Display of Recombinant Proteins on *Bacillus subtilis* Spores, Using a Coat-Associated Enzyme as the Carrier[▽]

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The display of proteins such as feed enzymes at the surface of bacterial spore systems has a great potential use for animal feed. Feed enzymes increase the digestibility of nutrients, leading to greater efficiency in the manufacturing of animal products and minimizing the environmental impact of increased animal production. To deliver their full potential in the gut, feed enzymes must survive the harsh conditions of the feed preparation and the gastrointestinal tract. The well-documented resistance of spores to harsh environments, together with the ability to use proteins that compose the spore as carriers for the display of passenger proteins, suggests that spores could be used as innovative tools to improve the formulation of bioactive molecules. Although some successful examples have been reported, in which abundant structural proteins of the *Bacillus subtilis* spore outer-coat layer were used as carriers for the display of recombinant proteins, only one convincing example resulted in the display of functional enzymes. In addition, no examples are available about the use of an inner-coat protein for the display of an active passenger enzyme. In our study, we show that the inner-coat oxalate decarboxylase (OxdD) can expose an endogenous phytase, a commonly used feed enzyme for monogastric animals, in an active form at the spore surface. Importantly, despite the higher abundance of CotG outer-coat protein, an OxdD-Phy fusion was more represented at the spore surface. The potential of OxdD as a carrier protein is further documented through the spore display of a bioactive heterologous passenger, the tetrameric β -glucuronidase enzyme from *Escherichia coli*.

Under extreme nutrient deprivation, *Bacillus subtilis* has the ability to enter a complex differentiation process that culminates with the formation of an extremely resistant spore. Spores consist of a central core compartment that contains a copy of the chromosome and is surrounded by a thick layer of a modified form of peptidoglycan known as the cortex. The cortex is covered by a multilayered protein coat, formed by an inner layer apposed to the cortex, and an outer layer. In most characterized strains of *B. subtilis*, the outer-coat layer is the outermost spore structure. The spore coat is formed through synthesis in the mother cell of over 70 polypeptides, which follow an ordered assembly program at the surface of the developing spore (13). Assembly is largely guided by a class of morphogenetic proteins that have important roles in the structural organization of the two main coat layers (8, 15, 17). SafA, for example, localizes at the interface between the cortex and inner coat and has an important role in assembly of the inner-coat layer (5). CotE, on the other hand, is found at the inner coat-outer coat interface and is essential for assembly of the outer-coat structure. When conditions become conducive for growth, spores germinate to regenerate vegetative cells, which rapidly resume the normal cyclic pattern of growth and division.

The *B. subtilis* spore coat has recently emerged as a nanostructure offering a novel and interesting surface for the display of biomolecules. Since *B. subtilis* presents a good safety

record as an additive in human and animal preparations (GRAS [generally regarded as safe]), one potentially valuable use of the spore coat display system is in the area of probiotics. In animal nutrition, feed enzymes are commonly used to improve the nutrition value of feeds, mainly by enhancing their digestibility and/or assimilation (4). Display of these enzymes at the spore surface could ensure *in situ* efficient enzymatic activity application at moderate cost. Examples of feed enzymes candidate for display are xylanase, hemicellulase, cellulase, protease, glycanase, or phytase. Phytase, in particular, is a commonly used feed enzyme for monogastric animals to improve nutritive value (34). Most of the phosphorus (50 to 80%) contained in feedstuffs of plant origin exists as the storage form phytate and is indigestible for nonruminant animals such as poultry and pigs, since they lack the enzyme to free phytate-bound phosphorus. Therefore, sufficient phytase needs to be added to the feed to decrease the supplementation of phosphorus to feedstuffs, thus reducing the environmental pollution in areas with intensive livestock production. However, despite successful spore display examples using the abundant structural coat proteins CotB (9, 16, 18), CotC (16, 30, 35), and CotG (16, 26) as an anchoring motif, a very limited number of studies are available regarding spore display of functional enzymes (25, 28, 39). In all reported cases of spore display, the common denominator governing choice of the carrier protein seems to have been its abundance, and its ability to ensure the highest level of surface exposure. CotB, CotC, and CotG, possibly the most represented protein within the coat, are outer-coat proteins, dependent on CotE for assembly (Fig. 1A). All three proteins also undergo extensive multimerization during their assembly at the spore surface (19, 40) (Fig. 1A). Thus far,

