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# Bacterial Species in Engineered Living Materials: Strategies and Future Directions

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## ABSTRACT

In recent years, there has been a notable increase interest in engineered living materials (ELMs) owing to their considerable potential. One key area of research within this field is the utilisation of various species of bacteria to create innovative living materials. In order to accelerate the advancement of bacterial-based living materials, a systematic summary of bacterial species and their design strategies is essential. Yet, up to this point, no applicable reviews have been documented. This review offers a concise introduction to living materials derived from bacteria, delves into the strategies and applications of each bacterial species in this realm, and provides perspectives and future outlooks in this field.

## 1 | Introduction

The development of engineered living materials (ELMs) is driven by the need for new materials capable of performing complex functions and adapting to dynamic conditions. ELMs are typically composed of matrices that provide structural support and a nurturing environment for living cells to grow and function. These advanced materials are engineered to interact with their surroundings, sense and respond to various stimuli, and execute specific tasks. One of the earliest documented examples of such living functional materials was developed in 2012 by Wendy J. Stark's group, who encapsulated *Penicillium chrysogenum* cells within an agar matrix to achieve sustained release of penicillin (Gerber et al. 2012). This work demonstrated the potential of integrating living organisms into materials for controlled substance delivery. Building on this foundation, Timothy K. Lu's team in 2014 made significant progress by applying synthetic biology techniques to engineer *E. coli* biofilms (Chen et al. 2014). Their research resulted in living materials that were not only responsive and tunable but also exhibited multiscale patterning capabilities. These advancements highlight critical milestone in ELMs' evolution, emphasising the role of genetic modification in enabling cells to perform specialised functions. The ubiquitous presence of bacteria in diverse natural environments underscores the suitability of bacterial biofilms as a readily available and renewable resource for ELMs creation and advancement. Biofilms are intricate communities of bacteria that adhere to surfaces and secrete an extracellular matrix composed of polysaccharides, proteins, and DNA (Eick 2021; Tursi and Tükel 2018). The building of the "house" by bacteria itself can protect bacteria from adverse environmental factors and immune responses. Furthermore, biofilms contain water channels, which help the distribution of nutrition and molecules (Wilking et al. 2013). Three-dimensional (3D) structures of biofilms in nature can become an Eden for bacteria in a bad environment, but they can be used as a platform for ELM design. Biofilm engineering for new living material refers to the harnessing of the biofilm formation process of bacteria. For instance, species of Comamonas are characterised as gram-negative and aerobic bacteria that are ubiquitous in the environment. Through the continuous expression of the YedQ protein, a biofilm of *Comamonas testosteroni* has been genetically engineered, and the engineered biofilm

has a higher degradation efficiency of 3-chloroaniline than that of the wild type (Wu et al. 2015). Curli fibre is a major component in *E. coli* biofilms, and decorating the curli fibre with an α-amylase allows the generated biofilm to maintain the activity of amylase to the substrate 4-Nitrophenyl-α-D-maltopentao side under denaturing environmental conditions (Botyanszki et al. 2015). The genus of *Komagataeibacter* forms dense bacterial cellulose (BC) sheets when cultured in static condition (Azeredo et al. 2019). When co-cultured with *Saccharomyces cerevisiae* yeast, the modified yeast strains release cellulase into the BC of *Komagataeibacter rhaeticus*, which, in turn, reduces the stiffness of the BC materials (Gilbert et al. 2021).

Over the last decade, ELMs have infiltrated numerous aspects of our daily existence, showcasing their versatility (Rivera-Tarazona et al. 2021). Within the ELM domain, classifications include archaeal, eukaryotic, bacterial, synthetic, and cross-domain ELMs (Lantada et al. 2022). Although substantial research has focused on archaeal, eukaryotic, synthetic, and cross-domain ELMs (Lantada et al. 2022; Huber et al. 2022), this review narrows its scope to exclusively cover bacterial ELMs, an area that has garnered significant interest. Bacterial ELMs can be categorised into two main types: those derived from natural biofilms and those from hybrid living materials. Hybrid living materials refer to a class of ELMs formed by encapsulating cells within an inorganic or organic matrix, thereby combining biological components with synthetic elements to create innovative functional materials (An et al. 2023). Over the past decade, significant advancements have been made in developing strategies for manipulating bacterial biofilms to fabricate biofilm-based living materials. These approaches leverage the inherent structure and functional properties of biofilms to create materials that can perform specific tasks. Meanwhile, parallel progress has been observed in the engineering of hybrid living materials, where researchers have focused on integrating live cells with non-biological matrices to produce composites with tailored functionalities. Despite these advances, the field of biofilm and hybrid material engineering is still in its infancy, presenting ample opportunities for growth and innovation. In this review, we first discuss the bacterial species used in natural biofilm-based living materials and bacterial species used in hybrid living materials and then provide perspectives on the bacterial species used in ELMs.

## 2 | Bacterial Species Used in Natural Biofilm-Based Living Materials

To date, many different living materials have been generated based on the natural biofilm formation of *E. coli*, *Bacillus subtilis*, *Komagataeibacter rhaeticus*, *Geobacter sulfurreducens*, *Corynebacterium glutamicum*, *and Caulobacter crescentus*. Figure 1 illustrates the scheme of creating diverse ELMs through natural biofilm engineering.

### 2.1 | *E. coli*

*E. coli* serves as a model organism and is widely present in laboratories around the world. Its resilience, versatility, broad metabolic capabilities, and ease of cultivation have made *E. coli* an extensively studied and well-understood bacterium. Additionally, the *E. coli* genome is relatively simple, allowing scientists to easily perform genetic modifications. While certain strains of *E. coli* can be pathogenic, the strains commonly used in laboratories are typically harmless and are considered generally regarded as safe (GRAS) microorganisms. These characteristics have made *E. coli* the most extensively utilised organism for the construction and study of ELMs. *E. coli* can form biofilm virtually everywhere, and the biofilm matrix components include proteins, cellulose, and extracellular DNA (Tursi and Tükel 2018). Compared to cellulose and extracellular DNA, proteins are highly programmable. Amyloids, a type of protein polymer, are crucial for the structural integrity of biofilms. They were originally recognised as virulence factors in *E. coli* (Olsén et al. 1989). In *Enterobacteria*, *csgBAC* and *csgDEFG* operons are responsible for the synthesis of curli amyloid fibres (Bhoite et al. 2019). csgD functions as a transcriptional regulator that promotes the transcription of the *csgBAC* operon. The proteins csgE, csgF, and csgG collectively form the secretion system responsible for transporting both csgB and csgA proteins to the bacterial outer membrane. The csgA protein, which serves as the building block for amyloid fibres, is exported by the secretion apparatus in an unfolded state. When csgA is secreted into the extracellular space, it undergoes self-assembly to form nanofibers, a process assisted by the csgB protein (Hammer et al. 2012).

Based on these findings, subsequent research in the field of ELMs gradually revolved around this system. To harness the unique characteristics of the curli system in *E. coli*, various strains have been selectively engineered or chosen for distinct applications. In 2014, researchers explored the development of strong underwater adhesion materials by utilising *E. coli* NEB C3016, a modified version of the *E. coli* BL21 strain. The study aimed to investigate how the combination of two different adhesion systems within this bacterial strain could lead to the creation of powerful adhesives designed to work effectively in water. Since the underwater attachment function of mussel foot proteins (Mfps) in *Mytilus galloprovincialis* depends on the presence of abundant 3, 4-dihydroxyphenylalanine, these researchers fused the Mfps domains to the csgA protein at the C-terminal; after expression and purification, the researchers reported that the fusion proteins exhibit strong and multifunctional underwater adhesive properties (Zhong et al. 2014). Researchers using *E. coli* DE3 (BL21) have found that adding fusion domains does not interfere with the standard β-sheet structures, but they have shown that it does impact the rate of assembly, appearance, and rigidity of the resulting fibrils (Cui et al. 2019). Therefore, the robustness of this system needs more consideration before conducting additional experiments.

In another study, researchers employed a genetically modified bacterial strain, *E. coli* PHL628-Δ*csgA*, derived from the *E. coli* K-12 MG1655. The *E. coli* PHL628 possesses an *ompR234* mutation where the original ompR regulatory protein undergoes a single amino acid substitution; specifically, leucine (L) at position 43 is replaced by an arginine (R) residue. This particular alteration results in the overexpression of curli (Prigent-Combaret et al. 2000; Vidal et al. 1998). In order to further engineer this *E. coli* strain, the native *csgA* gene was deleted from *E. coli* PHL628, resulting in *E. coli* PHL628-Δ*csgA*. This deletion allows for adverse peptides to be fused to csgA by

