Conversion of the OmpF Porin into a Device to Gather Amyloids on the E. coli Outer Membrane

Sol Vendrell-Fernández, Paloma Lozano-Picazo, Paula Cuadros-Sánchez, María M. Tejero-Ojeda, and Rafael Giraldo*

Cite This: https://doi.org/10.1021/acssynbio.1c00347

ABSTRACT: Protein amyloids are ubiquitous in natural environments. They typically originate from microbial secretions or spillages from mammals infected by prions, currently raising concerns about their infectivity and toxicity in contexts such as gut microbiota or soils. Exploiting the self-assembly potential of amyloids for their scavenging, here, we report the insertion of an amyloidogenic sequence stretch from a bacterial prion-like protein (RepA-WH1) in one of the extracellular loops (L5) of the abundant Escherichia coli outer membrane porin OmpF. The expression of this grafted porin enables bacterial cells to trap on their envelopes the same amyloidogenic sequence when provided as an extracellular free peptide. Conversely, when immobilized on a surface as bait, the full-length prion-like protein including the amyloidogenic peptide can catch bacteria displaying the L5-grafted OmpF. Polyphenolic molecules known to inhibit amyloid assembly interfere with peptide recognition by the engineered OmpF, indicating that this is compatible with the kind of homotypic interactions expected for amyloid assembly. Our study suggests that synthetic porins may provide suitable scaffolds for engineering biosensor and clearance devices to tackle the threat posed by pathogenic amyloids.

KEYWORDS: OmpF, porin loop grafting, amyloid adhesins, homotypic amyloid recognition, amyloid and prion bioremediation, Escherichia coli

INTRODUCTION

Amyloids are β-sheet-structured protein aggregates that are involved in prevalent neurodegenerative and systemic diseases but that recently have also been recognized to confer alternative functional states to some proteins.1–3 Amyloids have yet a hidden post-life as recalcitrant contaminants in natural environments, such as those associated with microbiota in soils and in the gut. The mammalian prion protein (PrP), when in its infectious assembled conformation (PrPSc) and with origin in the excreta and corpses of animals affected by scrapie in sheep and chronic wasting disease in cervids, is persistent in soils constituting an environmental contaminant and a potential zoonotic threat.5,6 On the other hand, amyloid fibers scaffolding biofilms common to γ-proteobacteria, such as curli (CsgA protein) in Escherichia coli,6 are a major component among the extracellular proteins secreted by bacteria in the gut.5 Recent evidence on the ability of curli amyloids to cross-seed in other proteins the nucleation of cytotoxic amyloid fibers3–12 constitutes a matter of concern adding to the emerging clues on a link between gut microbiota, neuroinflammation,12–15 and cancer.16,17 In view of the challenges posed by the ubiquitous presence of amyloids, a suitable targeting tool that acts as a biosensor of their presence and leads to their neutralization would be of utmost interest.

Synthetic biology (SynBio) of bacterial adhesion is a topic in expansion. Curli fibers have been engineered to modulate the

Received: July 26, 2021
assembly of multicellular architectures or the harvesting of metal ligands residues that build a precise conformational geometry. The fusion of the intimin (Neae) domain of autotransporters to single-domain antibodies (VHH, aka nanobodies) is a powerful approach to tailor adhesins on the bacterial surface for the recognition of a given protein sequence, on the basis of the specificity of the antibody variable domains for antigen recognition. However, it is less likely to be operational for binding to large polymorphic supramolecular assemblies such as those built on amyloidics, for which the development of conformation-specific full-length antibodies is still challenging.

In Gram-negative bacteria, the outer membrane (OM) constitutes the natural interface of the cell with the outside world; thus, it might be the platform of choice to implement tools for amyloid management through SynBio. Porins are the major protein components of the OM, where they fold as β-barrels embedded across its asymmetric bilayer (phospholipids toward the periplasm and lipopolysaccharide outward), constituting pores that act both as gatekeepers preserving the intake of ions and small molecules (with an upper limit of ca. 600 Da), including antibiotics. Porins also are receptors for bacteriophages and the channels for the import of bacteriocins. The structural basis for porin insertion and folding into the bacterial OM by the BAM chaperoning machinery has been recently elucidated as for the equivalent SAM complex in the mitochondrial OM. The newest additions to our exhausted antibiotic arsenal against multi-resistant Gram-negative bacteria are peptidomimetic β-strand-like molecules, which target BAM. They work either by establishing hydrogen bonds with strand β1 in BamA, the core β-barrel subunit, dumping its closure on the terminal strand β16, or through binding to some (L4, L6, and L7) of its eight extracellular loops, thus inhibiting the correct insertion of porins into the membrane.

SynBio of bacterial OM β-barrel proteins has found inspiration in the CsgG or FapF channels for the secretion of fibrillar pili or in the N-terminal domain of autotransporters. De novo design of β-barrels as successions of antiparallel β-hairpins, self-closing by zipping the first and last β-strands up, is among the latest accomplishments of protein engineering. Besides this, the insertion of antigenic peptides in the external loops boosts their immunogenic potential as vaccine candidates, but this often results in the aggregation of the chimeric protein, unable to be properly inserted into the OM, as amyloid-like (often cytotoxic) inclusion bodies.