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FIG. 1. (A) Inner- and outer-coat carrier proteins. Morphogenetic proteins SafA (A) and CotE (E) have central roles in the assembly of the inner and outer-coat layers, respectively, and control the assembly of the indicated proteins. The location of SafA and CotE at the cortex-inner coat and inner coat-outer coat interfaces, respectively, is indicated. The following coat carrier proteins, and their locations, are represented: B, CotB; C, CotC; D, OxdD; and G, CotG. D is presumed to be homohexameric; B forms covalently cross-linked dimers, whereas C and G undergo extensive multimerization (n) and cross-linking at the spore surface. The passenger proteins phytase (P) and β -glucuronidase (U) are shown as fusions to D or G. (B) General strategy used to construct the various carrier-passenger fusions. The indicated pDG364 derivatives, carrying the various gene fusions, were linearized and transferred to the nonessential *amyE* locus through a marker replacement (double-crossover) recombination event. Promoters are represented by arrows, and transcriptional terminators are represented by hairpin structures. L10, linker made of 10 alanine residues; *cat*, gene encoding chloramphenicol acetyltransferase. The various genes, color-coded to match panel A, are not drawn to scale.

the display of antigens, enzymes, or other functional components on spores using inner-coat proteins as carriers has not been reported.

The 43.4-kDa product of the *oxdD* gene, OxdD, is a minor component of the spore coat (6). OxdD is a component of the inner-coat layers, dependent on morphogenetic protein SafA for assembly (6, 33) (Fig. 1A). OxdD is highly similar to OxdC, a homohexameric enzyme (EC 4.1.1.2), which is specifically produced during growth of *B. subtilis* under acidic conditions (37). Both OxdD and OxdC show oxalate decarboxylase activity (6, 37).

We show here that *B. subtilis* OxdD can be used as an original anchoring motif to display proteins of biological interest at the surface. Because of its relatively low abundance and internal localization, the use of the coat-associated OxdD enzyme as a carrier protein for spore display could afford a higher degree of protection to a passenger protein, while minimizing any impact on the spore coat assembly process. First, as a step in the development of spore display for use in probiotics, OxdD was fused to *B. subtilis* phytase (Phy), a monomeric enzyme (EC 3.1.3.8) encoded by the *phy* gene with optimal activity at pH 7 to 7.5 (23). We show that an OxdD-Phy fusion protein is exposed at the spore surface and results in recombinant spores with phytase activity. We also show that the spore coat structural protein CotG can expose the endogenous phytase, but despite the higher abundance of this protein, the resulting recombinant spores show reduced levels of phytase compared to those of a strain expressing *oxdD-phy*. The potential use of OxdD as a carrier for the display of functional enzymes at the spore surface is further illustrated by the display of a fusion of OxdD to β -glucuronidase (GusA)

from *E. coli*, a homotetrameric enzyme encoded by the *uidA* gene (21, 22).

MATERIALS AND METHODS

Bacterial strains, media, and general techniques. The *B. subtilis* strains used in the present study are listed in Table 1. They are derived from strain PY79 (1A747; *Bacillus* Genetic Stock Center). *E. coli* K-12 was used to PCR amplify the *uidA* gene, encoding for β -glucuronidase (GusA). The high-fidelity *Herculese* polymerase (Stratagene) was used to generate PCR products. Tryptone blood agar broth (BD Difco) was used as the standard solid medium for routine propagation of all *B. subtilis* strains. Difco sporulation medium (DSM) was used to induce sporulation by nutrient exhaustion (32).

Construction of *B. subtilis* strains expressing CotG-Phy OxdD-Phy and OxdD-GusA fusion proteins. A schematic view of the cloning strategy used for the translational fusions is presented in Fig. 1B. The original promoter of the spore-

TABLE 1. Bacterial strains

Strain	Genotype or phenotype ^a	Origin or reference
<i>E. coli</i> SD58	pET16::phy/overproduction of His ₆ -Phy lacking its signal peptide, under the control of P _{T7lac}	This study
<i>B. subtilis</i> PY79	Wild type	BGSC ^b
SD48	PY79 Δ amyE::cotG-phy; Cm ^r	This study
SD50	PY79 Δ amyE::oxdD-phy; Cm ^r	This study
SD53 (AH2873)	Δ oxdD::oxdD-gfp; Sp ^r	6
SD60	PY79 Δ amyE::oxdD-uidA; Cm ^r	This study
AH7666	PY79 Δ phy; Sp ^r	This study