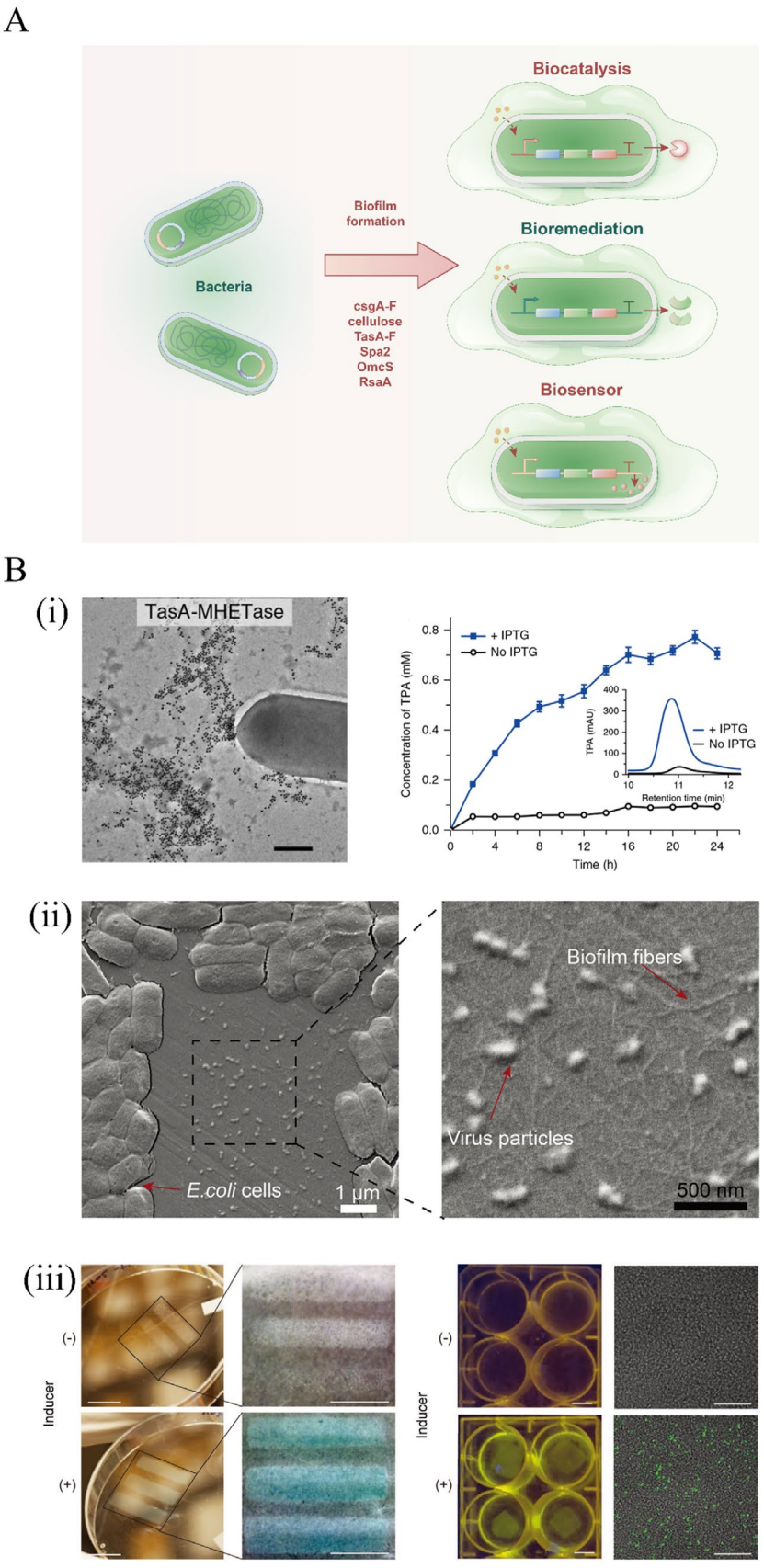


**FIGURE 1** | Legend on next page.

**FIGURE 1** | Bacterial ELMs derived from natural biofilms. (A) Schematic illustration of the strategies for creating diverse types of ELMs by leveraging natural bacterial biofilms. These ELMs are developed through: The secretion and assembly of csgA-F and TasA-F fusion amyloid proteins by *E. coli* and *B. subtilis* respectively, where "F" represents various functional protein domains or peptides; the secretion and assembly of cellulose by *K. rhaeticus*; the assembly of Spa2 fusion proteins within pili structure of *C. glutamicum*; the expression OmcS protein on the outer membrane of *G. sulfurreducens*; and the surface display of engineered RsaA proteins on *C. crescentus* cell surface. (B) Representative examples of engineered biofilms: (i) *B. subtilis* biofilm with MHETase activity. Reprinted with permission from Ref (Huang et al. 2019). Copyright2018 The Author(s), under exclusive licence to Springer Nature America Inc. (ii) *E. coli* biofilms designed to disinfect virus from river water. Reprinted with permission from Ref (Pu et al. 2020). The Authors 2020. This article is published with open access at Wiley-VCH Verlag. (iii) *E. coli* biofilms acting as biosensor. Reprinted with permission from Ref (Moser et al. 2019). Copyright2019 WILEY-VCH Verlag GmbH&Co. KGaA, Weinheim.

constructing the corresponding plasmids in vitro. After plasmid transformation, transformants with new functions can be generated. Studies have shown that a spontaneous covalent bond forms between SpyTag and SpyCatcher (Zakeri et al. 2012; Reddington and Howarth 2015). When SpyTag is combined with csgA through fusion, it is subsequently expressed and assembled by *E. coli* PHL628-Δ*csgA*, and the resulting fibres exhibit close resemblance to the native curli fibres. Upon further fusing SpyCatcher to an amylase, the immobilisation reaction was found to be robust. Additionally, the amylase-modified biofilms maintained their functionality even when subjected to diverse pH levels. This system is notable as it introduces a new, versatile approach for immobilising enzymes at specific sites (Botyanszki et al. 2015). A further extension of this study has explored the ability of the biofilm-based material to immobilise more enzymes by using the *E. coli* PHL628-Δ*csgA*. To accomplish this objective, the researchers designed and tested a series of conjugation pairs. In each instance, one part of the conjugation pair was connected to the C-terminal region of csgA, whereas the other part was attached to the N-terminal region of enzymes. They reported that the resulting material exhibited outstanding properties, including the targeted purification and immediate immobilisation of multiple enzymes straight from unrefined cell extracts (Nussbaumer et al. 2017).

In 2020, *E. coli* MG1655*PRO*Δ*csgAompR234* was utilised where the gene *csgA* had been knocked out, featuring constitutive substantial expression of both tetR and lacI proteins from a *PRO* cassette, as well as harbouring an *ompR 234* mutation. This *E. coli* strain was employed to purify virus-contaminated river water (Pu et al. 2020). Given that C5 is a well-characterised peptide with known affinity for influenza viruses, it has been demonstrated to specifically interact with the haemagglutinin membrane protein of the influenza virus (Matsubara et al. 2010). In this research, C5 was concatenated with the csgA protein, forming a fusion protein. The resulting materials effectively facilitated the direct extraction of virus particles from liquid mediums. Of note, the disinfection efficiency achieved levels undetectable by the qPCR assay's sensitivity threshold. Due to concern that the endogenous *csgB* gene might have an impact on the expression of the fusion proteins, a further deletion of the *csgB* gene in *E. coli* MG1655*PRO*Δ*csgAompR234* results in the generation of *E. coli* MG1655*PRO*Δ*csgBAompR234*, which is utilised for the production of conductive protein nanofibers. Because aromatic amino acid contents contribute to the electrical conduction of the PilA protein of *Geobacter sulfurreducens* (Reardon and Mueller 2013; Tan et al. 2016). By mimicking the motif present in the PilA protein of *Geobacter sulfurreducens*, a group of researchers has effectively incorporated conductive peptide motifs, consisting of different tyrosine and tryptophan residue combinations, onto the C-terminal region in csgA. This novel coupling has empowered the once non-conductive *E. coli* biofilm to gain conductivity (Kalyoncu et al. 2017).

Although the *E. coli* strains mentioned above have been used in several studies, there may be other elements of the two operons responsible for curli formation in the *E. coli* genome that may have some unknown effects on curli production. To address this issue, researchers have utilised an *E. coli* strain where all operons are deleted in *E. coli* PQN4 to conduct additional studies. New research has uncovered a significant increase in mercury pollution in the environment compared to previous estimates (agles-Smith et al. 2016). This highlights the necessity for novel methods to clean up contaminated areas. The *mer* operon is a commonly observed and long-standing bacterial operon present in plasmids and transposons, where the MerR protein controls the expression of the *mer* operon (Summers 1992). Once $Hg^{2+}$ binds to MerR, a structural transformation occurs, causing the liberation of the *mer* operon. Recent research has also shed light on the potential protective role of curli in bacterial defence, suggesting that it may effectively safeguard bacteria within biofilms by adsorbing heavy metal (Hidalgo et al. 2010). Substituting the *mer* operon genes with a yellow fluorescent protein gene and curli operons, mercury-inducible biosensors that can report and sequester mercury have been developed (Tay et al. 2017). In addition to the design for reporting and binding of mercury, this *E. coli* strain can also be used for abstracting various rare earth elements (REEs). This is particularly significant due to the growing need for REEs in recent decades. Lanthanide-binding tags (LBTs) are oligopeptides that exhibit strong binding for many lanthanides (Nitz et al. 2004). LBTs have been fused to the csgA protein, resulting in the construction of a fibre with selective REEs binding activity that shows a preference for lanthanides; the recovery procedure for this fibre is simple, requiring only the use of a dilute acid wash (Tay et al. 2018). Additionally, the application of *E. coli* PQN4 has been broadened into the field of bioelectronics. By strategically modifying multiple amino acid residues in the core region of the csgA protein, engineered curli fibres have been developed that exhibit significantly enhanced electrical conductivity in comparison to their wild-type counterparts. These modified curli fibres thus possess the capability for electron conduction, thereby expanding their potential applications in this cutting-edge field (Dorval Courchesne et al. 2018). Leveraging microbial engineering to fabricate materials with adverse functions has been established by these strains. Building 3D structures

in any pattern is a bottleneck in this field. A team has recently created a customisable biological substance for 3D printing of living materials. In pursuit of this objective, the researchers utilised the crosslinking of a fibrin-inspired fusion proteins to create a bioink. The process of fibrin polymerisation is partly facilitated by noncovalent interactions occurring between an alpha-chain domain located at the N-terminal of one fibrin monomer and a gamma-chain domain situated at the C-terminal of an adjacent monomer (Pratt et al. 1997). By integrating these two domains separately at the N-terminus and C-terminus of csgA, csgA-α and csgA-β fusion proteins are generated. Introducing specially engineered *E. coli* PQN4 to the ink enables the fabrication of 3D-printed living materials with specific functions, such as releasing the anticancer drug azurin and regulating their own cell growth (Duraj-Thatte et al. 2021).