Trying to overcome these limitations, grafting amyloidogenic sequences in the complementarity-determining regions of nanobodies has revealed the potential of homotypic, self-recognizing amyloid-based devices. A similar strategy inserting hydrophobic amyloidogenic sequences into porin loops is specially challenging, due to the expected generation of local traps in the rugged folding landscape of the porin during the BAM-assisted insertion of β-strands within the OM. However, among natural porins, Omp-Pst1/2 in the marine bacteriumProvidencia stuartii include each an amyloidogenic loop mediating co-axial channel assembly in trans and thus planktonic cell aggregation into floating communities, potentially enabling intercellular communication. In some pathogenicE. coli strains, porin heterotrimers are used as receptors in contact-dependent bacterial growth inhibition exerted by toxins as CdiA, implicating the extracellular loops L4 and L5. Therefore, engineering-tailored amyloidogenic loops into a porin of choice might be an attainable goal.

In this article, we report the conversion of the OmpF porin in the OM ofE. coli into a scavenger of a model amyloid peptide, gaining function as a synthetic amylo-adhesin. More specifically, grafting an amyloidogenic hydrophobic peptide sequence into the fifth extracellular loop in OmpF (L5) enables its homotypic recognition, either by harvesting the peptide on the bacterial OM or by enticing bacteria to attach to a surface functionalized with a protein displaying that peptide. These findings constitute a proof of concept of the potential that engineered OM bacterial porins have as probes for tagging extracellular amyloidics in their way toward bioremediation.

## RESULTS AND DISCUSSION

Expression of OmpF Variants with an Amyloidogenic Stretch Grafted into Extracellular Loops. TheE. coli OM porin OmpF (an antiparallel 16-stranded β-barrel with an elliptical section and 3.2 Å radius at the narrowest point through its path) was the scaffold of choice for inserting an amyloidogenic sequence due to its high copy number (ca. 20,000 molecules per cell), clustered as trimers in the membrane plane, which assures high density and valence of potential binding sites, its non-essentiality, due to its partial functional redundancy with other related porins, such as OmpC, that allows for obtaining null strains; and its known three-dimensional structure which, combined with numerous biophysical studies, has unveiled its function as an electro-selective import channel. To avoid any interference between the host native porin and the engineered OmpF variants, we first built a ΔompF null mutant in theE. coli MDS42 chassis. This is a derivative of the common MG1655 K-12 strain, with a reduced genome including each an amyloidogenic hydrophobic peptide on the bacterial OM or by enticing bacteria to attach to a surface functionalized with a protein displaying that peptide. These findings constitute a proof of concept of the potential that engineered OM bacterial porins have as probes for tagging extracellular amyloidics in their way toward bioremediation.

For the engineering of the external loops in OmpF, theompF gene was deleted in MDS42 by replacement with a linear kanamycin-resistance cassette including genomic sequences flanking ompF (Figure S1a), using λRed recombination (Figure S1b). Gene replacement in the resulting MDS42 ΔompF strain was then confirmed by PCR (Figure S1c).

For the engineering of the external loops in OmpF, theompF gene was first synthesized in vitro, including a hexa-histidine tag between the N-terminal signal sequence, which is cleaved during Sec-dependent export through the inner membrane, and the start of the sequence coding for the matured OmpF (Figure S2). This gene was cloned into a plasmid vector including the low-copy number replicon RK2, an IPTG-inducible P lac promoter, and its repressor (lacIq), thus becoming decoupled from the natural regulation of ompF expression, through OmpR and MucF, in response to variations in the environmental osmotic conditions. This parental ompF construct was used as the platform to explore the insertion of a DNA sequence encoding the amyloidogenic hydrophobic stretch from the model bacterial prion-like protein RepA-WH1 into the sequences coding for the eight extracellular loops in the β-barrel porin. In our design, the first and the last (the eighth) of the extracellular loops in OmpF were discarded.
because such hydrophobic insertion could interfere with the correct BAM-mediated assembly of the porin, which involves first the binding to the C-terminal strand and last the closure of the barrel by its antiparallel antiparallel bonding with the N-terminal strand. The second loop was dismissed because it bridges contiguous subunits for the assembly of the porin as functional trimers. The third loop was not altered because it bends inward the porin barrel, to constrain the transit of ligands across the channel, while the fourth was untouched since it contributes to hold the bent conformation of L3. Finally, the seventh loop was not modified either because it has the shortest extension (just a three-residue tight turn). Thus, the fifth (L5) and sixth (L6) loops were chosen to be independently mutated by removing their three central (mainly polar) residues and substituting them by a nine-amino acid insertion (hydrophobic and amyloidogenic) from RepA-WH1 (Figure 1b). This stretch had been extensively characterized as a “chameleon” sequence, that is, an active α-helical segment keen to adopt a β-strand conformation upon self-assembling as an amyloid, either when isolated or within the full-length protein or if artificially arranged in tandem repeats. The wild-type Cys29 residue was mutated to Ala because, while it is not expected to alter the aggregation propensity of the sequence, it should avoid the possible oxidation of the native residue to cystine once exposed to extracellular medium, which is known to interfere with, for example, amyloid assembly of CsgA. To check that the engineered loops including the grafted amyloidogenic stretch would be exposed and accessible, the structures of the mutant porins were modeled on those of the wild-type OmpF (Figure 1c).

