Functional amyloids are considered as common building block structures of the biofilm matrix in different bacteria. In previous work, we have shown that the staphylococcal surface protein Bap, a member of the Biofilm-Associated Proteins (BAP) family, is processed and the fragments containing the N-terminal region become aggregation-prone and self-assemble into amyloid-like structures. Here, we report that Esp, a Bap-orthologous protein produced by Enterococcus faecalis, displays a similar amyloidogenic behavior. We demonstrate that at acidic pH the N-terminal region of Esp forms aggregates with an amyloid-like conformation, as evidenced by biophysical analysis and the binding of protein aggregates to amyloid-indicative dyes. Expression of a chimeric protein, with its Esp N-terminal domain anchored to the cell wall through the R domain of clumping factor A, showed that the Esp N-terminal region is sufficient to confer multicellular behavior through the formation of an extracellular amyloid-like material. These results suggest that the mechanism of amyloid-like aggregation to build the biofilm matrix might be widespread among BAP-like proteins. This amyloid-based mechanism may not only have strong relevance for bacteria lifestyle but could also contribute to the amyloid burden to which the human physiology is potentially exposed.

**INTRODUCTION**

In the last two decades, an increasing amount of studies have shown that amyloid structures are not restricted to protein aggregation disorders but also, they can be found as functional amyloids for a number of relevant biological processes in eukaryotic cells, fungi, and bacteria. In bacteria, one of the most predominant processes in which functional amyloids have a biological role is in the context of multicellular behavior and biofilm formation. Biofilms are the most common bacterial growth in natural environments, medical, and industrial settings. In biofilms, bacteria are embedded in an extracellular matrix that provides structural integrity, protects bacteria from the action of the immune system and antimicrobial treatments, and favors nutrient adsorption and keeps extracellular enzymes close to the cells. Amyloids are especially well suited for assembling the biofilm matrix in the extracellular environment because they provide a stable chassis for its inherent resistance against protease digestion and denaturation. Amyloids can also modify the viscoelastic properties of the extracellular matrices helping biofilms to adapt and respond to environmental fluctuations. Therefore, proteins with amyloid or amyloid-like conformation have been proven widespread in biofilms of many unrelated bacteria. The most studied and characterized bacterial amyloid system is the curli fimbriae present in Escherichia coli and in other enterobacterial species, which is composed of the major structural subunit CsgA. Other examples of biofilm-related amyloids are FapC in Pseudomonas spp., TasA in Bacillus subtilis, chaplins in Streptomyces coelicolor or the phenol-soluble modulins (PSMs) in the pathogenic bacteria Staphylococcus aureus. In all these examples, functional amyloid assembly involves specialized machinery that controls the expression, secretion and polymerization of protein subunits. A more simplistic type of amyloid-like material is the one formed by the biofilm-associated protein (Bap) of S. aureus. This surface protein undergoes partial proteolytic cleavage that releases fragments containing the N-terminal region of Bap only under specific environmental conditions. This region has a molten globule-like conformation that, when the pH becomes acidic, switches to a β-sheet-rich conformation and polymerizes to form amyloid-like fibers. The N-terminal region of Bap contains two EF-hand calcium-binding motifs. Binding of calcium to the EF-hand motifs stabilizes the molten globule conformation. This prevents β-sheet transition and the subsequent self-assembly into amyloid fibers, hindering biofilm formation. Therefore, inactivation of the EF-hand calcium-binding domains by site-directed mutagenesis did not affect biofilm formation in the presence of calcium. The enterococcal surface protein Esp is a Bap-orthologous protein. Esp is a high molecular weight protein of 1873 amino acids. It contains well-differentiated modules including the N-terminal domain, sharing 26% sequence identity with Bap; the central core region with two domains (A and B) and a series of tandem repeats sharing 23% sequence identity with C-repeats of Bap; and the C-terminal domain that includes the LPXTG-like motif found in surface-associated proteins. Esp contributes to biofilm formation by E. faecalis. It was demonstrated that the minimal region contributing to Esp-mediated biofilm enhancement was confined to the N-terminal domain. However, the molecular mechanisms by which the N-terminal region of Esp performs this function is unknown. Based on our previous results obtained with Bap of S. aureus, we hypothesize that these proteins could mediate intercellular connection through a common molecular
mechanism of amyloid-like conformation. By using biophysical
in vitro assays, cell-based and microscopic approaches and dye-
binding analyses, we demonstrated that the N-terminal domain of
Esp formed aggregates with amyloid-like properties when the pH
of the media became acidic. We further showed that the N-
terminal domain of Esp is functional as it was able to induce
multicellular behavior in bacteria lacking the
\textit{esp} gene through the
formation of amyloid-like material, both when it was exogenously
added to the culture and when it was expressed from a plasmid.
Overall, our results suggest that the mechanism of amyloid-like
aggregation might be a widespread mechanism of the BAP-like
proteins to build the biofilm matrix.