*E. coli* JF1Δ*csg* is a distinct strain that has been specifically engineered for controlled expression and secretion of the csgA fusion proteins, with meticulous attention to avoiding interference from its native curli production system. In order to accomplish this, the researchers have methodically knocked out all the endogenous curli-associated genes within the *E. coli* JF1Δ*csg* genome (Moser et al. 2019). In this particular strain of *E. coli*, a light regulation system is employed to carefully manage the activation of the *csgBAC* operon via blue light. Furthermore, green light and red light are utilised to modulate the expression of csgA fusion proteins labelled with HA and His tags, respectively. The operon, encompassing the genes that govern the secretion apparatus of curli (*csgE, csgF, csgG*), is subjected to precise regulation by the IPTG inducer. The *csgD* was excluded because it encodes a regulatory protein. Thus, the *E. coli* JF1Δ*csg*, equipped with this advanced light control system, can be effectively utilised for the precise patterning and immobilisation onto various surfaces such as textiles, ceramics, and plastics, demonstrating the adaptability of this method in the fields of materials science and bioengineering. To explore additional functions, this strain has also been combined with other *E. coli* strains to form two strain systems. By combining with the *E. coli* MG1655*PRO*Δ*csgAompR234*, a living glue system with autonomous mechanical repair properties, has been developed (An et al. 2020). In this dual-strain living glue system, *E. coli* MG1655*PRO* Δ*csgAOmpR234* was used to produce csgA-Mfp3s fusion proteins and constitutively produced acyl-homo-serine lactone (AHL), and the *E. coli* JF1Δ*csg* was used to produce tyrosinase, which can enhance the adhesion ability of bacteria biofilm. Upon the addition of horse blood to induce the system, the *E. coli* MG1655*PRO* Δ*csgAOmpR234* strain is triggered to produce csgA-Mfp3s fusion proteins and continuously generates AHL. In response to the AHL signal, *E. coli* JF1Δ*csg* strain is activated to synthesise tyrosinase. Through the cooperation of these two strains, the small holes in the microfluidic device can be repaired when horse blood is pumped into the system. Although both cellulose and extracellular DNA constitute crucial elements of the biofilm matrix in *E. coli*, there have been no reports of living materials based on these two components to date. It is noteworthy that cell-surface lipopolysaccharides have been effectively restructured using metabolic pathway engineering. The inherent GDP-fucose de novo pathway in *E. coli* has been substituted with a GDP-fucose salvage pathway originating from *Bacteroides fragilis*. Many different fucose analogs can be added into the polysaccharides to decorate the cell surface (Yi et al. 2009). This strategy will be helpful to build living materials based on polysaccharides with more functions in the future.

### 2.2 | *B. subtilis*

Another popular model organism, *B. subtilis*, has been extensively harnessed for enzyme, chemical, and antimicrobial material production (Su et al. 2021; Su et al. 2020). As a model organism, numerous genetic manipulation techniques have emerged over the past decades (Liu et al. 2019). Moreover, under very harsh conditions, it forms spores (Errington 2003), which, along with its ability to develop biofilms on diverse surfaces (Arnaouteli et al. 2021; Arkatkar et al. 2010), have inspired the development of *B. subtilis*-based ELMs. The biofilm matrix of *B. subtilis* has been reported to include DNA, polysaccharides, and proteins. TasA is an amyloid protein distinct from curli types and is one of the main components of the *B. subtilis* biofilm matrix. Studies have shown that it is essential for maintaining the structural integrity of the biofilms (Romero et al. 2010). The tapA-sipW-tasA operon directs the synthesis of TasA (Winkelman et al. 2013). With an understanding of the role of the TasA protein in *B. subtilis* biofilms and how it is synthesised by the tapA-sipW-tasA operon, researchers have begun exploring the fusion of TasA with other functional proteins through genetic engineering to create living materials with new properties. By fusing TasA with mCherry protein and MHETase, the resulting fusion proteins produced by *B. subtilis* 2569Δ*tasA*Δ*sinR*Δ*eps* can equip living materials with additional capabilities, such as red fluorescence and MHETase enzymatic activity (Huang et al. 2019). In another study, a strain *B. subtilis* 1935Δ*eps*Δ*bslA* was used to fabricate living materials for bioremediation. By fusing the TasA protein with metallothionein, the engineered biofilm was shown to have the ability to remove $Pb^{2+}$, $Hg^{2+}$, and $Cu^{2+}$ ions from water (Zhu et al. 2024). While progress has been made in harnessing the TasA protein from *B. subtilis* for the construction of living functional materials, a literature review indicates that these are among the few instances reporting the utilisation of the TasA protein specifically for the production of such materials. However, there is an emerging perspective suggesting that exploring non-amyloid proteins for the constitution of the extracellular matrix in *B. subtilis* could present a strategic option for the design of next-generation ELMs. Utilising non-amyloid proteins might unlock new possibilities by virtue of their distinct properties and functions, which could enhance the versatility, robustness, and tunability of these materials. The EutM protein is a type of a bacterial microcompartment shell protein from *Salmonella enterica* (Schmidt-Dannert et al. 2018). This protein offers great flexibility for engineering, readily accepting N- and C-terminal fusions without loss of function (Zhang et al. 2018; Zhang et al. 2019). A recent study has genetically modified *B. subtilis* to display SpyTags on flagella for the purposes of cellular attachment and linkage with EutM-SpyCatcher scaffold building blocks to form the protein matrix of ELMs (Kang et al. 2021). Because CotB1p is a silica biomineralisation peptide, after the expression of EutM-SpyCatcher, EutM-CotB1p

fusion proteins, and the display of SpyTags on flagella, and upon incubation with silica, a biocomposite of ELMs with improved mechanical properties was developed.

### 2.3 | *K. rhaeticus*

*K. rhaeticus* is also regarded as a model organism for studying BC production. Cellulose, a major biopolymer produced by plants, is an important component of the biofilm matrix in many bacterial species (Lamas et al. 2016; Da Re and Ghigo 2006; Matthysse et al. 2005). The main distinction between BC and plant cellulose is that BC is an ultrapure nanocellulose that does not contain polymers like pectin and lignin. Moreover, BC has strong, flexible, and hydrophilic characteristics (Iqbal et al. 2014), allowing many potential applications for BC (Tang et al. 2022). Bacteria belonging to the *Acetobacteraceae* family, such as *Acetobacter and Komagataeibacter*, are famous for their cellulose production (Gupte et al. 2021; Avcioglu 2022). Biofilm formation, a common trait among many species in this family, is predominantly comprised of cellulose as the primary biofilm matrix. (Jacek et al. 2019; Subbiahdoss et al. 2022; Fontana et al. 1990) *K. rhaeticus* belongs to the genus *Komagataeibacter* and is generally considered a GRAS organism. The cellulose produced by this bacterium can self-organise into ordered structures without the need for an external template, which is particularly useful for constructing materials with complex shapes and functions. Moreover, this bacterium can grow under static culture conditions, eliminating the need for complex agitation equipment. These characteristics have motivated researchers to explore the development of ELMs based on *K. rhaeticus*. Currently, living functional materials are created by harnessing the extracellular matrix component cellulose produced by *K. rhaeticus* iGEM. Under static conditions, *K. rhaeticus* iGEM cultured flat pellicles, consisting of a substantial amount of thick BC. Researchers have adapted an AHL-based cell-to-cell communication system, originally designed for *E. coli*, to enable the production of sender and receiver pellicles (Walker et al. 2019). The sender pellicles produce the AHL synthase LuxI, while the receiver pellicles function as a receiver and express the transcriptional activator LuxR. Upon binding with AHL produced by sender pellicles, LuxR gets activated and subsequently boosts the activity of the Plux promoter. This activation event leads to the upregulation of a red fluorescent protein in the receiver pellicles. When cultured under shaking conditions, *K. rhaeticus* iGEM can form millimetre-scale rounded BC spheroids. These BC spheroids equipped with the AHL-based cell-to-cell communication system produce sender spheroids and receiver spheroids. Sender spheroids and receiver spheroids can function as interactive entities capable of interacting and responding to each other. Furthermore, these spheroids can also be used to regenerate damaged BC materials, further expanding the scope of this innovative technology in the living materials field (Caro-Astorga et al. 2021). Alteration of BC properties will increase the functionality of BC-based ELMs. The modification of BC characteristics has been achieved by the introduction of another polymer at the genetic level. For instance, the *crdS* gene is a curdlan synthase gene, and the introduction of the gene *crdS* into *G. xylinus* AY201 leads to the intracellular polymerisation of uridine diphosphate glucose, allowing the secretion of cellulose along with curdlan (Fang et al. 2015). Comparison of the resulting polysaccharides to normal BC pellicles has indicated that the nanofiber structure in the pellicle remains intact, and the water permeability of the bio-nanohybrids is minimal. Another method is the addition of *N*-acetyl-glucosamine to cellulose fibres, which not only renders BC vulnerable to lysozyme but also undermines its highly ordered structure (Yadav et al. 2010). Even though the above two studies have modified BC material properties, the limited availability of genetic tools and expertise for promoting the secretion of recombinant proteins from bacteria generating BC severely impedes their real-world implementation. To introduce more functions to cellulose-based living materials, an ELMs system inspired by kombucha tea has been developed. The drink known as kombucha is the result of fermentation brought about by a mixed community of bacteria and yeast often referred to as a symbiotic culture (Jayabalan et al. 2010; Villarreal-Soto et al. 2018). One research group has employed bacterial co-culture with *Saccharomyces cerevisiae* yeast to secrete TEM1 β-lactamase into *K. rhaeticus* cellulose, creating catalytic materials that can be cultured and preserved at room temperature (Gilbert et al. 2021).

### 2.4 | *Geobacter sulfurreducens*

Besides utilising these model organisms for developing living materials, there has been a growing interest in engineering living materials derived from non-model microorganisms, such as *G. sulfurreducens*. This Gram-negative, electroactive bacterium not only survives but also remains metabolically active in low-oxygen environments (Engel et al. 2020). Moreover, its metabolism is highly flexible, enabling it to utilise a variety of organic compounds, including acetate and lactate, as energy sources (Speers and Reguera 2012). Additionally, in recent years, the development of synthetic biology has led to the creation of genetic tools for manipulating bacteria in the *Geobacter* genus, making genomic editing of *G. sulfurreducens* more straightforward (Liu, Min, et al. 2023). The combination of these diverse biological characteristics makes *G. sulfurreducens* an ideal candidate for the development of ELMs. Currently, the development of ELMs using *G. sulfurreducens* is based on its ability to form biofilms. The typical features of *G. sulfurreducens* biofilms include a matrix made of polysaccharides, which contains pili and cytochromes. This structural composition of biofilms not only confers excellent electrical conductivity but also enhances hydrophilicity. Utilising this hydrophilic property of the biofilms, one research group induced electricity generation through water evaporation (Hu et al. 2022). Specifically, the process begins with forming a *G. sulfurreducens* biofilms on a glass slide. Once the biofilm has been established, a bottom copper electrode is fixed onto the biofilm, followed by the placement of a top copper electrode above it. This setup completes the fabrication of a power-generating device. When one end of the *G. sulfurreducens* biofilm formed on glass slides is immersed in water while ensuring that the bottom copper electrode remains above the water level, the device can generate power as water continuously evaporates. Under these conditions, the maximum power density achieved by this setup can reach approximately 685.12 $\mu W/cm^2$. OmcS protein is one of the many cytochromes produced by *G. sulfurreducens*. It is not only essential for interspecies electron transfer (Yalcin and Malvankar 2020), but research has also shown that overexpressing the OmcS protein in the cyanobacterium *Synechococcus*

*elongatus* PCC 7942 can significantly enhance its ability to generate photo-current (Sekar et al. 2016). A recent research group hypothesised that OmcS may have the potential to function as a photoconductor. To test this hypothesis, they selected *G. sulfurreducens* CL-1, which is capable of overexpressing the OmcS protein, for their experiments. After this strain formed a biofilm, upon photoexcitation, the living biofilm demonstrated the ability of photoconductivity, and the conductance of the biofilm followed Ohm's law (Neu et al. 2022).

