Optimal membrane fluidity modulates biofilm formation in *Escherichia coli*

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ABSTRACT

Changes in membrane composition govern the fitness and survival of bacteria in response to environmental stresses. One critical parameter is membrane fluidity, which dictates overall membrane integrity, adaptability to environmental fluctuations (e.g., temperature changes), and the functionality and interactions between integral and peripheral membrane proteins. In recent years, substantial evidence has shown that membrane fluidity is finely tuned for biofilm formation. While high membrane fluidity promotes robust biofilm formation in Klebsiella pneumoniae, this is challenged by reports from multiple species indicating that biofilms are enriched in saturated fatty acids (i.e., reduced fluidity). We hypothesize that this paradox arises from the complex relationship between lipid composition and biofilm formation. Our findings demonstrate that an optimal balance of lipid fluidity is required for biofilm formation. We propose that the precise fluidity required for biofilm growth, relative to that required for planktonic growth, may differ between species, and is readily adjusted during lifestyle transitions.

RESULTS AND DISCUSSIONS

Microbes in natural environments often exist in communities, called biofilms, where cells are encased in an extracellular polymeric matrix (EPS) that provides protection from external stresses such as detergents and antibiotics. This EPS, is primarily composed of polysaccharides, proteins, lipids, and extracellular DNA, with its composition and spatial organization tightly regulated by molecular signals [1]. Identifying the molecular cues that bacteria use to regulate biofilm formation and dispersal is critical for both combating infections and harnessing beneficial biofilms. The contribution of bacterial cell membrane fluidity, as a modulator of these processes, remains an underexplored area.

The bacterial membrane plays a vital role in supporting essential processes, including energy generation, fitness in different environments and changes in environmental conditions necessitates membrane lipid adjustments for survival and fitness [2]. Changes in membrane lipids serve as a molecular cue in the Envelope Stress Response (ESR) that can influence various pathways involved in biofilm development [3]. A typical bacterial phospholipid contains two fatty acid (FA) chains, and the chemical nature of these FAs dictates the membrane fluidity [2]. Previous studies have demonstrated that biofilms of *Salmonella enterica, Staphylococcus aureus, Listeria monocytogenes,* and *Pseudomonas aeruginosa* contain elevated levels of saturated fatty acids (SFAs) compared to their planktonic counterparts [4]. This suggests that bacterial membrane composition and fluidity may play roles in biofilm formation, although the underlying mechanisms remain unclear [4,5].

Most bacterial species *de novo* synthesise the acyl chains to be integrate into membrane lipids by the highly conserved type II fatty acid synthesis pathway distinctive from that used by eukaryotes [2]. As such, the pathway had served as an important target for antimicrobial discovery [6,7], with the recent progress shown the potential of potentiating susceptibility to otherwise ineffective antibiotics [8,9]. In *Escherichia coli*, the major unsaturated fatty acids (UFAs) are palmitoleate (C16:1) and *cis*-vaccenate (C18:1). Bacterial phospholipids exhibit chemical asymmetry, with SFAs typically occupying the *sn*1 position and unsaturated fatty acids (UFAs) occupying the *sn*2 position of the glycerol backbone owed to the substrate preference of acyltransferases involved in phosphatidic acid synthesis [2,10]. The UFA/SFA ratio that affects membrane fluidity is predominantly determined by the diversity of FAs at the *sn*2 position [2,10]. The only exception to this substrate selectivity at position *sn*1 is the integration of *cis*-vaccenate, which leads to higher membrane fluidity that is imperative for bacterial fitness under certain conditions, such as low temperature growth [11]. As such, this phospholipid asymmetry plays a pivotal role in modulating membrane fluidity, which, in turn, may influences biofilm formation. In *E. coli,* UFA synthesis is mediated by ketoacyl-ACP synthase I (FabB), while the conversion of palmitoleate to *cis*-vaccenate requires ketoacyl-ACP synthase II (FabF) [12,13]. Thus, FabB and, to a lesser extent, FabF, are key players governing membrane fluidity.

The *E. coli* K-12 strain AR3110 exhibits a distinct macrocolony architecture when grown on agar surfaces, characterized by a radial, rugose, three-dimensional structure with delaminated buckling and skin-like elasticity [14]. This strain also forms a robust pellicle highly resistant to mechanical disturbance at the liquid-air interface (Fig. 1B). Remarkably, both the rugose colony architecture and pellicle formation disappeared in the Δ*fabF* mutant, suggesting that reduced membrane fluidity underlies these phenotypic changes (Fig. 1A and 1B).