The expression of the porins (Figure 2a) with their L5 or L6 loops grafted with the amyloidogenic sequence and of the parental OmpF was first assessed through biochemical fractionation. This included the analysis of the cytosolic proteins present in the soluble cell lysate, the membrane proteins solubilized upon extraction of cell remnants with detergents (Triton X-100 and SB12), and the final insoluble materials, including both the OM and intracellular aggregates (Figure 2b). OmpF was identified, by western blotting with an anti-His tag antibody, as distributed between the detergent-soluble and -insoluble fractions. The antibody also highlighted the integrity of the distinct OmpF variants, with minor proteolytic degradation apparent as bands with higher.
electrophoretic mobility. The porin fraction that appears proteolyzed, evident even for OmpF-WT although it is more conspicuous for OmpF-L5 and especially for OmpF-L6, would correspond to protein molecules oversaturating the BAM machinery plus aggregates not competent for insertion at the OM, thus tagged for degradation. Immuno-fluorescence confocal microscopy of the cells, permeabilized to make accessible to the antibody the His-tag placed toward the periplasm, located both the OmpF-WT control and most of the OmpF-L5 as clusters distributed along the bacterial

Figure 2. Engineered variants of OmpF are expressed in the OM of an *E. coli* ΔompF strain. (a) SDS-PAGE time course analysis of whole bacterial cells expressing H₆-tagged OmpF porin (arrows), either the WT or variants with the amyloidogenic stretch inserted in the external loops L5 or L6. (b) Biochemical fractionation of the expressed OmpF variants (arrows). SDS-PAGE (top panel) displayed above the western blots of the same samples incubated with anti-His tag antibodies. The fraction soluble with non-ionic (Triton X-100) and zwitterionic (SB12) mild detergents probably corresponds to the porins as inserted in the OM. (c) Confocal microscopy sections of *E. coli* cells induced for 2h and incubated with primary anti-His tag and secondary Alexa-488 antibodies (green). OmpF is located at the bacterial envelope, with a homogenous, clustered distribution for the WT and the L5 mutant, while forming polydisperse aggregates for L6.
envelope, while OmpF-L6 exhibited a more heterogeneous distribution with most of the signal found as cytosolic aggregates (Figure 2c). These results are compatible with the insertion of OmpF-WT and OmpF-L5 into the OM.

Grafting of amyloid stretches in porins can interfere with their correct insertion in the OM, that is, by promoting cytoplasmic aggregation as shown here for a fraction of the OmpF-L6 mutant, with the likely outcome of triggering a protective response against envelope stress.71 A recent study on the BamA-promoted insertion of BamA itself illustrates that deletion of each of its eight extracellular loops can lead to the stall of porin folding, enhancing BamA differential suszepti-

Figure 3. Targeting OmpF at the E. coli OM with an amyloidogenic peptide. (a) Epifluorescence microscopy reveals that the amyloidogenic stretch in the prion-like protein RepA-WH1, terminally labeled with rhodamine (amyl_Rh peptide, 1.4 μM) (Figure S5a), marks (orange) bacterial cells that express the porin with the same sequence grafted within an external loop (+OmpF-L5) but not control bacteria in which no porin was expressed (ΔOmpF) or those exposed the wild-type porin (+OmpF) and, to a less extent, cells expressing the porin with a different engineered external loop (+OmpF-L6). A helical-prone soluble peptide from a different region in the same protein (alpha1_Rh, 1.4 μM; Figure S5a) does not bind to any of the recombinant bacteria. Left-hand panels are superpositions of DIC and DAPI staining and rhodamine emission; right-hand panels show the isolated rhodamine (TRITC) channel. White frames are zoomed-in (3×) views. (b) Quantitative analysis of the bacterial cells targeted by the amyl_Rh peptide in (a). Bars show the average percentile values (orange, labeled cells; gray, unlabeled), and whiskers show the standard deviations, from three independent experiments. The total number of cells of each type counted is indicated. One-way ANOVA was performed, including Tukey’s test for multiple comparisons within a 95% confidence interval: ****, *p* < 0.0001; ***, *p* < 0.001. Statistical analysis was done with GraphPad Prism v.6. (c) Confocal microscopy section of OmpF-L5 cells titrated with the amyl_Rh peptide. The DAPI (DNA, blue) and rhodamine (bound peptide, orange) channels are shown together with their superposition.
bility to the periplasmic protease DegP, with increasing sensitivity to proteolysis from L4 to L8. Nevertheless, as illustrated here with OmpF-L5, a majority of which managed to be inserted at the OM (Figure 2b,c), grafting a foreign amyloidogenic sequence into a loop can be compatible with the export of functional porins to the OM. In this sense, the expression of the distinct OmpF variants in bacteria cultured in the export of functional porins to the OM. In some cases, the expression of either the OmpF-WT allele or the amyloidogenic insertions, especially L5, did result in some delay in achieving the exponential phase, this did not substantially alter the bacterial growth rates (Figure S3), endorsing them as functional porins.