### 2.5 | *Corynebacterium glutamicum*

Following *G. sulfurreducens*, another non-model organism that has garnered attention is *Corynebacterium glutamicum. C. glutamicum* is a Gram-positive bacterium renowned for its pivotal role in amino acid production. This microorganism is a GRAS organism and features a flexible metabolism, enabling it to grow on various carbon sources (Becker et al. 2005). Moreover, *C. glutamicum* exhibits excellent compatibility with a wide range of genetic tools and methods, such as plasmid construction, gene editing (e.g., CRISPR/Cas9), and homologous recombination (Zha et al. 2023). These attributes make it particularly convenient for scientists to engineer *C. glutamicum* to achieve specific functions. Additionally, the cell-surface protein structures known as pili in *C. glutamicum* are composed of Spa1, Spa2, and Spa3 proteins. These pili components offer unique opportunities for constructing ELMs. Specifically, one research group developed ELMs by forming fusion proteins with Spa2, the major component of *C. glutamicum* pili. By fusing Spa2 with enzymes like TrEgl and SdBgl, two enzymes that collaborate to degrade cellulose into glucose, and coassembling them in pili on the cell surface, it becomes possible to generate ELMs that efficiently convert cellulose into glucose (Huang et al. 2024).

### 2.6 | *Caulobacter crescentus*

Expanding the range of non-model organisms used in ELMs research, *C. crescentus* presents distinctive features. This Gram-negative bacterium is well-known for its extensive use in cell cycle research. It exhibits high sensitivity to environmental changes and can survive in nutrient-poor conditions, such as those found in rivers and streams (Govers and Jacobs-Wagner 2020). Additionally, researchers have developed a variety of tools and techniques to engineer its genome (Guzzo et al. 2020). These characteristics make it very suitable as a cellular component in ELMs. Because it possesses a Surface-layer protein RsaA with a well-characterised atomic structure, using it as a basis for constructing ELMs would be an excellent choice. One group initially fused the RsaA protein with SpyTag peptides for expression on the surface of CB15NΔ*sapA* cells. Since SpyCatcher can form an isopeptide bond with SpyTag peptides, this allows for their conjugation. Subsequently, the team linked SpyCatcher to elastin-like polypeptides and quantum dots separately, then incubated these complexes with cells expressing the RsaA-SpyTag fusion proteins. As a result, soft materials and hard materials were formed on the cell surfaces, respectively (Charrier et al. 2019). In a follow-up study, this group replaced residues 251–689 of the native RsaA with an elastin-like polypeptide and a SpyTag. As a result, they were able to successfully display this bottom-up de novo (BUD) protein on the cell surface. The matrix formed by the BUD protein can encapsulate cells to form centimetre-scale ELMs. Notably, by fusing the oxidoreductase PQQ-glucose dehydrogenase with SpyCatcher and subsequently incubating this fusion protein with ELMs formed based on the BUD protein, the resulting materials exhibit PQQ-glucose dehydrogenase activity (Molinari et al. 2022).

## 3 | Bacterial Species Used in Hybrid Living Materials

Not limited to biofilm engineering for constructing ELMs, the development of these materials can also involve encapsulating bacteria in artificially formed extracellular matrices. This approach enables the creation of hybrid living materials with a wide range of functions. Figure 2 illustrates the scheme of creating diverse hybrid living materials that combine engineered bacteria with different polymers. The bacterial species used in hybrid living materials include *E. coli*, *Shewanella oneidensis, B. subtilis, Lactococcus lactis, Methylobacterium radiotolerans, Azospirillum brasilense*, *Bradyrhizobium* sp., *Synechococcus elongatus*, *Pseudomonas aeruginosa*, *Lactobacillus casei*, *Lactobacillus plantarum*, *Lactobacillus acidophilus*, *Lactobacillus gasseri*, *Bifidobacterium adolescentis* and *Lactobacillus bulgaricus*.

Agarose is commonly utilised as a matrix polymer due to its biocompatibility, inertness, gelling ability, and high water content, which aids in hydrating bacterial cells. To form hybrid living materials, researchers have selected it as the matrix for many bacteria species. To detect the heavy metals' presence in the environment poses a significant difficulty for European initiatives focusing on the health and security of the general public. Biosensors, as a robust alternative to physicochemical methods, can be employed for heavy metal and toxic organic substance detection. In order to form biosensors, a combination of agarose and *E. coli* was chosen. Specifically, the *E. coli* DH1 strain carrying the pBzntlux plasmid was selected because it contains a cadmium-responsive promoter that induces bioluminescence in the presence of cadmium. Immobilising bioluminescent bacteria *E. coli* DH1 pBzntlux in an agarose matrix enables continuous cadmium detection (Affi et al. 2009). Another selected bacterium is *Shewanella oneidensis*; it is a model organism extensively used in bioelectrochemical systems (BESs). Within BESs, the interaction between *S. oneidensis* and the electrode surface is pivotal in attaining high system efficiency. Favouring efficient direct electron transfer, the biofilm formation of *S. oneidensis* is a promising approach. Yet, naturally formed biofilms within the systems tend to be thin. To address this limitation, researchers have directly embedded *S. oneidensis* into a 3D artificial biofilm matrix based on agarose, offering scalability and biocompatibility suitable for industrial applications (Knoll et al. 2022). In the field of ELMs, a significant challenge is maintaining the viability of cells within materials when exposed to harsh conditions outside the laboratory. Spores, which are structures used by some bacteria for reproduction or surviving adverse conditions, exhibit tolerance to high temperature, desiccation, and radiation. Due to the ability of *B. subtilis* to form spores, this bacterium has also been selected for the development of hybrid

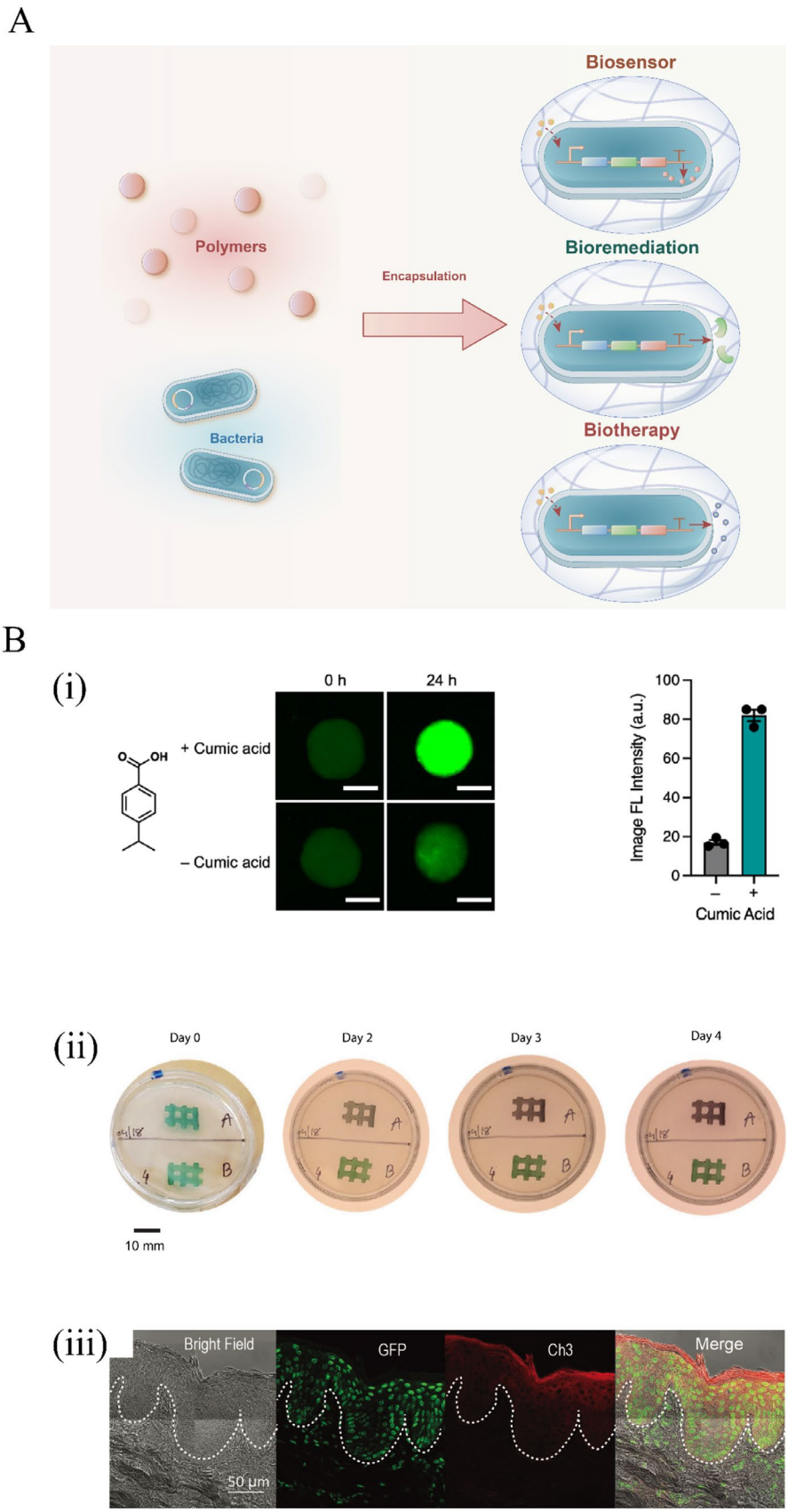


**FIGURE 2** | Legend on next page.

**FIGURE 2** | Bacterial ELMs derived from hybrid living materials. (A) Schematic illustration of using bacteria to create diverse hybrid living materials by encapsulating engineered bacterial cells within matrices such as agarose, alginate, Pluronic F-127, APBA and gelatin. (B) Representative examples of hybrid living materials include: (i) *B. subtilis* encapsulated in APBA functioning as biosensors. Reprinted with permission from Ref (Jo and Sim 2022). Copyright2022 American Chemical Socity. (ii) *S. elongatus* PCC 7942 encapsulated in alginate to form a living material exhibiting laccase activity against ABTS. Reprinted with permission from Ref (Datta et al. 2023). The Authors 2023. This article is published with open access at Springer Nature. (iii) *B. subtilis* encapsulated in Pluronic F-127 for treatment of *C. albicans* infections. Reprinted with permission from Ref (Lufton et al. 2018). Copyright2018 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

living materials. Lina et al. proposed a 3D printing method that combines agarose with *B. subtilis* PY79 spores to construct 3D objects (González et al. 2020). Through genetic engineering, the cells of the materials can respond to vanillic stimuli and synthesise lysostaphin, which is an antibiotic that specifically kills *Staphylococcus aureus* without affecting *B. subtilis*. Additionally, the spore-containing material remains stable at room temperature for extended periods.