^a Cm^r, chloramphenicol resistance; Sp^r, spectinomycin resistance.
^b BGSC, *Bacillus* Genetic Stock Center (<http://www.bgsc.org>).

anchoring motif (300 bp of DNA upstream from *oxdD* start codon, or 465 bp of DNA upstream *cotG* start codon) was used to synchronize expression with the passenger protein during sporulation. Passenger proteins were fused in frame with the carboxyl terminus of the anchoring motif (either OxdD or CotG), deleted of its stop codon. *B. subtilis* native phytase Phyl (GenBank accession number BG11198), was fused to either OxdD (BG13484) or CotG (BG11017) (numbering according to Genolist, Institut Pasteur, 1999–2001; Subtilist [http://genolist.pasteur.fr/Subtilist], and Colibri [http://genolist.pasteur.fr/Colibri]). The region encoding the signal peptide of Phyl (encompassing the 26 first residues; Swiss-Prot P42094) was excluded. A linker made of 10 alanine residues was introduced between the carrier and passenger proteins. Gene fusions *cotG-phy* (2,173 bp long) and *oxdD-phy* (2,594 bp long) were synthesized as synthetic genes and sequenced by DNA2.0, Inc. (California). They were then cloned between the BamHI and HindIII sites of pDG364 suicide vector (7), resulting in plasmids pSD21 (*cotG-phy*) and pSD22 (*oxdD-phy*). To construct a fusion of the *Escherichia coli uidA* gene, coding for β -glucuronidase (GusA; GenBank accession number EG11055) to the 3' end of the *oxdD* gene, we first introduced a NheI restriction site into the 10-residue linker of pSD22, leading to the replacement of the sixth alanine residue by a serine. Then, the *uidA* gene of *E. coli* was cloned between NheI and HindIII sites of plasmid pSD22, to yield pSD27, carrying a 3,339-bp open reading frame coding for a OxdD-GusA fusion protein under the control of the native *oxdD* promoter. After linearization with XhoI restriction endonuclease, pSD21, pSD22, and pSD27 were used to independently transform *B. subtilis* PY79 with selection for chloramphenicol (5 μ g/ml). Transformants that resulted from a marker replacement recombination event (double crossover) at the nonessential *amyE* locus were identified and designated SD48 (expressing the CotG-Phyl fusion), SD50 (OxdD-Phyl), and SD60 (OxdD-GusA).

Construction of SD58 (*E. coli* His₆-Phyl, N terminus). A 1.1-kb PCR fragment containing the *phy* gene without its first 84 codons (corresponding to the signaling peptide) was amplified by using primers phy-for 5'-GGAATTCATATGG TGAATGAGGAACATCATTTCAAAG-3' and phy-rev 5'-ATCGCTCGAGG CCGTCAGAACGGTCTTTCAGCTTCCTC-3', which generated NdeI and XhoI restriction site ends, respectively. Chromosomal DNA of *B. subtilis* 168 was used for amplification. The PCR fragment was digested and inserted into pET16b (Novagen) to create pSD26. Selection of transformants was done on LB plates containing 100 μ g of ampicillin/ml. Plasmid pSD26 carries an N-terminal fusion of *B. subtilis* phytase (without its signaling peptide) fused to a His tag. After purification, pSD26 was introduced into *E. coli* BL21 competent cells (Invitrogen) to allow efficient overexpression of the heterologous protein. Selection of transformants was done in LB plates containing 100 μ g of ampicillin/ml and 50 μ g of chloramphenicol/ml. The resulting strain was named SD58.

Overproduction and purification of *B. subtilis* phytase. A starter culture of strain SD58, grown overnight at 37°C with shaking, was used to inoculate 100 ml of fresh LB broth supplemented with ampicillin and chloramphenicol, which was incubated at 37°C and 150 rpm until reaching an optical density at 600 nm of ~0.6. At this point, IPTG (isopropyl- β -D-thiogalactopyranoside) was added to a final concentration of 1 mM, and the culture was incubated for another 4 h. The culture was then centrifuged 20 min at 8,000 $\times$ g and 4°C, and the cell pellet was resuspended in 4 ml of ice-cold lysis buffer (20 mM phosphate, 300 mM NaCl, 1 mM CaCl₂, 10 mM imidazole, 1 mM phenylmethylsulfonyl fluoride, 1 $\times$ anti-protease EDTA free solution). The cell lysate was obtained by passage through a French press cell at 19,000 lb/in², and the supernatant fraction was used to purify the His₆-tagged phytase on a Ni²⁺-nitrilotriacetic acid (NTA) column (Qiagen). The column was operated according to the manufacturer's instructions. Bound phytase was eluted at an imidazole concentration of 300 mM. All purification steps were carried out at 4°C.