As observed for the *P. stuartii* porins Omp-Pst1/2, an indication for the exposure of loops with amyloidogenic potential is the tendency of bacterial cells to aggregate as clumps. Indeed, this was the case for *E. coli* grown on glass slides upon expression of OmpF-L5, to a larger extent than for those bacteria expressing OmpF-L6, the WT porin, or the non-complemented parental null strain: their distinct ability to form biofilms, as evaluated through crystal violet staining (Figure S4a), and cell aggregates visible under the microscope (Figure S4b), both correlated with the expression of OmpF-L5. Although these studies did not allow us to unambiguously determine that the engineered OmpF-WT and OmpF-L5 were correctly inserted at the OM, this was further assessed by testing the ability of these cells and thus of the engineered porin extracellular loops to bind a homotypic amyloid peptide target.

**Binding of an Amyloidogenic Peptide to the Outer Membrane is Dependent on Having the Same Sequence Grafted into the L5 Loop in OmpF.** The extracellular accessibility of the loop-grafted OmpF porins and their ability to bind to the homotypic amyloidogenic sequence were challenged by incubation with two different peptides. The first one (RepA-WH1_alpha1) was a control comprising a segment close to the RepA N-terminus that, according to recent NMR studies, is highly soluble and *α*-helical albeit with a dynamic propensity to unfold, whereas the second peptide (RepA-WH1_amyl) includes the same amyloidogenic stretch grafted into the OmpF loops but also its two natural flanking charged residues within RepA (Figure S5a). Both peptides were designed with the same number of amino acid residues (17), including acetylated and amidated N- and C-termini, respectively, to mimic an internal protein chain context, and also an initial Tyr (for quantitation by UV light absorption) plus a Gly2 linker and a final Gly2-Lys tail. The ε-NH2 group in the C-terminal Lys is the only free amino group in both peptides and thus was used for the covalent attachment of a fluorescein rhodamine probe through N-hydroxysuccinimidyl (NHS) ester chemistry. Upon purification (Figure S5b) and mass spectrometry characterization (Figure S5c) of the fluorescent-labeled peptides, these were probed by incubation with bacterial cells expressing (or not) the distinct OmpF porin variants (Figure 3).

Epifluorescence microscopy revealed that the rhodamine-tagged amyloidogenic peptide efficiently labeled bacteria expressing the porin with the same homotypic sequence grafted into its fifth extracellular loop (OmpF-L5; 56% of the cells, when titrated with 1.4 μM probe, but down to 0.25 μM was tested as positive in binding) and to a much lesser extent (5%) those cells expressing OmpF-L6 (Figure 3a,b). However, nearly no signal from this peptide was detected in the absence of complementation with the engineered porins (ΔompF) or when complemented through the expression of OmpF-WT (Figure 3a,b). Under the same incubation conditions, the control non-amyloidogenic peptide did not label any cell (Figure 3a). Confocal microscopy confirmed that the amyloidogenic peptide decorated the surface of bacteria that expressed OmpF-L5, with a preference for the cell poles (Figure 3c). Regarding the binding affinities of the engineered bacterial cells for the labeled amyloidogenic peptide, albeit the number of molecules of OmpF per cell is difficult to assess, yet considering a bacterium as the modular functional unit, achieving ca. 50% of cell binding with about 1 μM peptide probe provides a first, rough glimpse for the affinity of their interaction. A rigorous appraisal of the binding affinity of the OmpF-L5 to the amyloidogenic peptide will require assays in which the porin trimers were reconstituted in model membranes with a defined stoichiometry.