RESULTS
Computational algorithms predict Esp aggregation tendency
The enterococcal surface protein Esp possesses high structural
similarities to the staphylococcal Bap. The N-terminal domain
(Esp\_N) of Esp shows 26\% identity with the N-terminal domain of
Bap (Fig. 1a). Bearing in mind that Bap adopts an amyloid
conformation to mediate biofilm formation, we have used four
existing computational algorithms commonly used to predict the
amyloidogenic propensity of a protein. The selected predictors,
including WALTZ\textsuperscript{18}, AGGRESCAN\textsuperscript{19}, TANGO\textsuperscript{20}, and FoldAmyloid\textsuperscript{21},
rely on different principles (\(\beta\)-sheet formation, aggregation
propensity, hydrogen-bonding interactions, and expected packa-
ging density). The aggregation properties predicted by these
algorithms for Esp were normalized and they were compared in
Fig.1b. All the algorithms identified a high number of amyloid-
prone segments at the Esp\_N region (Fig. 1b and Table 1 and
Supplementary Fig. 1A). The alignment of peptides predicted to
be amyloidogenic by at least 3 of the 4 algorithms showed no
clear conserved domains among them (Supplementary Fig. 1B),
suggesting that the presence of aggregation-prone regions rather
than amino acid motifs is conserved at the N-terminus of Esp.

| Amino acids | Sequence                          |
|-------------|-----------------------------------|
| 21–49       | SVGVASVLGVGLVFATGIVN              |
| 158–174     | TNIVKWYIRANDGLFA                 |
| 211–221     | RLMYRIYLVSH                     |
| 231–239     | IESTGYLET                     |
| 323–332     | YVSGIQMHMV                    |
| 440–448     | TYGTVYYLQ                     |
| 667–686     | NDAFSVLQDY                   |
| 698–705     | NGTIVFFT                     |
| 1050–1058   | KGTVVTTYSD                 |
| 1685–1694   | GTMYFWFEKV                 |
| 1701–1711   | NKKATVVVYYP               |
| 1718–1726   | VEVVIVSD               |
| 1843–1865   | VDSNIIYTAGLLIGTLGGLGY     |

Esp\_N expressed with the curli-dependent amyloid generator (C-
DAG) system forms amyloid fibrils
To explore the amyloid-forming propensity of Esp\_N, we used the
C-DAG system\textsuperscript{22}. Amino acids 67–511 from Esp were cloned into
the pExport plasmid that allows the expression of the epitope of
interest fused to the first 42 residues of the signal sequence of
CsgA (ssCsgA) and the 6xHistidine tag (Fig. 2a). We also cloned
into the pExport plasmid a C-repeat of Esp (amino acids
1148–1228), which showed no amyloidogenic-prone region
prediction on it, and one A-repeat and part of the B-domain
(amino acids 913–1060) that possess only one aggregation-prone
peptide (Fig. 1a). Besides, plasmids carrying the \textit{S. aureus} Bap\_B or
Bap\_A regions fused to ssCsgA were included as positive and
negative controls, respectively (Fig. 1a). The resulting plasmids
were introduced in the carrier \textit{E. coli} VS39 strain, which is deficient in the curli system but expresses \textit{csgG} gene that is required for the protein export. In the presence of arabinose, chimeric proteins are expressed and subsequently guided by the ss\textit{csgA} epitope at the extracellular space through a pore-like structure that is formed by the \textit{CsgG} protein in the outer membrane (Fig. 2a). The presence of extracellular amyloid-like aggregates was detected by analyzing the capacity of the strains to bind Congo Red dye (CR). As shown in Fig. 2b, \textit{E. coli} cells expressing Esp\textsubscript{N} domain were able to bind CR as the strain producing Bap\textsubscript{B} of \textit{S. aureus}, while no binding to CR was observed in \textit{E. coli} strains expressing Bap\textsubscript{A}, Esp\textsubscript{C}, and Esp\textsubscript{AB} chimeras. We also quantified the CR attached to the cells using a quantitative CR-binding assay. After incubation of the proteins using the C-DAG system, \textit{E. coli} cells were incubated in a solution containing CR. The colorant bound to the amyloids-like material was solubilized from the cells using ethanol–acetone (Fig. 2c). Results showed that \textit{E. coli} strain that expressed Esp\textsubscript{N} was able to bind significantly higher levels of CR in comparison to \textit{E. coli} strain that express Esp\textsubscript{C} and Esp\textsubscript{AB} (Fig. 2d). Interestingly, electron microscopy analysis of the \textit{E. coli} strain exporting the Esp\textsubscript{N} domain revealed the presence of extracellular dense aggregates surrounding the cells, that are most likely fibrous (Fig. 2e, f). Esp\textsubscript{N} represented 500 nm. Scale bar of panels \textbf{f}, \textbf{h}, \textbf{i} represents 200 nm. Scale bar of panel \textbf{g} represents 100 nm.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig2}
\caption{Expression of Esp\textsubscript{N} using the curli-dependent amyloid generator (C-DAG) system. \textbf{a} Representative scheme of C-DAG. Potential amyloid domains are expressed under the control of the inducible PBAD promoter, which allows the expression of the epitope fused to the first 42 residues of the signal sequence of CsgA. CsgG is expressed under the control of an IPTG-inducible promoter. Outer membrane (OM), cytoplasmic membrane (CM), periplasmic space (PS). \textbf{b} Variation in colony color phenotype of \textit{E. coli} cells exporting the N-terminal domain of Esp (Esp\textsubscript{N}), a C-repeat (Esp\textsubscript{C}), and AB-repeats (Esp\textsubscript{AB}) on agar supplemented with CR. \textit{E. coli} expressing Bap\textsubscript{B} and Bap\textsubscript{A} from \textit{S. aureus} was used as positive and negative controls respectively. \textbf{c} CR solubilized using ethanol–acetone from \textit{E. coli} cells expressing Esp\textsubscript{N}, Esp\textsubscript{C}, Esp\textsubscript{AB}, Bap\textsubscript{B}, and Bap\textsubscript{A}. \textbf{d} Quantification of CR. Absorbance was measured at 500 nm. Bars represent the standard deviations of the results of six independent experiments ($n=6$). Statistically significant differences were determined using Mann–Whitney test *$p<0.05$, **$p<0.01$. Transmission electron micrographs of negatively stained fiber-like structures formed by \textit{E. coli} cells that express Esp\textsubscript{N} \textbf{e}, \textbf{f} and Bap\textsubscript{B} \textbf{h}. \textit{E. coli} cells that express Bap\textsubscript{A} \textbf{i}, Esp\textsubscript{C} \textbf{j} and Esp\textsubscript{AB} \textbf{k} did not show the presence of fibers as shown by transmission electron microscopy. Immunogold labeled of fiber-like structures of samples from \textit{E. coli} cells that express Esp\textsubscript{N} using anti-His antibodies \textbf{g}. Scale bar of panels \textbf{e}, \textbf{j}, \textbf{k} represents 500 nm. Scale bar of panels \textbf{f}, \textbf{h}, \textbf{i} represents 200 nm. Scale bar of panel \textbf{g} represents 100 nm.}
\end{figure}
Recombinant Esp_N assemblies into β-sheet-rich structures with amyloidogenic properties at acidic pH