Isolation of *B. subtilis* extracellular proteins. The extracellular proteome was prepared essentially as described by Antelmann et al. (1). Briefly, cultures (20 ml) and its derivative bearing a Δ phy mutation (AH7666), were grown in a synthetic medium containing 15 mM (NH₄)₂SO₄, 8 mM MgSO₄ · 7H₂O, 27 mM KCl, 7 mM sodium citrate · 2H₂O, 50 mM Tris-HCl (pH 8) supplemented with 0.16 mM KH₂PO₄, 2 mM CaCl₂ · 2H₂O, 1 μ M FeSO₄ · 7H₂O, 10 μ M MnSO₄ · 4H₂O, 4.5 mM glutamic acid, 780 μ M tryptophan, 860 μ M lysine, and 0.2% (wt/vol) glucose. Samples were collected 1 h after the end of the exponential phase of growth by centrifugation for 30 min at 21,000 $\times$ g and 4°C. Proteins present on the culture supernatant were then precipitated during 1 h at 4°C with trichloroacetic acid (TCA) at a final concentration of 5% and recovered by centrifugation during 30 min at 21,000 $\times$ g and 4°C. The protein pellet was once washed with 70% ice-cold ethanol and finally resuspended in 200 μ l of a buffer containing 10 mM Tris (pH 8), 10 mM MgCl₂, 500 mM EDTA, 200 mM NaCl, 10% glycerol, 1 mM PMSF, and 1 $\times$ anti-protease EDTA free solution.

Construction of AH7666 (*B. subtilis* wild type with *phy* deleted). Long flanking homology PCR was used to delete the endogenous copy of *phy* in PY79 background (38). The primers P1-phy (5'-ATATCTGCCTAAAAAAGTGC-3') and P2-phy-spec (5'-ACATGTATTACGAACGAAAATCGAAATAGAAAG CAGCTTGTGCAGC-3') were used to amplify a fragment upstream of the *phy* gene, while the primers P3-phy-spec (5'-ATTTTAGAAAAACAATAAACCCCTT GCACCTTCATTGGTACCCTCC-3') and P4-phy (5'-CACCTGTTTAGGTG TAAGCAG-3') were used to amplify a fragment downstream of *phy*. Both PCR products were bridged by a fragment containing the spectinomycin cassette (11). The final PCR product was then used to replace the endogenous *phy* gene by the spectinomycin cassette via double crossover.

Spore purification and analysis. Spores were harvested by centrifugation of DSM cultures 24 h after the onset of sporulation. After washing, spores were purified with a 20 to 50% Gastrografin (Schering) step gradient, as previously described for Renocal-76 gradients (12). Purified spores were suspended in cold buffers (as specified below) supplemented with protease inhibitors (Complete EDTA-free; Roche). Samples of the purified spore suspension were used to analyze the coat polypeptide composition as described before (12). The total cell count, as well as the titer of heat or lysozyme-resistant spores, was determined in samples of DSM cultures 24 h after the onset of sporulation (12).

Detection of β -glucuronidase (GusA) activity by fluorescence microscopy. *In situ* detection of β -glucuronidase activity was performed using the fluorogenic substrate C₁₂FDGlcU, a lipophilic analog of fluorescein di- β -D-glucuronic acid containing a 12-carbon aliphatic chain supplied with the ImaGene Green C₁₂FDGlcU GUS gene expression kit from Molecular Probes. Cleavage by β -glucuronidase releases the yellow-colored, green-fluorescent compound 5-decanoylamino fluorescein (maximal excitation wavelength = 495 nm, maximal emission wavelength = 518 nm). This substrate was used on purified spores of PY79 and SD60, according to the indications of the manufacturer. Before adding the substrate, part of the spore suspension was washed with water and incubated for 1 h at 37°C in a 0.1% trypsin solution (Amimed), as a control. Spores were washed three times with phosphate-buffered saline (PBS) at pH 7.4 (Roche). The spores were then examined by fluorescence microscopy (Eclipse E600; Nikon) using phase-contrast optics and a standard filter for the visualization of green fluorescence emission (Ex = 490 nm, Em = 520 nm), reflecting GusA activity at the spore surface. Images were acquired with a digital camera (CoolSnap EZ; Visitron Systems) and processed with ImageJ (W. S. Rasband, ImageJ, 1997–2008 [http://rsb.info.nih.gov/ij/]; National Institutes of Health, Bethesda, MD) for quantification of the fluorescence signal. Background was measured for each picture and subtracted from the fluorescence intensity of the samples. The quantification was made on 150 spores per strain.