Aside from the SFA/UFA ratio, membrane fluidity is also influenced by the profile of phospholipid headgroups. In this work, rather than using standard FA profiling, we utilised DPH fluorescence anisotropy, a common method for measuring membrane fluidity. A reduced anisotropy correlates with higher membrane fluidity, while higher anisotropy indicates a more ordered and less fluid membrane. The measured anisotropy in the Δ*fabF* mutant was significantly lower compared to the wild-type AR3110 (Fig. 1C), which supported the hypothesis that membrane fluidity is crucial for biofilm formation in *E. coli* (Fig. 1C).

Cerulenin inhibits both FabF and FabB, enzymes crucial for the synthesis of UFAs, thereby reducing membrane fluidity [15]. To investigate the role of membrane fluidity in biofilm formation, AR3110 were treated with subinhibitory concentrations of cerulenin in both rugose and pellicle biofilm models (Supplemental material, Fig. S1). This treatment reduced membrane fluidity to levels similar those observed in the Δ*fabF* mutant (Fig. 1C). The macrocolony of AR3110 typically exhibits a skin-like consistency, which prevents full resuspension of the biomass in solution (Supplemental

material, Fig. S2). However, both the Δ*fabF* mutant and cerulenin-treated wild-type macrocolonies acquired a buttery consistency, allowing for easy resuspension (Supplemental material, Fig. S2). The data shown in Fig. 1A and 1B demonstrated the absence of rugose macrocolony architecture and pellicle formation in both the Δ*fabF* mutant and cerulenin-treated wild-type AR3110, suggesting that reduced membrane fluidity hinders the development of stable biofilm structures.

The ectopic expression of FabB counteracted the fluidity reduction of AR3110 caused by cerulenin treatment (Fig. 1F), thereby restoring pellicle formation in cerulenin-treated AR3110 (Fig. 1D). This observation suggests that a reduction in membrane fluidity antagonises biofilm development. While this manuscript was being prepared, a similar observation was reported in *Klebsiella pneumoniae* [16]. FabR negatively regulates the expression of FabA and FabB in known *Enterobacteriaceae* species, including *K. pneumoniae*. Consequently, the activity of UFA synthesis in the *K. pneumoniae* Δ*fabR* mutant is elevated [16], leading to higher membrane fluidity compared to the WT during biofilm growth. In agreement to our observation, *K. pneumoniae* Δ*fabR* mutant was found to produce robust biofilm capable of withstanding mechanical disruptions [16]. Dramé *et al.* [16] and this work collectively suggest that high membrane fluidity is beneficial for the robust development of microbial biofilms.

An intriguing aspect of biofilm lipid composition is the observed higher SFA content in biofilms compared to planktonic cells in several species, including *S. enterica* [4]. This observation seems to contradict the idea that increased membrane fluidity is essential for biofilm formation. However, we hypothesize that this paradox arises from the complex relationship between lipid composition and biofilm formation. While membrane fluidity is crucial, an overabundance of UFAs may lead to excessive fluidity, compromising biofilm robustness.

In this work, we ectopically overexpressed FabB to suppress the biofilm-inhibitory activity of cerulenin (Fig. 1D). Strikingly, in the absence of cerulenin treatment, AR3110 cells ectopically expressing FabB exhibited a significantly more fluid membrane (Fig. 1F), and a pellicle was no longer formed in liquid culture (Fig. 1E), supporting our hypothesis that an optimal balance of lipid fluidity, instead of simply high fluidity, is critical for biofilm formation.

To this end, we argue that the precise UFA/SFA ratio optimal for biofilm growth relative to that required for planktonic growth may vary across species if not at strain levels, and is readily adjusted to facilitate the transition into biofilm lifestyle [4,5,16]. Our findings highlight the critical role of optimised membrane fluidity for robust biofilm development, and these insights could pave the way for future studies exploring lipid composition as a potential target for biofilm control in clinical and industrial applications.

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Author contributions

YH conceptualised the project, designed and conducted the experiments, and contributed to data collection and analysis; JQ contributed to experimental resources; YH and MT supervised the study and obtained the funding. YH wrote the manuscript, and all authors edited the manuscript.

Competing interests

MT is an employee of the GSK group of companies. All remaining authors declare no competing interests. This research was conducted in the absence of any commercial or financial relationships that could be constructed as a potential conflict of interest.