To study the biophysical properties of the N-terminal domain of Esp we purified a recombinant Esp_N domain (rEsp_N) (Supplementary Fig. 3). rEsp_N protein was incubated in phosphate-citrate buffer solutions with different pH. As it was previously observed for Bap_B14, rEsp_N formed aggregates at acidic pH (pH 4.0–4.2) (Fig. 3a). At pH 4.2, rEsp_N formed a subtle but visible ring of protein adhered to the walls of the tube. Interestingly, the process was reversible and rEsp_N aggregates dissociated completely when the pH was raised to 7 (Fig. 3b). Analysis of the structure of the rEsp_N aggregates by scanning electron microscopy showed the formation of a dense mesh-like network (Fig. 3c).

We determined the relative size of rEsp_N aggregates by static light scattering (Fig. 4a), showing the presence of significantly big aggregates of rEsp_N at pH 4.2 that completely disassembled when buffer was changed to pH 7. We also showed that these self-assembled rEsp_N aggregates bound strongly to bis-ANS dye evidencing their exposure of hydrophobic patches to solvent (Fig. 4b).

In order to test whether the aggregates formed by rEsp_N have amyloid-like properties, we analyzed the secondary structure content by far-UV circular dichroism (CD) of the rEsp_N at pH 4.2 and pH 7. Results showed that rEsp_N domain showed a preponderance of β-sheet content at acidic pH (Fig. 4c, d). We also analyzed the amide I region of the attenuated total reflectance-Fourier transform infrared spectroscopy (ATR-FTIR) spectrum in the range 1700–1600 cm⁻¹ of the rEsp_N aggregates (acidic pH) and its soluble state (neutral pH). The overall analysis of both spectra showed a left shift absorbance in the 1610–1630 cm⁻¹ range of the β-strand region of the amide I spectrum, which is attributed to amyloid-like β-strand interactions (Fig. 4e).

Deconvolution of the spectra showed that the majoritarian band of rEsp_N aggregates was detected at 1620–1630 cm⁻¹, which is typically assigned to the presence of inter-molecular β-sheets that contributes 38.5% of the area (Fig. 4f). The dominated structure of the soluble Esp_N (neutral pH) spectra is detected at 1640 cm⁻¹ assigned to the presence of intra-molecular β-sheets covering 37.91% of the area. The second majoritarian structures appear at 1640 cm⁻¹, covering 33.9% of the area and at 1620–1630 cm⁻¹ that contributes 29.01% of the area for the Esp_N aggregates and the soluble protein, respectively. A representation of the second derivative values, which are necessary for the deconvolution method of the bands, is shown in Supplementary Fig. 4. Overall, our data are consistent with the assembly of Esp into supramolecular β-sheet-enriched aggregates.

To confirm that the detected β-sheet-enriched aggregates display amyloid-like properties, we analyzed if rEsp_N aggregates could bind amyloid-indicative dyes, such as CR, Thioflavin-T (Th-T), and Proteostat. CR binding to rEsp_N aggregates was determined by analyzing the absorbance recorded from 380 to 680 nm. As it is characteristic for amyloid proteins, incubation of CR in the presence of rEsp_N aggregates formed at pH 4.2 resulted in a red shift of its spectrum compared to the protein incubated at pH 7 and the CR buffer. Moreover, the reversion of the aggregates at pH 7 displayed no red shift in the spectrum (Fig. 5a). Th-T fluorescence emission was enhanced in the presence of rEsp_N aggregates at the intensity of Th-T fluorescence spectral maximum of 488 nm. In contrast, no change in Th-T fluorescence was observed when the protein was incubated at pH 7.0 (Fig. 5b).