The alginate exhibits a high degree of biocompatibility and low cytotoxicity, and it also displays a crosslinking behaviour when exposed to divalent cations. Given these characteristics of alginate, different bacterial species have been chosen to form hybrid living materials in combination with it. *Lactococcus lactis* is a Gram-positive bacterium widely used in food fermentation processes. Due to its long-term use in the food industry, it presents a safe choice for bioengineering applications. This bacterium is a facultative anaerobe, capable of growing under both aerobic and anaerobic conditions. It also coexists well with the human body and possesses an efficient protein secretion mechanism that can be utilised for the production and release of necessary enzymes and functional proteins into the surrounding environment. Because *L. lactis* produces lactic acid during metabolism from carbon sources, which can be detrimental to cell growth due to accumulation, one research group selected *L. lactis* NZ9020, which does not produce lactic acid due to a lactate dehydrogenase deficiency, for constructing ELMs. When *L. lactis* NZ9020 expressing the III7-10 fragment of human fibronectin ($FN_{III7\text{-}10}$), *L. lactis* NZ9020 expressing human bone morphogenetic protein-2 (BMP-2), and human bone marrow-derived mesenchymal stem cells (hBM-MSCs) were encapsulated together using alginate and co-cultured in liquid medium, the *L. lactis* was able to trigger the differentiation of hBM-MSCs within the material (Witte et al. 2019). The pink-pigmented methylobacteria, *Methylobacterium radiotolerans*, is a Gram-negative α-proteobacteria of the genus *Methylobacterium*. This bacterial species is not only tolerant to radiation, but it can also colonise nodules and other plant tissues. The application of *M. radiotolerans* to jatropha has been observed to increase seed yield. However, traditional liquid and solid formulations encounter the issue of decreased bacterial viability during storage. Scott et al. found that encapsulation of this bacterium by alginate prolongs its viability during storage (Strobel et al. 2018). For bacteria of the *Azospirillum* genus and *Bradyrhizobium* sp., which are capable of assisting plants in nitrogen fixation, there is also an issue of reduced bacterial cell viability during storage. Similarly, encapsulating *A. brasilense* Az39 and *Bradyrhizobium* sp. SEMIA6144 in a matrix composed of alginate results in an improved cell survival rate and enhanced effectiveness in promoting the growth of *Arachis hypogaea* (Cesari et al. 2020). Additionally, given the unique ability of Cyanobacteria to grow in low-cost media and utilise $CO_2$ as a carbon source, coupled with the genetic engineering capabilities available for cyanobacteria that enable modular plasmid design and gene expression, the cyanobacterium *S. elongatus* PCC 7942 is chosen for ELMs fabrication. By immobilising genetically engineered *S. elongatus* in alginate, it can be effectively used in bioremediation efforts (Datta et al. 2023). In the development of living materials for bioremediation, the CotA laccase from *Bacillus subtilis* was constitutively expressed in the *S. elongatus* PCC 7942. When embedded within an alginate hydrogel matrix and printed, this responsive biomaterial demonstrates the capability of decolorizing indigo carmine.

Another man-made matrix polymer, Pluronic F-127, possesses a lower critical solution temperature, which enables it to rapidly solidify when the temperature drops below a certain threshold (Lee et al. 2010). Both *B. subtilis* and *E. coli* have been explored for forming hybrid living materials with this polymer. Microorganisms often release secondary metabolites such as antibiotics into their environment to defend against other microorganisms and maintain ecological balance. Over the past decade, there has been extensive research into using beneficial live bacteria for disease treatment. One research group chose to encapsulate *B. subtilis* within Pluronic F-127 to develop a formulation aimed at treating *C. albicans* infections. *B. subtilis* was selected because it is generally recognised as a safe microorganism that can produce various antimicrobial substances. In the *C. albicans* infection model, the formulation containing live *B. subtilis* 3610, which persistently releases the antifungal agent surfactin, resulted in no inflammation in mice (Lufton et al. 2018). 3D printing technology allows scientists to precisely design and manufacture objects with complex geometries and internal structures tailored to specific needs. Materials generated using this technology have been applied in various fields, including drug delivery and tissue engineering. A recent study selected Pluronic F-127 as one of the main components of bioink, and by utilising Aerotech's three-axis robotic deposition stage, it was possible to print high-resolution living materials. The incorporation of *E. coli* DH5α*PRO*, a cell type suitable for efficient plasmid transformation, enables the 3D material to produce green fluorescent protein when induced by AHL or IPTG (Liu et al. 2018).

APBA is an organic compound with a molecular formula of C8H10BNO3. It is a white crystalline powder commonly used in the organic synthesis of pharmaceuticals. The boronic acid functionality of APBA allows it to form reversible covalent bonds with specific biomolecules, making it a valuable tool in drug research. To further improve the current application of APBA in ELMs, a programmable living material has been created featuring a dynamic covalent connection between a tailored *B. subtilis*

and APBA (Jo and Sim 2022). This breakthrough was inspired by the discovery that APBA and APBA-based polymers have the ability to establish covalent connections with the diols present on the cell surface of *B. subtilis*. In this innovative system, *B. subtilis* PY79 underwent genetic modification to enable the production of red fluorescent protein (RFP) integrated with the N-terminal signalling sequence of gene *AmyQ* (Phan et al. 2006), enabling the newly synthesised RFP to be secreted into the extracellular space. When these living materials containing the engineered *B. subtilis* PY79 were cultivated in suitable media, the fluorescence levels of the material and gathered liquid supernatant resulting from RFP production gradually increased, demonstrating the capability of materials to generate and release recombinant RFP. Furthermore, by engineering *B. subtilis* PY79 to express green fluorescent proteins in response to specific small molecules like cuminic acid and IPTG, biosensor living materials can be developed.

Gelatin, derived from collagen present in animal connective tissues, is a protein known for its capacity to form a gel upon hydration. This characteristic stems from the capability of the protein to absorb water and create a three-dimensional network of molecules. Quorum sensing (QS) serves as a cell-to-cell communication mechanism utilised by numerous bacteria to synchronise their actions and control gene expression based on population density. *Pseudomonas aeruginosa* possesses a complex QS system, which it uses to regulate the expression of a wide array of genes through multiple signalling molecules. These genes are involved in various behaviours, including biofilm formation and antibiotic resistance. Understanding *P. aeruginosa*'s QS system is crucial for deciphering how pathogenic bacteria coordinate collective behaviour to adapt to environmental changes. This area of study has garnered significant interest in the medical field because it is closely related to the infection process. When *Pseudomonas aeruginosa* are connected by gelatin and printed using the Micro-3D technique, the resulting materials can be utilised to investigate the QS-mediated communication behaviour of cell aggregates as small as 500 cells (Connell et al. 2014). However, the applications of gelatin as a gel matrix are limited by its network stiffness. On the other hand, the utilisation of an alginate matrix is restricted due to its sensitivity to low pH conditions, which may lead to undesired structural collapse. *Lactobacillus casei* is a common lactic bacterium that can grow over a relatively wide range of pH conditions and is widely found in nature, including in the gastrointestinal tracts of humans and animals. As a probiotic, it has immune-modulating properties and contributes to improving gut health. However, it may also experience reduced viability during storage and use. By combining alginate and gelatin, a dual-network hydrogel system is formed, capitalising on their complementary properties. This integration enhances the viability of probiotic bacteria *L. casei* ATCC 393 compared to non-encapsulated free cells (Li et al. 2009). In a similar manner, for *L. casei* 01, the incorporation of prebiotics, specifically starch, into the alginate matrix can improve the durability and protective capabilities of the matrix (Ta et al. 2021). Furthermore, egg lecithin, a natural emulsifier and stabiliser derived from eggs, has been explored for its effects on the survivability of alginate-starch encapsulated bacteria. Bacteria of the genera *Bifidobacteria* and *Lactococcus* also play a promoting role in maintaining host health. Encapsulating *Lactobacillus*, *Bifidobacterium* species, and *Lactococcus lactis* in freeze-dried beads containing lecithin demonstrates promising stability in terms of survival (Donthidi et al. 2010).

## 4 | Perspectives

Over the past 10 years, the field of ELMs has grown due to the combination of techniques employed in synthetic biology and material science. The pursuit of additional materials with diverse functions similar to living organisms on the earth will continue to stimulate the development of this area. For bacterial cells, significant efforts have been dedicated to *E. coli* for the fabrication of ELMs. Specially, modular engineering has been used to design amyloid proteins with diverse functions. Amyloid proteins are controlled by the *csg* operons, and the monomeric unit of amyloid is the csgA protein, which is secreted in an unfolded conformation. When the csgA protein is fused with various protein domains or peptides, novel fusion proteins with unique functions can be generated. As a proof of concept, *E. coli* NEBC3016 was the first *E. coli* strain used to express strong and multifunctional underwater materials in vitro. The *E. coli* PHL628-ΔcsgA has been used to obtain materials with biocatalytic function. Subsequently, *E. coli* PQN4, *E. coli* JF1Δ*csg*, *E. coli* MG1655*PRO*Δ*csgA*, and *E. coli* MG1655*PRO*Δ*csgBAompR234* were engineered to secrete and assemble fusion proteins with diverse functions (Table 1). The characteristics of these strains used in ELMs are that the *csgA* gene, *csgA/csgB* genes, or all curli genes are deleted. As csgC effectively prevents the formation of curli amyloid proteins by inhibiting primary nucleation through electrostatically guided interactions between molecules (Taylor et al. 2016). Perhaps further deletion of such a gene in the *csgBA* mutant strain, *E. coli* MG1655*PRO*Δ*csgBAompR234*, will stimulate amyloid protein secretion. However, the inhibition of csgA amyloid protein formation by csgC may serve as a protective mechanism for bacteria. Therefore, it is essential to evaluate the impact of such a modification on bacterial viability and stability before proceeding with genetic manipulation. Compared to directly deleting *csgC*, upregulating *csgE*, *csgF*, and especially *csgG* might provide a more straightforward and safer approach to increase the yields of secreted curli biofilms. To date, however, no attempt at generating such an *E. coli* strain has been reported.