Assay of *B. subtilis* phytase activity. Whole-cell extracts were prepared from 24-h DSM cultures of strains SD48, SD50, and PY79. Cells and mature spores were harvested by centrifugation (20 min, 8,000 $\times$ g) and suspended in a buffer containing 100 mM Tris-HCl (pH 7.4; Fluka), a cocktail of protease inhibitors (Complete EDTA-free), and 2 mM CaCl₂ (Sigma), required to maintain active conformation of *B. subtilis* phytase (24, 36). The mixture was passed twice in French cell press (Sim-Aminco) at 900 lb/in² to release both forespores and the cytoplasm content of the mother cells. Spores were purified from whole-cell extracts as described previously (40). Phytase activity was measured by incubating either 10 μ l of whole-cell extracts or 10 μ l of pure spores suspensions with 40 μ l of 2 mM sodium phytate (Sigma) in the above-mentioned buffer. After 30 min of incubation at 55°C, the reaction was stopped by adding 50 μ l of 5% TCA (Merck) (36). After centrifugation (4 min, 8,000 $\times$ g), 50 μ l of the supernatant was diluted in 500 μ l of water. The released orthophosphate (P_i) was photometrically measured at 700 nm by monitoring the production of phosphomolybdate for 15 min at 50°C after the addition of 500 μ l of the color reagent. The color reagent was prepared freshly by mixing 1 volume of 2.5% ammonium molybdate (Fluka), 1 volume of 10% sodium ascorbate (Fluka), and 3 volumes of 1 M H₂SO₄ (Fluka) (27). One unit of phytase was defined as the amount of enzyme required to release 1 μ mol of P_i from sodium phytate in 1 min. The total amount of protein was assayed with the bicinchoninic acid method (Pierce), in order to calculate specific activity. The dry weight was measured after drying spores suspensions for 24 h at 80°C.

Immunodetection of spore displayed phytase. An anti-phytase rabbit polyclonal antibody was produced by immunizing rabbits with a mix of two synthetic phytase-specific peptides (NH₂-CAEPGGSGKQVVDRA-COOH and NH₂-C HKQVNPRLKDRSDG-COOH; Eurogentec). Before immunolabeling, part of the spores was incubated at 37°C for 1 h in a 0.1% trypsin solution (Amimed), as a control. They were washed three times with PBS (pH 7.4; Roche) supplemented with 2% bovine serum albumin (Sigma) and then probed with primary and secondary antibodies. Immunostained spores were mounted on agarose-

TABLE 2. Heat resistance and lysozyme resistance of various *B. subtilis* strains

Strain	Relevant genotype	Sporulation (CFU/ml) ^a		
		Viable cells	Heat-resistant cells	Lysozyme-resistant cells
PY79	Wild type	4.2×10^8	3.2×10^8	2.5×10^8
SD48	$\Delta amyE::cotG-phy$	4.2×10^8	4.8×10^8	4.0×10^8
SD50	$\Delta amyE::oxdD-phy$	4.5×10^8	4.3×10^8	3.2×10^8
SD60	$\Delta amyE::oxdD-uidA$	4.8×10^8	2.6×10^8	2.9×10^8

^a The total viable, heat-resistant, or lysozyme-resistant cell enumeration was determined 24 h after the onset of sporulation in liquid medium (DSM), as described in Materials and Methods.

coated microscope slides and observed by fluorescence, as described above. The fluorescence signal was quantified on 150 spores per strain.