Finally, staining with Proteostat showed that the dye was significantly concentrated at the rEsp_N aggregates (Fig. 5c). The fact that rEsp_N aggregates bind all these amyloid-indicative dyes confirmed the amyloid-like features of Esp.

In addition, we tested the aggregation kinetics of rEsp_N at pH 4.2 and pH 7 by measuring Th-T fluorescence signal over time. We observed a very high Th-T signal from the very beginning of the reaction when the protein was incubated in acidic buffer. The rapid aggregation of rEsp_N gave a kinetics missing the lag-phase that is typical of the sigmoidal aggregation kinetics of the amyloid proteins (Fig. 5d). Taking together all these results showed that the N-terminal domain of Esp self-assembled in structures with amyloidogenic properties at acidic pH.

Heterologous expression of Esp_N induces multicellular behavior through the formation of fibrillar structures

We generated a chimeric protein comprising the Esp_N domain (amino acids 50–743) tagged with the 3xFLAG amino acid sequence and linked to the R domain of the clumping factor A (R_CfA) that contains the LPXTG motif for anchoring to the peptidoglycan. The chimeric protein was expressed from the pCN51 vector under the control of the Pcad-cadC promoter. This plasmid was transformed into the biofilm negative S. aureus Δbap strain². As a control, we expressed the entire Esp protein from the Δbap_bap plasmid. The localization of both Esp_N-R_CfA chimeric protein and full Esp on the bacterial cell wall was confirmed by immunofluorescence using anti-esp antibodies (Fig. 6a). As shown in Fig. 6b, bacterial cells expressing the Esp chimeric protein induced cell aggregation (or cell clumping) and biofilm formation similarly to those cells expressing the whole Esp protein. This result confirmed that the N-terminal domain of Esp is sufficient to encourage cell aggregation, similar to the full Esp.

According to the in vitro assays with the rEsp_N protein, Esp-mediated multicellular behavior was only observed under acidic conditions, when bacteria were grown in media supplemented with glucose, TSB-gluc (Fig. 6b, c), or LB-gluc (Supplementary

Fig. 3  Recombinant rEsp_N protein forms reversible aggregates in acidic phosphate–citrate buffer.  a 2 μM of purified rEsp_N protein was incubated in phosphate buffer solutions at different pH values. Aggregates started to be visible in buffers with pH 4 and 4.2 (indicated by a red arrow).  b Reversion assay showed complete disassembly after phosphate–citrate buffer exchange from pH 4.2 to pH 7.  c Scanning electron microscopy of rEsp_N aggregates. Scale bar represents 1 μm.
To validate the functionality of rEsp_N, we performed an exogenous complementation assay that consisted in determining the capacity of rEsp_N to induce bacterial clumping when exogenously added to a bacterial culture. Specifically, we extracellularly added rEsp_N to a bacterial culture of the E. faecalis 23 strain that lack the esp gene and therefore is unable to induce bacterial aggregation. As a control, we also used the S. aureus Δbap strain. As shown in Fig. 7a, the addition of rEsp_N promoted bacterial aggregation in both strains (Fig. 7a). By using immunofluorescence labeling with anti-His antibodies we were able to detect rEsp_N protein in the clusters formed by both of E. faecalis and the S. aureus Δbap strains (Fig. 7b). Finally, we analyzed the cell aggregates by SEM and TEM. We detected extracellular tangles of fiber-like material on the cell surface of E. faecalis 23 connecting and attaching cells together (Fig. 7c, d). All these results showed that rEsp_N is biologically active and induce cell-to-cell aggregation in E. faecalis.

DISCUSSION

Amyloid fibers have been recently discovered as the main group of proteins composing the extracellular matrix of many unrelated bacteria. Two different types of biofilm-related amyloid machineries are described so far. The first group consists in intrinsic bacterial amyloids, where the amyloid state is the primary functional form of the proteins. Examples of amyloids of this group are: curli (csaABC-csgDEFG) in enterobacteria and Fap (fapA-F) in Pseudomonas and chaplins in S. coelicolor. The second type are facultative bacterial amyloid-like proteins where the protein adopts a functional globular folded structure and depending on proteolytic processing and environmental triggers,
it can also switch to an amyloid conformation. It seems that the facultative amyloids play a dual role in biofilm formation acting as adhesins in their native conformation and as matrix scaffolds when they polymerize into amyloid-like fibrillar structures. An example of this type of proteins is Bap of S. aureus. Bap is secreted and covalently anchored to the cell wall. At its native conformation, Bap is involved in primary adhesion to an abiotic surface and host cell ligands. Then, Bap is processed releasing to the media fragments containing the N-terminal region to the media, whilst part of the C-terminal repeat region remains anchored to the membrane. When the pH becomes acid the N-terminal domain of Bap switches from its partially ordered native state to an aggregation-prone conformation that facilitates polymerization into amyloid-like fibrillar structures.