To extend the spectrum of bacteria employed in the engineering of amyloid proteins, the TasA protein from *B. subtilis* has also been incorporated. By fusing the TasA protein with various proteins or protein domains, programmable, printable, and 3D objects with diverse functions have been developed. To enable broader applications and industrial-scale production of living materials utilising *B. subtilis* biofilms in the future, it is essential to enhance the biofilm formation capability of *B. subtilis* through genetic engineering. Studies have shown that integrating the signal peptide of the gene *amyQ* with red fluorescent protein and microbial transglutaminase promotes the release of both proteins in *B. subtilis* (Jo and Sim 2022; Mu et al. 2018). To enhance the release of TasA proteins, future efforts can be directed to explore the possibility of integrating the signal peptide of the gene *amyQ* with the TasA protein. Another way to enhance matrix production is to identify new strains that can produce more amyloids. Conducting PSI-Blast searches in the NCBI RefSeq protein database utilising csg proteins from *E. coli* MG1655 as query sequences has successfully identified

**TABLE 1** | Bacterial species used in the natural biofilm-based living materials.

| Strain name | Year | Description | Features | Advantages | Application |
|---|---|---|---|---|---|
| *E. coli* PHL628-Δ*csgA* (Botyanszki et al. 2015; Nussbaumer et al. 2017) | 2015, 2017 | *E. coli* MG1655 with a deletion of the *csgA* gene and an *ompR234* mutation | Deletion of *csgA*; *ompR234* mutation | Capable of fusing various peptides or proteins to curli fibres, enhanced biofilm formation | Biocatalysis |
| *E. coli* MG1655*PRO* Δ*csgA ompR234* (Pu et al. 2020; An et al. 2020) | 2020, 2020 | *E. coli* MG1655 with constitutive high level expression of *tetR* and *lacI* from *PRO* cassette, with *csgA* knock out, and with *ompR 234* mutation | Constitutive expression of TetR and LacI proteins; deletion of *csgA* gene; *ompR234* mutation | Robust regulatory protein expression, enhanced genetic manipulation flexibility, improved biofilm formation | Bioremediation; Biosensing |
| *E. coli* MG1655*PRO* Δ*csgBAompR234* (Kalyoncu et al. 2017) | 2017 | *E. coli* MG1655 *PRO* Δ*csgA ompR234* with further deletion of *csgB* gene | Constitutive expression of TetR and LacI proteins; deletion of *csgA* and *csgB* gene; *ompR234* mutation | Improved control over gene expression, elimination of potential inference from endogenous csgB, improved biofilm formation | Bioelectronics |
| E. *coli* PQN4 (Tay et al. 2017) | 2017 | An *E. coli* MC4100 strain that has undergone genetic modification to eliminate the curli operon | Complete deletion of curli formation operons | Improved reproducibility, facilitate functional peptide integration | Bioremediation |
| *E. coli* JF1 Δ*csg* (Moser et al. 2019) | 2019 | Permanently delete all curli associated genes from the *E. coli* JF1 | Native curli operons knockout | Elimination of cross-talk | Biosensing |
| *B. subtilis* 2569 Δ*tasA*Δ*sinR* Δ*eps* (Huang et al. 2019) | 2019 | *B. subtilis* 2569 lacks the *tasA* gene, as well as the *sinR* gene and the *epsA*~*O* gene | Deletion of *tasA*, *sinR*, *epsA*~*O* genes | Facilitates detection of functionalized TasA variants, enhanced biofilm formation, simplified biofilm matrix composition | 3D printing |
| *B. subtilis* 1935 Δ*eps*Δ*bslA* (Zhu et al. 2024) | 2024 | Knocking out *epsA*~*O* and *bslA* genes of wild-type strain *B. subtilis* 1935 | Deletion of *epsA*~*O* genes; deletion of *bslA* gene | Reduced background interference, simplified biofilm architecture | Biosensing, bioremediation |
| *K. rhaeticus* iGEM (Caro-Astorga et al. 2021) | 2021 | BC-producing bacterium isolated from kombucha tea | High cellulose production; high resistance to toxic chemicals | Significant chemical tolerance, development of a genetic toolkit | 3D printing, patterns formation |
| *G. sulfurreducens* CL-1 (Neu et al. 2022) | 2022 | A *G. sulfurreducens* strain that Overexpresses OmcS nanowires | Overexpression of OmcS protein | Enhanced electron transfer capability, increased photo-current generation efficiency | Bioelectronics |

(Continues)

**TABLE 1** | (Continued)

| Strain name | Year | Description | Features | Advantages | Application |
|---|---|---|---|---|---|
| *C. glutamicum*Δ*spa2*Δ*dec* (Huang et al. 2024) | 2024 | Deletion of the *spa2 and dec* genes in *C. glutamicum* ATCC 14067 | Deletion of *spa2*; deletion of *dec* | Biomass-to-chemical conversion, colour change marker | Biocatalysis |
| *C. crescentus* CB15NΔ*sapA* (Charrier et al. 2019) | 2018 | *C. crescentus* CB15N with deletion of gene *sapA* | Deletion of *sapA* gene | Enhanced surface display stability, higher display efficiency | Soft materials |
| *C. crescentus* NA1000 Δ*sapA*::*PxylmKate2* (Molinari et al. 2022) | 2022 | Deletion of gene *sapA* and insertion of the *mKate2* gene, controlled by the xylose-inducible promoter *Pxyl* at the same locus in *C. crescentus* NA1000 | Deletion of *sapA* gene; Pxyl-mKate2 insertion | Enhanced surface display stability, visual tracking | Biocatalysis |

numerous curli-containing strains within the same genus (Dueholm et al. 2012). For instance, we can identify 30 strains containing curli within the genus *Salmonella*, 10 strains containing curli within the genus *Shigella*, and 4 strains containing curli within the genus *Citrobacter* (Figure 3). Importantly, strains of these genera have been shown to have biofilm formation abilities. (Choong et al. 2021; Chiang et al. 2021; Zhou et al. 2021) Over the last decades, numerous alternative amyloid systems beyond curli type have also been discovered in various bacterial biofilms. Chaplins, a functional amyloid secreted by *Streptomyces* spp., assemble at the air-liquid interface to form hydrophobic amyloid protein sheets (Elliot et al. 2003). The *fap* operon in *Pseudomonas* species secretes amyloid proteins that function not only in initial adhesion but also in mature biofilm formation (Dueholm et al. 2013). In a similar manner, *Staphylococcus* species release Bap proteins, which assemble into amyloid-like fibres outside the cells and aid in the construction of the biofilm matrix (Taglialegna et al. 2016). However, it is worth mentioning that, among all functional amyloids distinct from curli types, only the TasA protein from *B. subtilis* has been recently modified for the development of ELMs. This may be attributed to the well-known pathogenicity of *Pseudomonas* and *Staphylococcus*, as well as the difficulty in lab handling of *Streptomyces* (Serra et al. 2015; Shepherd et al. 2010). To address the issue of pathogenicity of *Pseudomonas* and *Staphylococcus*, future efforts should be directed to construct both attenuated strains based on living materials (Valentine et al. 2020; Collins et al. 2002). In addition to constructing ELMs around amyloid proteins, such as those found in biofilm matrices, researchers have explored using non-amyloid proteins like Spa2, OmcS and RsaA for ELM construction. Non-amyloid proteins are also present within the extracellular matrices of various other bacteria; for instance, the biofilm matrix of *B. subtilis* contains the amphipathic surface protein BslA (Morris et al. 2024), while that of *Pseudomonas aeruginosa* includes lectin proteins (Metelkina et al. 2022). Although these non-amyloid proteins have been identified, they have not yet been utilised in the development of ELMs. Future engineering efforts focused on utilising non-amyloid proteins from biofilm matrices promise to significantly expand the diversity and potential applications of ELMs.

Cellulose is also an important extracellular component in biofilms of many bacterial species (Hung et al. 2013; Simm et al. 2014). BC-based ELMs have been developed with the ability to regenerate in response to damage. Additional species that can produce cellulose will broaden the uses of BC-based ELMs. *K. rhaeticus* is an α-proteobacteria in the family *Acetobacteraceae*. In one selected proteobacterial genome, many bacteria have BC synthesis (*bcs*) genes. Some α-proteobacteria, such as *Gluconacetobacter xylinus* E25, *Gluconacetobacter hansenii* ATCC 23769, *Methlobacterium extorquens* PA1, and *Sinorhizobium melilo* GR4, have been shown to produce cellulose under experimental conditions (Figure 4) (Römling and Galperin 2015). However, compared to protein modular engineering, modification of cellulose at the genetic level is difficult due to the complicated regulatory process. Living functional materials based on cellulose are currently produced by utilising BC secreted to encapsulate bacterial cells, yet there have been no reports of genetic modification of the cellulose metabolic pathway at the genomic level. In addition, the structure and morphology of BC closely resemble those of natural collagen, which makes