RESULTS AND DISCUSSION

Among spore-associated enzymes, OxdD resides in the inner-coat layers of *B. subtilis* spores (6) (Fig. 1A). A passenger protein fused to this enzyme could in theory be more protected than when fused to a carrier located in the outer coat. Here, we investigated the behavior of the inner-coat protein OxdD, by comparison to the outer-coat protein CotG, as a carrier for the display of monomeric *B. subtilis* phytase. We also assessed the ability of OxdD to display the heterologous multimeric *E. coli* β -glucuronidase.

Spore display of *B. subtilis* phytase. Although green fluorescence protein (GFP) fused to OxdD has been successfully exposed at the surface of *B. subtilis* spores (6), spore display of a functional enzyme has not been reported. The *B. subtilis* Phy passenger enzyme, truncated for its 26-residue signal peptide, was translationally fused to the C terminus of the anchoring motif OxdD. A linker, made of 10 aliphatic amino acids, was introduced between the carrier and passenger proteins to minimize any potential steric effect that could disturb the correct folding of either protein (Fig. 1B). Since CotG had been previously described as the carrier for the spore display of recombinant proteins (16, 25, 28), a control fusion CotG-Phy was also designed (Fig. 1B). The translational *oxdD-phy* and *cotG-phy* fusions are carried by strains SD50 and SD48, respectively (Table 1).

Since in spores of *cotG* insertional mutants the normal structural organization of the outer coat is severely disturbed (14), we have chosen to allow expression of the wild-type *cotG* allele in strains expressing passenger-carrier fusions (strain SD48). A similar approach was used with OxdD as a carrier (strain SD50), since the function of OxdD in spore assembly is not yet fully elucidated (6). Both integrations at the *amyE* locus of the wild-type strain PY79 locus had no detectable effect on spore viability and resistance to heat and to lysozyme (Table 2). Moreover, examination of the collection of coat polypeptides extracted from spores of strains SD48 or SD50 resulted in a pattern that did not differ from that obtained for the wild-type PY79 spores (data not shown). We concluded that expression of the wild-type and engineered carrier proteins did not interfere in any detectable way on the assembly and function of the spore coat lattice.

To test whether the fusion proteins were presented at the spore surface, we conducted immunofluorescence microscopy experiments. For these studies, we used a rabbit polyclonal anti-Phy antibody (see Materials and Methods). This antibody

specifically recognized the purified His₆-Phy protein (Fig. 2A). In addition, it recognized the native Phy protein in extracts prepared from culture supernatants of the wild-type strain PY79, grown under conditions where Phy is known to accumulate (1), and did not react with any protein in extracts prepared in parallel from a *phy* deletion mutant (AH7666) (Fig. 2B). Spores were collected 24 h after the onset of sporulation, purified on density gradients (see Materials and Methods) and either directly analyzed or treated with trypsin as a control. Significant fluorescence was observed on purified spores harboring the CotG-Phy or OxdD-Phy fusions (Fig. 3A). As expected, no significant fluorescence was observed at the surfaces of spores of the wild-type strain PY79 strain. Importantly, treatment with trypsin resulted in loss of the fluorescence signal. Treatment with trypsin also resulted in the complete loss of fluorescence from spores of a strain (AH2873; Table 1) expressing an *oxdD-gfp* fusion (data not shown). As an additional control, the primary antibody was omitted to test for specific binding of the secondary antibody coupled with the fluorophore. No fluorescence was observed under these conditions. We then quantified the fluorescence signal for spores of strains SD48 and SD50 in comparison to wild-type PY79 spores. The results show that the average signals on SD48 (85 average units [AU]) and SD50 (91 AU) were ~3-fold higher

FIG. 2. Purification of *B. subtilis* phytase (Phy) and specificity of an anti-Phy antibody. (A) SDS-PAGE analysis (lanes 1 to 3) and immunoblot analysis (lanes 4 to 6) of extracts of *E. coli* strain SD58 noninduced (lanes 1 and 4), induced with 1 mM IPTG (lanes 2 and 5), and of the His₆-Phy protein (lanes 3 and 6), partially purified by Ni²⁺ affinity chromatography. The asterisk indicates a cross-reactive species or a degradation product of the His₆-phytase. (B) SDS-PAGE (lanes 1 and 2) and immunoblot analysis (lanes 3 and 4) of the cell culture supernatants of the PY79 wild-type strain of *B. subtilis* (lanes 1 and 3) and of strain AH7666, bearing a Δphy mutation (lanes 2 and 4).

