm (biofilm mass), we measured the amount of cells adhered to the surface of the microtitre plate wells (Pedrido *et al*., 2013). To this end, *B. subtilis* cells were grown in 96-well polyvinylchloride (PVC) microtitre plates at 30°C in LBY medium. The bacterial inoculum for the microtitre plates was obtained by growing the cells in LB to mid-exponential phase and then diluting the cells to an OD_{600} of 0.08 in LBY and biofilm formation was monitored by staining with CV. Growth medium and non-adherent cells were carefully removed from the microtitre plate wells. The microtitre plate wells was incubated at 60°C for 30 min. Biofilms were stained with 300 μl of 0.3% CV in 30% methanol at room temperature for 30 min. Excess CV was removed, and wells were rinsed with water. The CV from stained biofilms was then solubilized in 200 μl of 80% ethanol/20% acetone. Biofilms formation was quantified by measuring the Abs_{570nm} for each well using a Beckman Coulter Multimode Detector (DTX 880). Representative data of one of at least four independent experiments are shown.

DNase I footprinting

Plasmids containing the 350 bp *yloA* promoter fragment were linearized at the unique *EcoR*I site flanking the insert, labeled using α-^{32}P-dATP (Amersham) and the Klenow enzyme, followed by inactivation of the Klenow and release of the singly end-labeled fragment via digestion at the unique *BamH*I site on its opposite flank. Labeled DNA fragments were purified using standard polyacrylamide gel electrophoresis and electro elution techniques. Protein binding buffer composition (1X) was 50mM TRIS (pH8), 10 mM $MgCl_2$, 100 mM KCl, 10 mM 2-mercaptoethanol, 100 μg/ml BSA (bovine serum albumin). DNase I footprinting assays were performed at room temperature (22°C) and analyzed as described (Strauch *et al*., 1989, 2007).

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