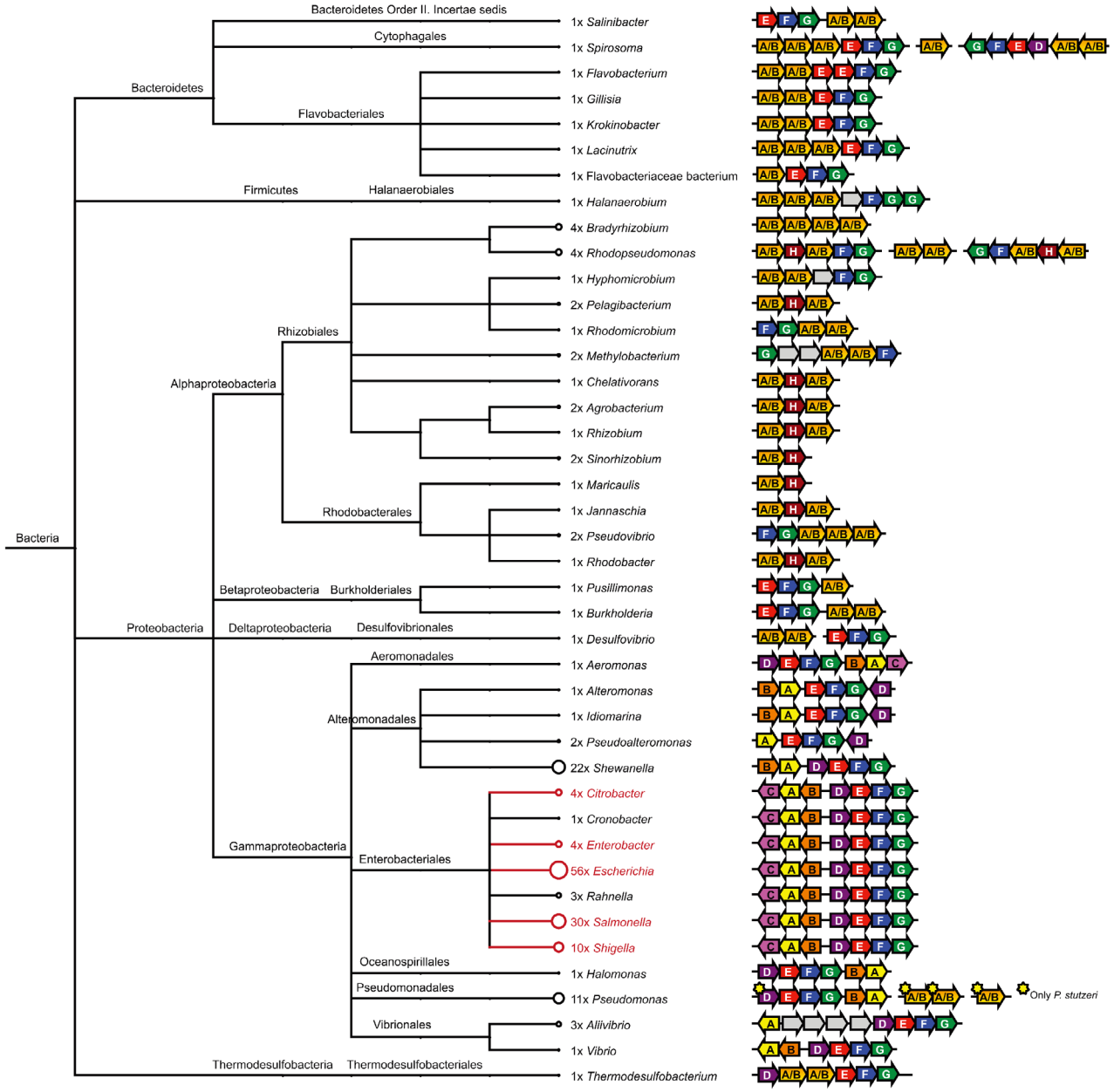


**FIGURE 3** | Phylogenetic distribution of curli systems and operon structure. The count of strains harbouring the curli system within each genus is denoted alongside the respective taxonomic units. The genera highlighted in red represent those where curli systems had been previously documented prior to August 30, 2012. Reprinted with permission from Ref (Dueholm et al. 2012). The Authors 2012. This article is published with open access at Public Library of Science.

it an enticing option for immobilisation. Immobilising *Bacillus subtilis* cells in BC using the 'adsorption-incubation' method led to living materials demonstrating high therapeutic efficiency in wound healing models (Savitskaya et al. 2019).

The strategy of encapsulating cells in polymers to form extracellular matrices effectively solves the problem of low extracellular matrix production of many bacteria under laboratory conditions, thereby expanding the range of bacterial species that can be applied in the field of ELMs. In addition to the species mentioned in the section of ELMs based on naturally synthesised biofilms, the *E. coli* DH1 pBzntlux, *S. oneidensis* MR-1, and *B. subtilis* PY79 have been reported to be encapsulated in artificial matrix polymers for various purposes (Table 2). The key to the success of this strategy is the selection of polymers and bacteria. Since these polymers are not molecules naturally produced by the corresponding bacteria, the interplay between cells and the extracellular matrix formed by these polymers differs from those when the extracellular matrix is naturally synthesised. It has been reported that when *Vibrio cholerae* is encapsulated in agarose, the interplay between the cell and the external environment determines the external morphology and internal structure of the cell cluster (Zhang et al. 2021). When cultured in a rigid environment, cells tend to form aggregates that exhibit oblate shapes and exhibit bipolar cellular ordering. In contrast, cells thriving in soft environments exhibit a spherical morphology and a disorganised cellular arrangement. When *Staphylococcus aureus* is encapsulated in agarose, a distinct oblate shape with a

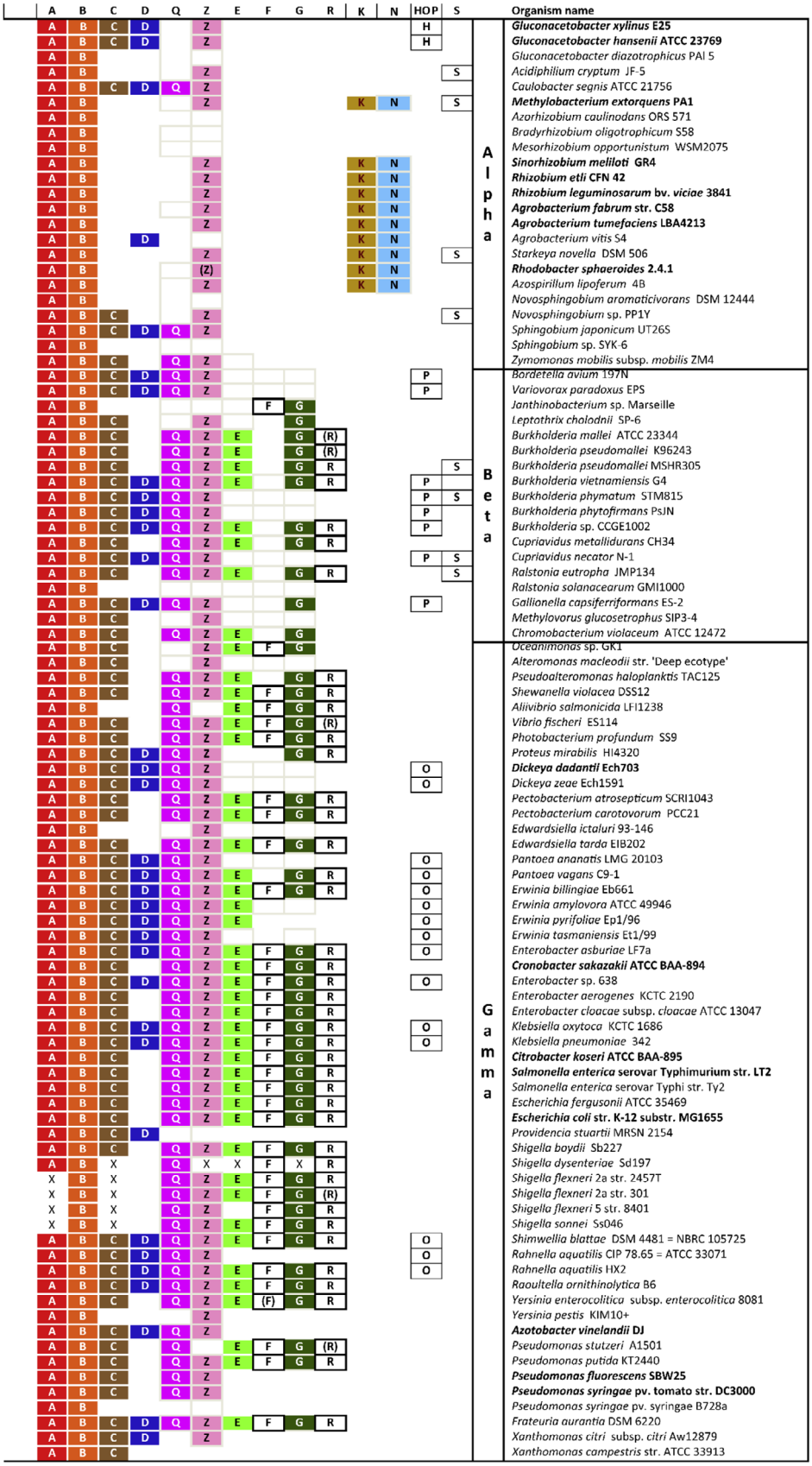


**FIGURE 4** | The presence of *bcs* genes in selected proteobacterial genomes. Note that some bacterial strains in the α-proteobacteria like *Gluconacetobacter xylinus* E25, *Gluconacetobacter hansenii* ATCC 23769, *Methylobacterium extorquens* PA1, *Sinorhizobium melilo* GR4 have been shown to produce cellulose in the experimental condition. Reprinted with permission from Ref (Römling and Galperin 2015). Copyright2015 Elsevier Ltd. Published by Elsevier Ltd. All rights reserved.