To get insights into the biochemical nature of the interaction between the amyloidogenic stretches in OmpF-L5 and the probe RepA-WH1_amyl, binding of this peptide was competed with epigallocatechin-3-gallate (EGCG) or resveratrol, two natural polyphenolic molecules known to inhibit amyloid aggregation, and at the same concentration that was reported to interfere with RepA-WH1 amyloidogenesis both in *vitro* and *in vivo*. Epifluorescence microscopy revealed that the polyphenols efficiently abolished labeling of the bacterial cells by the fluorescent peptide (Figure 4), providing a strong indication for the amyloid nature of the homotypic interaction between the grafted loop in OmpF and its targeted sequence.
Figure 5. Monitoring the adhesion of *E. coli* cells displaying the engineered OmpF porin to surfaces functionalized with a prion-like protein including a homotypic amyloidogenic stretch. (a) Schematic overview of the experimental setting (not drawn to scale). Incubation chambers were casted on NHS-activated slides, coated with an anti-fouling matrix to reduce unspecific attachment of bacteria. NHS chemistry enabled the covalent immobilization of dimers RepA-WH1(A31V)-mCherry (cyan-red), which includes the same amyloidogenic sequence (orange) grafted in OmpF-L5 or mCherry (red) as a control. Just two molecule orientations among the various possible ones are depicted. Upon quenching the excess of unreacted NHS groups with Tris (pink), cells were inoculated at the chambers in buffers of either acidic or basic pH and then extensively washed with the same buffer solutions. Bacteria were labeled with DAPI (blue) during fixation with PFA before observation. (b) Epifluorescence microscopy of the slides sketched in (a) after incubation either with bacteria displaying OmpF-WT or the grafted OmpF-L5. Bacteria expressing OmpF-L5, but not those bearing OmpF-WT, preferentially attached to the slides homogeneously coated with RepA-WH1(A31V)-mCherry (at pH 8.0) and, through a cell pole (arrows), to surfaces in which the amyloidogenic protein assembled as fibers (at pH 5.5), rather than to the slides which displayed mCherry at either of the two pHs. (c) Quantitation of the bacteria attached in 15 distinct representative fields (80.6 × 61.4 μm each) from two independent repeats of the experiment. One-way ANOVA analysis, with Tukey’s test for multiple comparisons within a 95% confidence interval, indicated that the differences observed for any pair of groups containing OmpF-L5 bound to WH1(A31V)-mCherry at both pH values are statistically significant with *p* < 0.0001. Analysis performed with GraphPad Prism v.6.
Bacteria Displaying the Grafted OmpF-L5 Target an Amyloidogenic Protein Immobilized on a Functionalized Surface. The ability of the engineered porin displayed on the bacterial OM to recognize the amyloidogenic sequence was also explored with the target attached to functionalized surfaces. Initial attempts were carried out with the RepA-WH1_amyl peptide (Figure S5a) immobilized through disuccinimidyl glutarate cross-linking to poly-Lys-coated glass slides, but they failed due to a high background (unspecific) deposition of bacteria whatsoever the variant expressed. A second round was performed with the peptide linked to glass slides coated with an anti-fouling, low cell adhesion polymer functionalized with NHS groups, but nearly no bacteria attached, probably due to poor accessibility of the peptide within the matrix to the extracellular loops in the porin. Thus, a third strategy was successfully tested on the same NHS-activated slides (Figure 5a), but relying on the immobilization of RepA-WH1(A31V)-mCherry, the protein including the amyloid peptide fused to a red fluorescent construct, extensively characterized on its ability to assemble cytotoxic intracellular, prion-like amyloid aggregates. For this otherwise soluble protein, amyloidogenesis is triggered by the binding of a number of ligands, while recent evidence shows that at acidic pH, the conformational flexibility of both the α1 and α5 helices and the amyloidogenic loop is enhanced, which might promote aggregation by decreasing the stability of the fold. As a control, the isolated mCherry tag was similarly fixed to the same kind of the surface. Incubations of the protein-functionalized slides with bacteria expressing either OmpF-L5 or the control OmpF-WT were carried out (Figure 5a), either under the same conditions that resulted in binding of the amyloidogenic RepA-WH1_amyl peptide to the bacterial envelope (pH 8.0; Figure 3) or at pH 5.5.

Under both buffer conditions, stable deposition of bacteria on the protein-coated surface was only appreciated for those cells expressing OmpF-L5 and when the protein targeted was RepA-WH1(A31V)-mCherry, with higher numbers of cells at pH 8.0 (Figure 5b,c). Interestingly, while red fluorescence was homogenously distributed on the surface for RepA-WH1(A31V)-mCherry at the basic pH, under the acidic conditions, the engineered OmpF-L5. The observed polar attachment to an immobilized target protein by the bacterial cell rods expressing this porin is compatible with such a scenario. On the contrary, on surfaces functionalized with the control mCherry protein, the coating was homogenous (so no fibers were assembled) at any pH of incubation, and cell deposition was much reduced, disregarding whether bacteria expressed the OmpF-L5 or WT (Figure 5b,c).

L4 and L5 loops in OmpF are those exhibiting the highest sequence variability, for example, in other Gram-negative bacteria such as Versinia spp., suggesting that they might be adapted to distinct ecological interactors, including bacteriophages. However, such sequence evolvability also indicates that porin loops are subjected to selective pressure, which raises a note of caution on the likeliness of the appearance of suppressor mutations, either in the loops or elsewhere, if binding to the amyloidogenic sequences would come at a cost to the fitness of the engineered bacteria.

The amyloidogenic stretch grafted into OmpF, in its natural context within the plasmid replication protein RepA, contributes to the formation by its WH1 domain of a functional amyloid bridge that zips up together plasmid molecules for inhibiting untimely replication firing. However, in the isolated RepA-WH1 domain and upon conformational selection of an oligomeric species, the amyloidogenic stretch becomes a major determinant of cytotoxicity. As a tandemly repeated engineered peptide module, the same sequence elicits the aggregation of chimeric translation termination factors, both in yeast and in bacteria, enabling synthetic stop codon read-through translation. By insertion within the OmpF loop L5, this versatile modular sequence has been now repurposed for yet another function, tagging bacterial cells for molecular self-recognition of amyloids, which poses advantages over the usage of deliverable proteins as biosensors of prions and eventually for their bioremediation. Either with origin in human microbiota or in natural environments, protein amyloids constitute a threat for human and animal health due to their ability to cross-seed amyloid diseases. Biosensing the presence of such amyloidogenic proteins is necessarily the first step toward their clearance. The general validation of the bacterial device presented here will need inserting amyloidogenic stretches from other prion-like proteins, in particular those present in natural environments, into the porin loops.