FIG. 3. Immunofluorescence detection of CotG-Phy and OxdD-Phy protein fusions at the surface of purified spores. FITC, fluorescein isothiocyanate. (A) Phase-contrast microscopy (PC) and fluorescence microscopy (FITC). Scale bars, 2 μ m. (B) Fluorescence intensity was quantified on 150 spores. AU, arbitrary units. Purified spores were submitted or not to trypsin proteolysis. noAb, control without primary antibody. Spores were purified from cultures of the following strains: PY79, wild type; SD48, CotG-Phy; and SD50, OxdD-Phy.

than the baseline signal observed for PY79 spores (29 AU) (Fig. 3B and 4A). In spite of a similar average intensity for SD48 and SD50, 75% of SD48 spores displayed at least 2-fold more fluorescence than the baseline (>60 AU), whereas 100% did so for SD50. Together, these results suggest that the OxdD-Phy fusion was more efficiently displayed at the spore surface.

Phytase activity at the surface of *B. subtilis* spores. Having determined that both CotG and OxdD served as carriers for the display of Phy at the surface of *B. subtilis* spores, we then wanted to determine whether the passenger protein was presented in an active form. For this purpose, we conducted measurements of Phy specific activity. We first measured Phy activity in whole-cell extracts prepared from samples directly collected from DSM cultures of strains SD48, SD50, and PY79, 24 h after the onset of sporulation (Fig. 4B). Although a specific activity of 3.1 U/mg of protein was obtained for PY79, reflecting the endogenous *B. subtilis* phytase activity, values of 11.4 U/mg and 6.9 U/mg were obtained for SD48 and SD50, respectively (Fig. 4B). For reference, a partially purified His₆-tagged version of the *B. subtilis* phytase missing its signal peptide (Fig. 2A) gave a specific activity of 23 U/mg of protein when produced in *E. coli* (data not shown). The phytase activity detected for SD48 and SD50 extracts over the activity obtained for PY79 cultures clearly demonstrated expression of a functional phytase during sporulation, from the sporulation specific promoters that drive expression of either *cotG* or

oxdD. Next, we sought to determine whether phytase activity could be detected on density gradient-purified spores—in other words, whether the CotG-Phy or OxdD-Phy fusion detected at the spore surface (see above) represented active enzyme (Fig. 4C). Since not all proteins are extractable from the spore coat, units of phytase activity in purified spores were normalized with respect to spore dry weight. Activities of 5.7×10^3 U/g (dry weight) and of 2.7×10^3 U/g (dry weight) were obtained for SD48 and SD50, respectively. No activity could be detected for PY79 spores or for spores of strains SD48 and SD50 that were treated with trypsin prior to the assay (data not shown). These results indicate that both the CotG or OxdD carriers resulted in the display of active phytase at the spore surface.

Although the OxdD-Phy fusion protein appeared more abundant than CotG-Phy at the spore surface, as assessed by immunofluorescence (see above), spores of SD50 (OxdD-Phy) showed a 2-fold-decreased specific activity compared to those of SD48 (CotG as the carrier). One possibility is that the OxdD-Phy fusion protein, presumed to be mostly associated with the inner-coat layers, is less accessible to the substrate than is CotG-Phy, thought to be mainly located in the outer coat.

Spore display of active *E. coli* β -glucuronidase. The results presented above show that the inner-coat protein, OxdD, can be used as a carrier for the display of active, monomeric

FIG. 4. Abundance of the displayed phytase at the spore surface and specific activity of *B. subtilis* phytase. (A) Immunofluorescence detection of CotG-Phy and OxdD-Phy protein fusions at the surfaces of purified spores (average signal). (B) Assay of whole-cell extracts prepared from *B. subtilis* cultures in sporulating medium, 24 h after the onset of sporulation. (C) Assay of purified spores from the same cultures. DW, dry weight. Spores were purified from cultures of the following strains: PY79, wild type; SD48, CotG-Phy; SD50, OxdD-Phy. One unit (U) of phytase was defined as the amount of enzyme required to release 1 μ mol of inorganic phosphate from sodium phytate in 1 min.