The results presented here, propose Esp as a novel facultative amyloid-like protein. The prediction of potentially amyloidogenic regions using several computational algorithms converged to indicate regions capable of adopting an amyloid conformation in the N-terminal domain of Esp (Esp_N). Esp undergoes a limited proteolytic or spontaneous cleavage at certain conditions, as shown by western-blot using antibodies against the N-terminal region of Esp (Supplementary Fig. 6). This processing would enable the release of the N-terminal domain of the protein that self-assembles into amyloid-like fibrillar structures. However, we are unable to determine which part of the N-terminal region of the protein are releasing the processed peptides that form part of the amyloid-like fibers. It is important to notice that sortase-mediated anchoring of Esp is not strictly required for multicellular aggregation, since extracellular addition of rEsp_N-purified protein lacking the LPXTG motif is biologically active and induces cell-to-cell aggregation. Although were able to detect extracellular fibrous dense aggregates in E. coli cells exporting the Esp_N domain by electron microscopy analysis, we did not prove in a truthful way that they are canonical amyloid fibers. Secondary structure data (CD spectra and FTIR) and binding to amyloid indicator dyes are not sufficient to affirm that Esp adopts a cross-β arrangement typical of amyloids.

Another coincidence between Esp and Bap is the fact that acidic pH conditions favor amyloid polymerization of both proteins. As occurs with Bap, the pH at which the N-terminal domain of Esp shows aggregation activity (pH 4.2) is close to the isoelectric point of the full protein (pI 4.5) and the isoelectric point of the Esp N-terminal domain (pI 4.68) and, therefore, lack of net charge at this pH favors the conversion of the protein from its native state to the amyloid-like state. Previous work established that high glucose concentration (0.5% (w/v) or higher) in the growth media promoted Esp-mediated biofilm formation without affecting esp expression. Our results provide a possible explanation to this situation: an acidification of the growth media as a consequence of glucose metabolism would favor Esp amyloid folding and aggregation, thus inducing biofilm formation without relevant changes in esp gene expression. Of course, we cannot exclude the possibility that other undetermined factors might participate in this process and interact with Esp. Another major point to be considered in the amyloidogenic capacities of Esp and Bap, is the fact that Esp does not contain any EF-hand motif in its N-terminal domain. For Bap, binding of calcium to the EF-hand motifs is a key process that regulates the formation of amyloid-like fibers. In the presence of calcium, the protein undergoes tertiary rearrangements that increase its stability in solution and thus decrease its aggregation behavior. In case of Esp, this process might be triggered by other metal ions or factors that require further investigation.

Bap and Esp belongs to the biofilm-associated protein (BAP) family. Members of this family have been identified in many unrelated bacteria including BapA of Salmonella enterica ser. typhimurium, Bap of Acinetobacter baumannii, LapA of Pseudomonas putida, BapL of Listeria monocytogenes, Bap of Burkholderia cepacia, and BpfA of Shewanella oneidensis. All the BAP proteins share several structural and functional features (Supplementary Fig. 7). They are high-molecular-weight proteins.
that contain a core domain of tandem repeats. They play a relevant role in bacterial infectious processes and confer upon bacteria the capacity to form a biofilm. Based on the functional similarities found between Esp and Bap, it is tempting to speculate that other members of the BAP family will also possess amyloid-like properties. In fact, the prediction of potentially amyloidogenic regions using WALTZ algorithm indicated that other BAP members might be capable of forming amyloid-like structures from the N-terminal domain of Esp (Esp_N) or the repeat units, in the case of Bap of *S. enteritidis* (Supplementary Fig. 7). It is known that repeating units may contain peptides with amyloidogenic behavior. In a previous work Lembré et al. demonstrated that a peptide derived from the repeats of the Bap homolog protein of *S. epidermidis* formed amyloid fibers. This amyloid domain has been proposed to mediate protein–protein interactions and the tertiary structure of Bap in *S. epidermidis*. Although the peptide is repeated 17 times in the protein Bap of the *S. epidermidis* strain C533, the peptide is present only one time in the Bap sequence of *S. aureus*. Our analysis confirmed the absence of amyloid-like domains in the central repeat regions of Bap of *S. aureus* and Esp of *E. faecalis*. Therefore, the presence of several aggregation-prone regions in a domain or the amyloidogenic peptides derived of the repeating units of a protein would be sufficient to promote an amyloid-like conformation of the BAP family of proteins. However, the putative amyloidogenic behavior of all these domains needs to be validated and will be the object of further research that will dig in the mechanistic insight to the likely aggregation mechanism of BAP family proteins.
METHODS

Oligonucleotides, plasmids, bacterial strains, and culture conditions

Bacterial strains, plasmids, and primers used in this study are listed in Tables 2 and 3. E. coli strains were grown in LB broth (Conda-Pronadisa). E. faecalis and S. aureus strains were grown in trypticase soy broth (Conda-Pronadisa), supplemented with glucose 0.5% (w/v) in E. faecalis or glucose 0.25% (w/v) in S. aureus, brain heart infusion (BHI) broth and B2 broth media (casein hydrolysate 10 g/l, yeast extract 25 g/l, NaCl 25 g/l, K2HPO4 1 g/l, glucose 5 g/l). When required for selection, medium was supplemented with appropriate antibiotics at the following concentrations: erythromycin (Em), 1.5 and 10 µg/ml; ampicillin (Amp), 100 µg/ml; chloramphenicol (Cl), 20 µg/ml.