### Experimental Section

#### Genomic Deletion of ompF

On the reduced genome E. coli strain MDS42, deletion of the ompF gene was carried out through λRed recombination. Bacteria were first transformed with the pKD46 plasmid (pSC101 replicon-repA<sup>β</sup>, Ap<sup>R</sup>, P<sub>lac</sub>, I-Sce, ParαBAD, λred/eco-bet-gam, and arac)#79 at 30 °C. Then, competent cells previously grown for 1 h in the presence of 0.15% arabinose for λRed induction were electroporated with a linear PCR fragment including the Km<sup>R</sup> aphII<sup>β</sup> gene from pWRG717, amplified with oligonucleotides including tails complementary to the genomic sequences flanking the ompF ORF (Figure S1a). Transformants were plated on LB agar supplemented with 50 μg·mL<sup>−1</sup> kanamycin and incubated at 37 °C for promoting the loss of pKD46. Positive clones replacing ompF with the Km<sup>R</sup> cassette (Figure S1b) were identified by colony PCR (Figure S1c).

#### Construction of Loop-Engineered OmpF Variants

For complementation of MDS42 ΔompF, a synthetic cassette (Figure S2) comprising a Ptac promoter, a translation initiation sequence (RBS), and ompF (WT) with its encoded signal sequence followed by a hexahistidine tag was generated by ATG/biosynthetics GmbH (https://www.atg-biosynthetics.com). This cassette was then cloned into the low-copy number vector pRK2-WH1-mCherry lacI<sup>β</sup>, in which the Ptac-WH1-mCherry SpeI-SphI fragment was replaced with the equivalent synthetic Ptac-ompF (Figure 1a). As a matter of fact, successful cloning required that the recipient bacterial cells carried pRL-lact, a helper plasmid providing extra levels of the Lac<sup>β</sup> repressor, that was then readily eliminated by retransforming the plasmid mix into naïve MDS42 ΔompF cells and then selecting for clones Ap<sup>R</sup> and Cm<sup>R</sup>.

Engineering the amyloidogenic stretch in RepA-WH1(A31V) into ompF loops LS and L6 was carried out by Pfu
DNAPol PCR extension of complementary mutagenic oligonucleotides (Figure 1b) on the pRK2-ompF(WT) lacI plasmid template (see above) plus removal of the parental plasmid strands by DpnI digestion. The oligonucleotides included an NheI site (GCTAGC) within the region encoding the amyloidogenic grafted to enable a first screening of the resulting clones by restriction analysis. Mutant constructs were checked by DNA sequencing (Macrogen; https://dna.macrogen.com).

OmpF Expression and Localization within Bacterial Cells. Expression of the OmpF variants was carried out in the MDS42 ΔompF strain from overnight inocula in LB medium, supplemented with 100 μg·mL−1 ampicillin, at 30 °C. When OD₆₀₀ nm reached 0.3, IPTG was added to 0.1 mM and grown for up to 4 h. Cells were harvested, and total protein contents were analyzed by (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) SDS-PAGE (12.5% PA) plus western blotting using a mouse monoclonal antibody recognizing the N-terminal His6-tag (1:20,000; Sigma) and a secondary HRP-conjugated goat anti-mouse antibody (1:20,000; Sigma), as described.81

The formation of bacterial cell aggregates on the expression of the distinct porin constructs was explored by displaying 300 μL aliquots of the induced (or not) bacterial cultures in Lab-Tek (Nunc) 8-well borosilicate lid-covered chambers and then leaving them to grow resting for 24 h at 30 °C. The culture supernatant was then carefully removed, rinsed twice with 200 μL of PBS, and finally fixed, for 30 min at room temperature (RT), with 100 μL of 2% paraformaldehyde (PFA) in PBS, supplemented with 1 μL of 0.5 mg·mL−1 4′,6-diamidino-2-phenylindole (DAPI; Merck) in H₂O. Finally, chambers were washed with 200 μL of PBS, disassembled, and mounted (Fluoromount-G; SouthernBiotech) on glass slides before observation under the microscope. In parallel, five replicas of the chambers, after removing the culture medium, were stained with crystal violet (0.1% in water, for 15 min) for biofilm detection. The excess stain was then removed, wells were washed there times with 300 μL of Milli-Q water by pipetting, and the remaining, well-attached crystal violet was left to dry for up to 10 min. The lysate was then centrifuged at 29,000 g (Fiberlite F13-14 rotor; Sorvall) for 30 min at 4 °C. Pellets were resuspended in 0.1 M KCl, 50 mM Tris-HCl pH 7.4, 2 mM DTT, 1 mM EDTA, 1% Triton X-100, 0.5% SB12 (N-dodecyl-N,N-dimethylammonio-3-propane sulfonate; Serva), and 10% glycerol, and sonication was carried out as mentioned above, followed by centrifugation under the same conditions.

Targeting of the engineered porins to the OM was assessed by immunofluorescence microscopy. Upon porin expression (see above), cells from 1 mL cultures were harvested, washed with cold 0.9% NaCl, and incubated with 8 μg·mL−1 lysozyme (Sigma) for 5 min at RT, to make the periplasm-oriented His6-tag accessible to the antibodies. Cells were gently washed in cold PBS before labeling with a monoclonal anti-His antibody (1:500; Sigma) and then with an anti-mouse Alexa 488-conjugated antibody (1:500; Thermo-Fisher), as described.81

Bacteria were then observed using confocal laser microscopy (see below).