phytase at the spore surface. We then wanted to test whether OxdD could be used for spore display of a larger, oligomeric enzyme. We tested whether *E. coli* β -glucuronidase, encoded by the *uidA* gene, could be presented in active form at the spore surface as a fusion to OxdD. The crystal structure of human GusA has been determined and suggests that the enzyme is likely to function as a 273-kDa homotetramer (3) with the catalytic site formed from a large cleft at the interface of two monomers (20). Presumably, the *E. coli* enzyme, which shares 50% amino acid identity with the human version, is also a homotetramer (10). To attempt the functional display of *E. coli* GusA at the surface of *B. subtilis* spores, the *uidA* gene was fused to the 3'-end of the *oxdD* gene with, as for the *oxdD-phy* fusion (see above), an interspersing 10 amino acid-linker (Fig. 1B). Ectopic integration of the gene fusion at *amyE* resulted in strain SD60 (Table 1). Expression of the OxdD-GusA fusion at

the *amyE* locus of PY79 did not measurably interfere with spore function or assembly (Table 2 and data not shown). Spores of strain SD60 were collected 24 h after the onset of sporulation in DSM, purified, and assayed for *in situ* detection of β -glucuronidase activity using the fluorogenic substrate C₁₂FDGlcU, (see Materials and Methods). In parallel, activity assays were conducted in trypsin-treated SD60 spores, and in spores of the wild-type strain PY79. Cleavage of the colorless C₁₂FDGlcU substrate into-5-dodecanoylamino fluorescein results in the production of a stable green-fluorescent product, allowing β -glucuronidase activity to be monitored by fluorescence microscopy. Fluorescence was emitted by SD60 spores (Fig. 5A). In contrast, no fluorescence was detected for PY79 spores, as well as for SD60 or PY79 spores treated with trypsin prior to the assay (Fig. 5A). Quantification of the fluorescence signal (Fig. 5B and C) showed that 40% of SD60 spores emit-

FIG. 5. Assay for the activity of GusA at the spore surface, through conversion of the colorless substrate C₁₂FDGlcU (see Materials and Methods) into a yellow fluorescent product. (A) Detection of a functional OxdD-GusA fusion by phase-contrast microscopy (PC) and fluorescence (FITC) microscopy. Bars, 2 μ m. (B) The fluorescence intensity was quantified on 150 spores. Purified spores submitted or not to trypsin proteolysis. Spores were purified from cultures of the following strains: PY79, wild type; and SD60, OxdD-GusA. (C) Average fluorescence intensity quantified on 150 spores. AU, arbitrary units.

ted at least 2-fold more fluorescence than the baseline (>60 AU). Possible explanations for the occurrence of nonfluorescent spores might be that the fluorogenic product of cleavage diffuses away from spores or that the high molecular weight (882 g/mol) and lipophilic nature (12-carbon aliphatic chain) of the fluorogenic substrate limits its penetration into the inner coat, where OxdD-GusA presumably resides. It can also be speculated that OxdD-GusA fusion has not assembled into an active form in nonfluorescent spores. In any event, our results show that active GusA can be displayed at the spore surface as a fusion to the inner-coat protein OxdD.

Concluding remarks. Further work will address the optimization of strain engineering to increase activity of the passenger enzyme fused to OxdD. For instance, improvement might result from increased multicopy-driven expression, from expression in protease-deficient *B. subtilis* strains or through the selection of passenger enzymes with higher specific activity (e.g., phytase from *Citrobacter braakii*). Nevertheless, based on the current activity measured at the surface of spores displaying *B. subtilis* OxdD-Phy (2.7×10^3 U/g [dry weight]), ~0.4 g of spores (dry weight) would represent 1,000 U of phytase. This amount of enzyme, added to 1 kg of feed in a daily swine diet, would replace 1 g of inorganic phosphorus supplementation and therefore could reduce total phosphorus excretion by 30 to 50% (29).

Based on the results presented here, spore-associated enzymes, such as OxdD, seem to be valuable carriers for spore display of passenger proteins. One question raised by our study is whether exposure of the spores to acid or proteases in the upper parts of the gastrointestinal tract would result in the inactivation of the displayed carrier-enzyme fusion protein. This may indeed happen to some extent. However, *B. subtilis* spores were recently shown to have the ability to germinate and sporulate upon exiting the stomach, leading to persistence of the *B. subtilis* strain in the gut (2, 31) and to constant surface display of freshly synthesized and fully active enzymes. Spore surface display thus provides the basis for novel means of formulation for proteins of interest such as feed enzymes (phytases, lipases, hemicellulases, and others).

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