Table 2. Strains and plasmids.

| Strains and plasmids                                                                 | Reference |
|------------------------------------------------------------------------------------|-----------|
| E. faecalis 23 E. faecalis esp−, non-biofilm forming                                 | 16        |
| E. faecalis 11279 E. faecalis esp−, medium biofilm forming                         | 16        |
| E. coli VS39 E. coli transformed with pVS76 (cat PlacUV5 csgG, pACYC184 ori; produces CsgG) | 22        |
| E. coli VS39 Esp_N VS39 complemented with pEXPORT:esp_N                              | This study|
| E. coli VS39 Esp_C VS39 complemented with pEXPORT:esp_C                              | This study|
| E. coli VS39 Esp_AB VS39 complemented with pEXPORT:esp_AB                            | This study|
| E. coli VS39 Bap_B VS39 complemented with pEXPORT:bap_B                              | 5         |
| E. coli VS39 Bap_A VS39 complemented with pEXPORT:bap_A                              | 5         |
| S. aureus V329 Δbap S. aureus bap−                                                 | 23        |
| S. aureus Δbap Esp_N S. aureus Δbap containing pCN51:esp_N                          | This study|
| S. aureus Δbap Esp S. aureus Δbap containing pCN51:esp                              | This study|
| S. carnosus TM400                                                                   | 5         |
| S. carnosus TM400 Esp_N S. carnosus TM400 containing pCN51:esp_N                   | This study|
| S. carnosus TM400 Esp S. carnosus TM400 containing pCN51:esp                        | This study|
| pET46-eK-LIC_Esp_N Plasmid for expression of Esp N-terminal                         | This study|
| pCN51:esp_N Plasmid for expression of Esp N-terminal domain fused to the R-domain of ClfA, under the control of the Pcad promoter | This study|
| pCN51:esp Plasmid for expression of Esp under the control of the Pcad promoter      | This study|
| pEXPORT:esp_N Expression vector for C-DAG system including the N-terminal domain of Esp | This study|

Fig. 7 Exogenous complementation of Esp-negative strains with purified rEsp_N. a Bacterial clumping of overnight cultures of esp-negative E. faecalis 23 and S. aureus Δbap grown under shaken conditions (200 rpm) at 37 °C in the presence of rEsp_N. Ø indicates no addition of rEsp_N to bacterial culture. b Immunofluorescence showing surface localization of rEsp_N. Bacteria were fixed and labeled with anti-His antibodies and DAPI. Scale bars represent 3 µm. c Scanning electron microscopy and d transmission electron micrographs of negatively stained E. faecalis 23 incubated with (right panel) and without rEsp_N (left panel). Scale bar represents 500 nm.

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DNA manipulations and bacterial transformation

General DNA manipulations were performed using standard procedures. Plasmids were purified using the NucleoSpin Plasmid miniprep kit (Macherey-Nagel) according to the manufacturer’s protocol. FastDigest restriction enzymes and Rapid DNA ligation kit (Thermo Scientific) were used according to the manufacturer’s instructions. Plasmids were transformed into *E. coli* XL1-Blue strain (Stratagene), *E. faecalis*, and *S. aureus* by electroporation. Briefly, *S. aureus* cells were cultured in B2 broth until the OD at 650 nm reaches 0.5. Cells were washed three times with glycerol 10% (v/v). Cells were resuspended in 15 ml of glycerol 10% (v/v) and incubated at 20 °C for 15 min. After incubation, cells were resuspended in 10% glycerol at the final concentration of 1 × 10^10 CFU/ml. For staphylococcal electroporation, 50 μl of the electrocompetent cells were mixed with 2 μg of plasmid and the mix was added to an ice-cold 0.1-cm gap electrode Gene Pulser Cuvette (Bio-Rad). One pulse was given at 100 Ω, 1.25 V, and 25 F capacity. Immediately after the pulse, 1 ml of SMMP media was added to the cuvette. After incubation at 37 °C for 2 h, the cell mixture was plated onto TSA plates with selective antibiotics. For *E. faecalis*, cells were cultured in BHI media overnight at 37 °C. Cells were washed three times with glycerol 10% (v/v) and were resuspended at the final concentration of 1 × 10^10 CFU/ml. For enterococcal electroporation, 50 μl of the electrocompetent cells were mixed with 3 μg of plasmid. The mix was added to an ice-cold 0.1-cm gap electrode cuvette. One pulse was given at 400 Ω, 1.25 V, and 25 F capacity. Immediately after the pulse, 1 ml of BHI media with subinhibitory concentration of chloramphenicol (0.2 μg/ml) was added to the cuvette. After incubation at 37 °C for 2 h, the cell mixture was plated onto TSA plates with the selective antibiotic.

### Generation of chimeric proteins

The N-terminal domain of the *esp* gene (*esp_N*) was amplified from *E. faecalis* 11279 using primers Esp_Ori_Bam and Esp_B_Kpn (Table 3). To allow anchoring of amplified *esp_N* domain to the bacterial cell wall, the R region of clumping factor A (*clfA*) gene containing an LPXTG motif was amplified from *S. aureus* Newman strain using primer K-3xFagCifA, containing the 3xFlag sequence and a recognition sequence for KpnI, and primer CifA-3C with a recognition sequence for EcoRI. The KpnI/EcoRI-restricted R-clfA was cloned into pCN51 vector (pCN51:esp_N-clfA). The *Esp_N-CifA* chimeric protein was expressed under the control of the *P_α1-α3-cifA* promoter, a cadmium inducible promoter. *Esp* gene was amplified using primers EF1-InPhu-pCN51-BamH and EF2-InPhu-pCN51-Kpn and the PCR product was cloned in pCN51 (pCN51:esp).