Peptide Labeling and Solution Binding Assays to Engineered Bacterial Cells. Peptides RepA-WH1_amyl and RepA-WH1_alpha1 (Figure S5a) were synthesized (AAPTec Focus XC; solid-phase Fmoc) at the Protein Chemistry Facility (CIB-CSIC). Six milligrams of each peptide was dissolved in 100 μL of 1,1,3,3,3-hexafluoro-2-propanol (HFIP; Fluka) and incubated for 1 h at 40 °C and 1400 rpm agitation in an Eppendorf Thermomixer, producing an aggregate-free solution. Peptides were then dried out at RT in a SpeedVac (Thermo) and then resuspended in dimethyl sulfoxide (DMSO; Merck) to a concentration of 4.5 mM (as determined by A₅₈₀ nm of the Tyr residue). For labeling the C-terminal Lys in the peptides with 5/6-carboxytetramethylrhodamine succinimidyl ester (NHS-rhodamine; Thermo), 500 μL reaction aliquots were casted including 300 μM peptide in 10% DMSO, 0.1 M Hepes pH 8, and 1.3 mM NHS-rhodamine and left to react at 25 °C with agitation (300 rpm) for 16 h in the dark. While the alpha1 peptide remained mostly soluble, the amyl peptide precipitated and was harvested by centrifugation (16,500×g, 5 min). The amyl peptide was solubilized in 10% HFIP and 0.5% trifluoroacetic acid (TFA), while the alpha1 was supplemented with these reagents. Labeled peptides were separated from the unreacted NHS-rhodamine by fast protein liquid chromatography in a 1 mL octyl-Sepharose hydrophobic interaction chromatography column (GE Healthcare) wrapped with aluminum foil, developing a 10 mL gradient between 0.1% TFA in H₂O and 75% acetonitrile, 0.1% TFA, and H₂O (Figure S5b). Peak fractions were evaporated (SpeedVac), resuspended in 100 μL of DMSO, and stored at −20 °C. The concentration of the labeled peptides was calculated by measuring the A₅₅₂ nm (ε₅₅₂ = 80,000 M⁻¹·cm⁻¹). The mass of the peptide adducts was determined by MALDI-TOF/TOF mass spectrometry at the Proteomics Facility (CNB-CSIC) (Figure S5c).

For assessing peptide binding to bacteria, IPTG-induced (or un-induced) cells were harvested after 2 h at 30 °C (see above), washed twice with 1 mL of cold 15 mM NaCl and 20 mM Hepes pH 8, and resuspended in the same buffer to OD₆₅₀ nm = 1. In 2 mL Eppendorf tubes, 200 μL of the bacterial suspension (ca. 1.6×10⁸ cells) was incubated with distinct concentrations (0.25–11 μM) of the rhodamine-labeled peptides in 1 mL final volume of 15 mM NaCl, 20 mM Hepes pH 8, and 9% DMSO. Binding of the amyloidogenic peptide to bacteria expressing OmpF-L5 was found to be sensitive to the salt concentration, standing up to 150 mM NaCl, while DMSO was required to keep the peptide in solution, being compatible with the interaction in the interval between 5 and 10%. Incubation proceeded during 16 h at 4 °C in a rotating wheel. Then, bacteria were sedimented and washed twice by pipetting with 1 mL of cold 15 mM NaCl and 20 mM Hepes pH 8. Samples were fixed with PFA supplemented with DAPI (see above), and 10 μL drops of the final cell suspension were spread on glass slides and mounted for microscopy. To test polyphenol interference with the binding of the amyloidogenic peptide to the cell surface,
EGCG or resveratrol was included at the incubation step at 250 \( \mu \text{M} \) (from 25 mM stocks in DMSO).

**Bacterial Cell Binding to Immobilized Fluorescent Protein Targets.** SecureSeal adhesive chambers (HybriWell) were mounted on 3D-NHS-functionalized slides (polyAn GmbH) coated with an anti-fouling, low-cell adhesion matrix. The protein which the amyloid peptide comes from, His\(_{6}\)-RepA-WH1(A31V)-mCherry, and a His\(_{6}\)-mCherry control were puriﬁed as reported and then placed with a thin tip in the chambers at a ﬁnal concentration of 20 \( \mu \text{M} \) in 100 \( \mu \text{L} \) of 154 mM NaCl, 50 mM Hepes pH 8, and 0.1 mM EDTA. Protein immobilization proceeded at RT, with orbital shaking (100 rpm) in the dark for 16 h. The protein solution was then removed, and any remaining unreacted NHS groups were quenched with 1 M Tris pH 8 for 2 h. Chambers were then washed twice for 30 min with 154 mM NaCl and 20 mM Hepes pH 8 (or 20 mM Mes pH 5.5). Bacterial cells, processed as indicated above for their incubation with the labeled peptides but in buffers either at pH 8.0 or 5.5 and at an OD\(_{600\text{ nm}}\) of 0.1 (100 \( \mu \text{L}, \text{ca.} 8 \times 10^6 \text{ cells} \)), were then placed into the chambers and incubated in a fridge for 16 h. Afterward, bacterial cell suspensions were drained, the adhesive incubation chambers were removed, and the slides were washed three times (for 20 min each at RT) with 4 mL of their corresponding pH 8.0 or 5.5 buffer, before PFA ﬁxation, DAPI staining, and mounting for microscopy (see above).