**TABLE 2** | Bacterial species used in hybrid living materials.

| Strain name | Year | Description | Features | Advantages | Application |
|---|---|---|---|---|---|
| *E. coli* DH1 pBzntlux (Affi et al. 2009) | 2009 | A bioluminescent *E. coli* strain that can be used to detect the presence of cadmium | Contains *lux CDABE* genes | High sensitivity | Biosensing |
| *S. oneidensis* MR-1 (Knoll et al. 2022) | 2022 | Model organism for exoelectrogenic electron transfer, low biofilm formation ability on electrodes | Diverse electron acceptor spectrun | Versatile use of electron acceptors | Bioelectronics |
| *B. subtilis* PY79 (González et al. 2020; Jo and Sim 2022) | 2020, 2022 | A derivative of *B. subtilis* ATCC23857 | Spore-forming capability | Tolerance to harsh conditions | 3D printing; biosensing |
| *L. lactis* NZ9020 (Witte et al. 2019) | 2019 | A strain lacks the *ldhA* and *ldhB* genes for lactate dehydrogenase | Lacks lactate dehydrogenase | Reduced lactic acid accumulation | 3D printing |
| *M. radiotolerans* (Strobel et al. 2018) | 2018 | Pink-pigmented methylobacteria | Radiation tolerance, ability to colonise nodules and plant tissues | Promotion of plant growth, strong environmental adaptability | Agriculture use |
| *Bradyrhizobium* sp. SEMIA6144, *A. brasilense* Az39 (Cesari et al. 2020) | 2020 | Rhizobacteria that are commonly utilised as liquid commercial inoculants for peanut plants | The ability to fix atmospheric nitrogen | Improve crop yields | Agriculture use |
| *S. elongatus* PCC 7942 (Datta et al. 2023) | 2023 | Model cyanobacteria | Photoautotrophic | Cost-effective cultivation, high genetic tractability | 3D printing, biosensing, bioremediation |
| *B. subtilis* 3610 (Lufton et al. 2018) | 2018 | Natural wild-type, nonmodified strain | Antifungal capability | High safety profile | Biotherapy |
| *E. coli* DH5αPRO (Liu et al. 2018) | 2017 | Molecular cloning strain | High efficiency in uptaking foreign DNA | High transformation efficiency | 3D printing |
| *P. aeruginosa* PA14 (Connell et al. 2014) | 2014 | Laboratory reference strain | Possesses complex QS system | Enhanced therapeutic development | Micro-3D printing |
| *L. casei* ATCC 393 (Li et al. 2009) | 2009 | Probiotic bacteria | Wide pH tolerance | Enhances immune function | Probiotic products |
| *L. casei* 01 (Ta et al. 2021) | 2021 | Probiotic bacteria | Wide pH tolerance, susceptibility to antibiotics | Safety for consumption | Probiotic products |

(Continues)

**TABLE 2** | (Continued)

| Strain name | Year | Description | Features | Advantages | Application |
|---|---|---|---|---|---|
| *Lactobacillus casei* ssp. *Casei* NCFB 161, *L. plantarum* DSM 12028, *L. acidophilus* NCFB 1748, *L. gasseri* NCFB 2233, *L. bulgaricus* NCFB1489, *B. adolescentis* NCIMB 2204, *Lactococcus lactis* ssp. *lactis* NCIMB 6681 (Donthidi et al. 2010) | 2010 | Probiotic bacteria | Adaptation to gut environment, metabolic diversity | Promotion of gut health | Probiotic products |

degree of hexagonal ordering has also been observed, differing from naturally occurring morphologies and order found during unconstrained growth. Crucially, multiple research studies have demonstrated a robust correlation between bacterial morphologies and their bioproduction capabilities (Jiang and Chen 2016; Wang et al. 2017; Choi and Lee 1999; Wang et al. 2014). Hence, a profound knowledge of the interplay between bacteria and the matrix would help a better choice of polymers and bacteria. It is commonly recognised that the level of oxygen concentration holds a pivotal position in the development of numerous aerobic or facultative anaerobic microorganisms. When *S. aureus* and *P. aeruginosa* are encapsulated in agarose and alginate, respectively, the growth of cells exhibits an oxygen-dependent phenomenon; aggregates are larger near the oxygen-supplied interface, and aggregates further from the oxygen-supplied interface are smaller (Pabst et al. 2016; Sønderholm et al. 2023). Deoxyviolacein (dVio), a natural pigment belonging to the violacein compound class, has garnered significant attention due to its promising potential for applications across diverse fields. When *E. coli* are encapsulated in agarose for production of dVio, the highest production was observed at the air-medium interfaces, while the lowest level of production was observed 1.5 mm away from both interfaces (Sankaran et al. 2019). Therefore, it is vital to check whether the pore size in the materials formed by wrapping cells with polymers satisfies the cellular requirement for oxygen.

The 3D structure of a natural biofilm, composed of polysaccharides, proteins, and DNA, affords physical protection to bacteria contained within. The barrier formed by the biofilm presents a formidable obstacle for antibiotics and other antimicrobial agents, hindering their penetration and access to the bacterial interior. This impediment results in a reduced effective concentration of these medications, diminishing their potency. Furthermore, certain bacteria embedded within the biofilm may exist in a state of slow growth or dormancy, rendering many antibiotics, which are primarily designed to target rapidly reproducing bacteria, less effective against them (Gebreyohannes et al. 2019; Stewart and Costerton 2001). In parallel, experiments have demonstrated that when encapsulated within an alginate matrix, *E. coli* exhibits substantial protection against antibiotics. Specifically, they can withstand concentrations up to 1000 times the minimum inhibitory concentration for ciprofloxacin and chloramphenicol (Pham et al. 2023). Additionally, it is noteworthy that the bacterial strains commonly utilised in the development of living materials are often genetically modified microorganisms (GMMs). Consequently, there is a critical need to thoroughly assess and manage the potential environmental release of such engineered cells. To keep bacteria from escaping the living materials and surviving in the environment, both chemical and physical containment measures have been implemented as barriers. One strategy that has been developed for chemical containment involves the use of *S. elongatus*. In *S. elongatus*, the gene *Synpcc7942_0766* is regulated by a theophylline-inducible riboswitch. When the substance theophylline is added, it induces overexpression of gene *Synpcc7942_0766*. The overexpression of the gene *Synpcc7942_0766* can lead to the excision of prophage, ultimately resulting in cellular lysis (Datta et al. 2023). Additional methodologies have been formulated for chemical confinement of GMMs, which could be implemented in living materials (Lee et al. 2018; Foo et al. 2014; Hirota et al. 2017).

However, in certain instances, the successful implementation of these strategies necessitates further genetic manipulation of the engineered bacteria. Multiple genetic operation steps are always labour-intensive and time-consuming. Interestingly, prophage-related genetic elements and residual prophage sequences have been widely identified in virtually all sequenced bacterial genomes, and it is not uncommon for bacteria to harbour multiple prophages within their chromosomal structures (Canchaya et al. 2003). Experimental evidence has shown that prophage-induced cell lysis has occurred in *P. aeruginosa* and *G. sulfurreducens* biofilms upon exposure to a quorum-sensing signal molecule, referred to as 2-heptyl-4-hydroxyquinoline, and mitomycin C, a known lytic inducer, respectively (Giallonardi et al. 2023; Liu, Ye, et al. 2023). Based on these findings, it is speculated that such a mechanism could potentially be harnessed in the future for containing bacteria escape in this field.

Strategies for physical containment of bacteria in living materials involve integrating different types of hydrogels. For instance, leveraging the hydrophilic and biologically inert properties of polyacrylamide (PAAM), it has been combined with alginate to create polyacrylamide-alginate (PAA) mixed hydrogels, which effectively encapsulated 2-phenylphenol living sensors (Luisi et al. 2022). The encapsulated *E. coli* NEB5α cells within the living sensors can remain viable for up to 2 weeks without any leakage. Alternatively, a deployable physical containment strategy (DEPCOS) system has been developed (Tang et al. 2021). This system comprises dual components: a core made of alginate-based hydrogel and an outer protective shell. The outer shell ingeniously integrates a stretchable polymer matrix (derived from polyacrylamide) with an energy-dissipating network (also sourced from alginate). This innovative design ensures both robust structural integrity and efficient energy absorption. The results have shown that the DEPCOS system provides an effective means of containing *E. coli*.

## 5 | Outlook

Bacteria, owing to their simple genomes, rapid reproduction, and the advanced state of genetic engineering technologies available for them, stand at the forefront of ELM research. Bacterial-based ELMs have garnered diverse applications in areas such as biocatalysis, environmental remediation, electrical conductivity, biosensing, biotherapy, and agriculture. However, much of the existing work has concentrated on single-cell types, which can complicate practical implementation due to the need for multifunctionality. Looking forward, integrating bacteria with other cell types such as archaea and various eukaryotic organisms like fungi, algae, and animal cells can pave the way for ELMs that exhibit enhanced multifunctionality. For instance, co-culturing *K. rhaeticus* with *S. cerevisiae* or combining *L. lactis* with human mesenchymal stem cells showcases innovative approaches that combine distinct biological attributes (Gilbert et al. 2021; Hay et al. 2018).

A significant challenge remains the inadequate natural production of extracellular matrix by bacterial cells. To tackle this challenge, future efforts should focus on the following areas. Firstly, the application of artificial intelligence, particularly machine learning techniques, to systematically explore literature for bacteria harbouring the csg operon that can produce increased csgA homologous proteins. Machine learning has proven invaluable in advancing multiple facets of healthcare, contributing to the discovery of new drugs, improving disease diagnostic accuracy, and facilitating disease prognosis (Carracedo-Reboredo et al. 2021; Yeoh et al. 2021; Silva et al. 2022). Essential genes are crucial for the development and continuance of all organisms, and machine learning techniques have also been employed in identifying essential genes (Aromolaran et al. 2021). Secondly, the identification of small biological molecules that can act as cross-link polymers for constructing artificial matrix components to envelop bacteria and create hybrid living materials is essential. Currently, the majority of hybrid living materials derived from artificial synthesis involve encapsulating bacteria with a single cross-link polymer. However, natural biofilms typically consist of a blend of various components such as proteins, cellulose, and DNA in conjunction with bacterial cells. To achieve a more accurate simulation of the structure of natural biofilms, the selection of diverse cross-link polymers incorporating different extracellular matrix molecules like proteins and DNA will enhance the future applications of ELMs.

Ethical considerations are paramount. While certain bacteria like *B. subtilis* and *L. casei* are typically considered GRAS organisms, they can become pathogenic under specific conditions (de Boer and Diderichsen 1991; de Seynes et al. 2018). Genetic manipulation, while innovative, may introduce unforeseen ecological impacts. Therefore, stringent measures and regulatory oversight are essential to ensure safety and compliance. Public engagement through education and outreach is also critical for building trust and promoting acceptance. In conclusion, advancing bacterial-based ELMs requires a balanced approach that considers human health, environmental protection, and public sentiment. Responsible progress should be pursued with these factors in mind, fostering innovation while safeguarding societal well-being.

### Author Contributions

**Hu Wang:** conceptualization, investigation, writing – original draft, writing – review and editing. **Chunzhong Li:** investigation, supervision. **Yanmin Wang:** investigation, supervision. **Huanming Zhang:** project administration, supervision, funding acquisition.

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### Conflicts of Interest

It is hereby stated by the authors that no conflicts of interest exist.

### Data Availability Statement

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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