### Construction of plasmids for Esp expression in C-DAG system

To obtain *E. coli* strains for C-DAG system, we PCR amplified the N-terminal region of *esp* (**esp_N**) with primers EspB-NotI and EspB-XhoI-3. Nucleotides from 2737 to 3192 for an A-repeat and the B-domain (**esp_AB**) were amplified using primer EF1-InPhu-pCN51-BamH and EF2-InPhu-pCN51-Kpn. The B-domain (**esp_C**) gene containing an LPXTG motif was amplified from *E. faecalis* VS39 strain. Induction of protein production and presence of amyloid-like material was assessed on solid medium containing 10 μg/ml CR by evaluating colony color phenotype. To quantify the amount of CR bound to fibers generated from arabinose-induced C-DAG strains, we resuspended and incubated *E. coli* cells in 1 ml of PBS containing 1.5 μl of CR 0.8% (w/v). The dye was solubilized from the cells using ethanol:acetone (80:20 v/v). Absorbance was measured at 500 nm using a Biospectrophotometer (Eppendorf). Independent experiments were performed six times. Mann–Whitney tests was used to assess significant differences (*p < 0.05) of absorbance among tested strains.

### Multicellular behavior phenotypes

For the biofilm formation assay strains were grown overnight at 37 °C and then diluted 1:40 in the corresponding media. Cell suspension was used to inoculate sterile 96-well polystyrene microtiter plates (Thermo Scientific). After 24 h of incubation at 37 °C wells were gently rinsed two times with water, dried, and stained with crystal violet for a few minutes. To quantify biofilm formation capacity, crystal violet adhered at the bottom of the wells was resuspended with 200 μl of a solution of ethanolaceton (80:20 v/v) and the absorbance was quantified at 595 nm.
Protein expression and production of anti-Esp antibodies

N-terminus of Esp (amino acids 50–743) was PCR amplified from E. faecalis 11279 using high fidelity Phusion DNA Polymerase (Thermoscientific) and primers Esp-LIC-Fw and Esp-LIC-Rv (Table 3) designed for use in the LIC cloning system. The resulting fragment was cloned in pET46-Ek/LIC vector (Novagen). Overnight cultures of E. coli BL21 DE3 containing the expression plasmid were diluted 1:100 and grown to an OD600nm of 0.6. Isopropyl B-β-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM and the cultures were shaken (150 rpm) at 18°C overnight. After centrifugation, pellets were resuspended, sonicated, and centrifuged. Supernatants were filtered (0.45 μm) and rEsp_N protein purified by Ni affinity chromatography using HisTrap™ FF columns (GE Healthcare). To achieve the highest purity, size exclusion chromatography was applied with a HiLoad 16/600 Superdex 200 pg column (GE Healthcare). The concentration of the purified protein was determined by the bicinchoninic acid (BCA) Protein Assay (Pierce, Thermo Scientific) using BSA as standard. Chicken polyclonal antibodies raised against purified rEsp_N protein were supplied by Davids Biotechnologie company. Antibodies were subsequently purified by chromatography.

Immunoblot analysis

One milliliter of an overnight culture was harvested, washed, and finally resuspended in 100 μl of PBS containing 30% (w/v) raffinose (Sigma), 5 μl of lyostaphin 1 mg/ml (Sigma), and 2 μl of DNase 1 mg/ml (Sigma). After 2 h of incubation at 37°C, cells were centrifuged. The supernatants from surface protein extracts were recovered and analyzed by SDS-PAGE (7.5% (w/v) separation gel; 5% (w/v) stacking gel). For Western blot analysis, protein extracts were blotted onto Hybond-ECL nitrocellulose membranes (Amersham Biosciences). Anti-Esp-purified antibody was diluted 1:10,000 with PBS-Tween 5% (w/v) skim-milk. Horse-radish peroxidase-conjugated goat anti-chicken (Abcam Ab97135) diluted 1:20,000 in PBS-Tween 5% (w/v) skim-milk was used as a secondary antibody for Esp detection and the subsequent chemiluminescence reaction was recorded.

Formation of rEsp_N aggregates and reversion assay

To determine the effect of the pH in protein aggregation, rEsp_N recombinant protein at a final concentration of 2 μM was incubated in phosphate-citrate buffer at pH ranging from 3.6 to 8. Macroscopy protein aggregates were visualized as a ring of protein adhered to the glass wall at the air–liquid interface after 24 h of incubation at 37°C under shaking conditions (200 rpm). For reversion assay of rEsp_N aggregates formed after 24 h of incubation at 37°C, the phosphate-citrate buffer at pH 4.2 was removed and exchanged for phosphate-citrate buffer at pH 7. After an overnight incubation at 37°C and shaking conditions, dissolution of rEsp_N aggregates was macroscopically determined.