**Microscopy.** Epifluorescence microscopy was performed with a Nikon Eclipse 90i equipped with CFI PLAN APO VC 100x/1.40 NA and 60x/1.40 NA oil immersion objectives and a Hamamatsu ORCA-R\(_2\) CCD camera. For the indicated ﬂuorophores, the ﬁlters (Semrock) were rhodamine (excitation: 543/22, emission: 593/40; 1 s exposure) and DAPI (ex: 360/40, em: 470/40; 0.4 s exp). Differential interference contrast (DIC) images were also captured. Confocal laser microscopy was carried out in a multispectral Leica TCS SP8X, equipped with a HC PL APO CS2 100x/1.40 NA oil immersion objectives and 63x/1.40 NA oil immersion objectives and a Hamamatsu ORCA-R\(_2\) CCD camera. For the indicated fluorophores, the filters (Semrock) were rhodamine (excitation: 543/22, emission: 593/40; 1 s exposure) and DAPI (ex: 360/40, em: 470/40; 0.4 s exp). Differential interference contrast (DIC) images were also captured. Confocal optical sections were set to 0.548 nm.

**CONCLUSIONS**

In summary, this study shows that inserting an amyloid-prone sequence into the OM porin OmpF enables the bacteria to become attached to the same sequence, either as a free peptide or forming part of a prion-like protein. This illustrates the potential of homotypic interactions in amyloid assembly as a means to implement specific molecular recognition between bacteria and extracellular proteins. These results open a way to the bioremediation by bacteria of potentially harmful amyloids present in natural environments.

**ASSOCIATED CONTENT**

**Supporting Information**
The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssynbio.1c00347.

Construction of a \( \Delta \text{ompF} \) mutant for expression of engineered OmpF variants, sequence of the synthetic \( \Delta \text{ompF} \) gene, growth curves of bacteria expressing the distinct OmpF, formation of biofilms and cellular aggregates by bacteria exposing the OmpF variants, and characterization of the rhodamine-labeled peptides (PDF).

**AUTHOR INFORMATION**

*Corresponding Author*

**Rafael Giraldo** — Department of Microbial Biotechnology, National Centre for Biotechnology (CSIC), 28049 Madrid, Spain; Department of Cellular and Molecular Biology, Centro de Investigaciones Biológicas (CSIC), 28040 Madrid, Spain; orcid.org/0000-0002-5358-7488; Email: rgiraldo@cnb.csic.es

**Authors**

**Sol Vendrell-Fernández** — Department of Microbial Biotechnology, National Centre for Biotechnology (CSIC), 28049 Madrid, Spain; Present Address: Genetics of Biofilms Unit, Institut Pasteur, 75015 Paris, France

**Paloma Lozano-Picazo** — Department of Cellular and Molecular Biology, Centro de Investigaciones Biológicas (CSIC), 28040 Madrid, Spain; Present Address: Centre for Biomedical Technology, Technical University of Madrid, 28223 Pozuelo de Alarcón—Madrid, Spain.

**Paula Cuadros-Sánchez** — Department of Microbial Biotechnology, National Centre for Biotechnology (CSIC), 28049 Madrid, Spain; Present Address: Hospital Emilio Quintero Cañizares, 546531 Ocaña, Colombia

**María M. Tejeiro-Ojeda** — Department of Cellular and Molecular Biology, Centro de Investigaciones Biológicas (CSIC), 28040 Madrid, Spain; Present Address: Dept. of Molecular Neurobiology, Cajal Institute (CSIC), 28002 Madrid, Spain.

Complete contact information is available at: https://pubs.acs.org/10.1021/acssynbio.1c00347

**Author Contributions**

S.V.-F. and P.L.-P contributed equally to this work. S.V.-F. carried out the work with peptides and on their binding to cells and analyzed data. P.L.-P. constructed the \( \Delta \text{ompF} \) bacterial strain and expression plasmids and developed, together with P.C.-S., the protocol for biochemical analysis of membranes. M.M.T.-O. built the L5 and L6 \( \Delta \text{ompF} \) mutants. R.G. conceived the project, performed experiments, analyzed data, and wrote the article with contributions from all the authors.

**Notes**

The authors declare no competing financial interest.

**ACKNOWLEDGMENTS**

We are indebted to Sylvia Gutiérrez-Erlandsson and Ana Oña-Blanco (CNB-CSIC) for their help with confocal microscopy, to Javier Varela (CIB-CSIC) for peptide synthesis, to Sergio Ciordia (CNB-CSIC) for mass spectrometry of the peptides, and to Cristina Fernández for the mCherry-tagged proteins. This work has been financed with grant RTI2018-094549-B-I00 from the Spanish MCIN/AEI (10.13039/501100011033 and FEDER “A way to make Europe”) to R.G. P.L.-P. was a recipient of a EU Young Employment Initiative Grant (YEI 2015-CS_MAD_CIB_091). P.C.-S. was a recipient of a grant from Fundación Carolina 2018–2019.
REFERENCES