Th-T binding

rEsp_N protein at 0.1 mg/ml was incubated at pH 7.0, pH 4.2, or pH-reverted from pH 4.2 to pH 7.0 and Th-T added at 25 μM final concentration before measuring. Fluorescence emission spectra were recorded in the range of 460–600 nm with an excitation wavelength of 445 nm using a 5 nm slit width for excitation and emission in a Jasco FP-8200 spectrophotometer (Jasco Corporation, Japan) at 25°C. Aggregation kinetics of 0.05 mg/ml rEsp_N protein in phosphate–citrate buffer at pH 4.2 and pH 7.0 were recorded for 1800 s under agitation (800 rpm) at 25°C in the presence of 25 μM Th-T. The kinetic traces were measured exciting at 340 nm and recording in the 320–340 nm range, using excitation and emission bandwidths of 5 nm at low sensitivity, in a Jasco FP-8200 fluorescence spectrophotometer (Jasco Corporation, Japan).

Bis-ANS binding

Bis-ANS-binding assay was performed incubating 0.1 mg/ml rEsp_N (stock 0.2 mg/ml) in phosphate–citrate buffer at pH 4.2 and 7.0 containing 10 μM bis-ANS. Samples were measured in a Jasco FP-8200 spectrophotometer (Jasco Corporation, Japan) at 25°C in the 400–600 nm range with an excitation wavelength of 370 nm using a slit width of 5 nm for excitation and emission.

ATR-FTIR spectroscopy

ATR FTIR spectroscopy analyses of Esp_N samples in phosphate–citrate buffer pH 4.2 and in buffer pH 7 were performed with a Bruker Tensor 27 FTIR Spectrometer (Bruker Optics Inc.) supplied with a Golden Gate MkII ATR accessory. Each spectrum consists of 32 acquisitions measured at a resolution of 1 cm⁻¹. Spectra were acquired, background subtracted, baseline corrected, and normalized, with the Min/Max normalization method, using the OPUS MIR Tensor 27 software. For the analysis of the IR spectra, the second derivative was calculated and plotted to define the number and position of band components under the absorbance spectra, according to the local minima. Next, spectra were smoothed using the non-parametric smoother Loess at a 5% level and automatically fitted with the “Peaks I residuals” method using PeakFit package v4.12 (Systat Software), placing initially as many peaks as local minima appeared in the secondary derivative of the absorbance spectra. The resulting Gaussian curves were plotted using the amplitude and center values of the fitted bands. In order to assign secondary structures to band components, frequencies in the range 1611–1630 cm⁻¹ were assigned to intermolecular β-sheet structures, 1630–1640 cm⁻¹ to intramolecular β-sheet structures, 1648–1657 cm⁻¹ to β-helical structures and 1662–1687 cm⁻¹ to β-turns, as described in the literature.

Immunofluorescence

Cells were grown overnight in the corresponding tested conditions and fixed during 10 min with 3% (w/v) paraformaldehyde (SIGMA). 200 μl of fixed bacteria were placed on coverslips and incubated for 30 min. After several washes with phosphate-buffered saline (PBS), cells were saturated with PBS–0.5% (w/v) BSA, and finally stained with anti-Esp or monoclonal anti-polyHistidine antibody (Sigma Aldrich H1029) antibodies diluted 1:1000. Alexa Fluor 488-conjugated goat anti-chicken (Abcam ab150169) or Alexa Fluor 488-conjugated goat anti-mouse (Invitrogen A-11029) diluted 1:200 were used as a secondary antibody and DAPI diluted 1:200 was used to label bacteria.

Microscopy analysis

For TEM, cells were grown overnight in the corresponding tested conditions, washed twice with PBS and then fixed with 2% (v/v) paraformaldehyde (SIGMA) for 1 h at room temperature. Formvar/ carbon-coated nickel grids were deposited on a drop of fixed sample during 5 min and rinsed three times with PBS. Negative staining was performed using 2% (v/v) uranyl acetate (Agar Scientific, Stansted, UK). Observations were made with a JEOL 1011 transmission electron
microscope. For Esp immunogold labeling, grids coated with the sample were washed and incubated for 45 min on a drop of PBS containing 1:10 antibody against Esp. After washing with PBS, grids were incubated for 45 min with gold-conjugated (6 nm) goat-anti-mouse IgG (H&L) secondary (AURION 810.022). Grids were stained with 2% (v/v) uranyl acetate as described above. For scanning electron microscopy, cells on thermoxan coverslips were fixed for 1 h in 0.07 M sodium cacodylate buffer (pH 7.3) containing 1.2% glutaraldehyde and 0.05% Ruthenium red (RR). The samples were then washed in the same buffer containing 0.05% RR and post-fixed in 1% osmium tetroxide in cacodylate buffer. Coverslips were treated with the critical point drying method and observed on a Zeiss JSM 982 scanning electron microscope at the Laboratoire de Biologie Cellulaire et Microscope Electronique, UFR Medecine (Tours, France).

Reporting summary
Further information on experimental design is available in the Nature Research Reporting Summary linked to this paper.

DATA AVAILABILITY
The data that support the findings of this study are included in the article, its supplementary information files, or are available from the corresponding author upon reasonable request.

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AUTHOR CONTRIBUTIONS
A.T., L.M.-C., I.L., J.V. conceived and designed the experiments. A.T., L.M.-C., P.D.-M., S.N., J.A.G., J.V. conducted experiments. S.N., S.V., J.A.G. assisted with the biophysical experiments. J.A.G., S.V., I.L., J.V. analyzed the data and wrote the manuscript. All authors read and approved the final manuscript.

COMPETING INTERESTS
The authors declare no competing interests.

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