Data availability

All data generated or analyzed in this study were included in this article.

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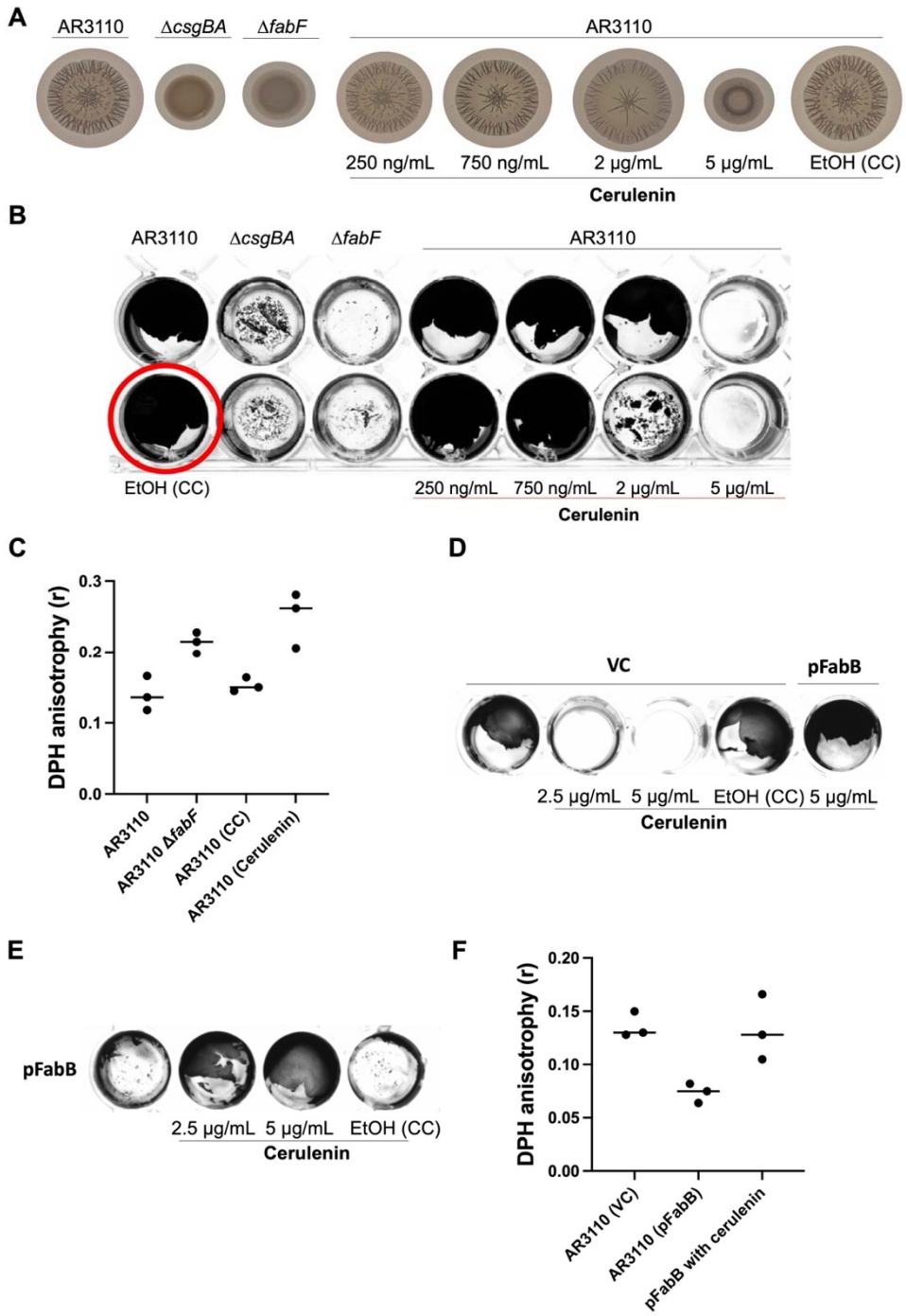

Fig 1. Optimal membrane fluidity governs *E. coli* biofilm rugosity and pellicle formation. (A) The *fabF* null mutation or cerulenin treatment eliminated rugosity in AR3110 macrocolony biofilms grown on YESCA agar at 26°C; (B) The *fabF* null mutation or cerulenin treatment abolished pellicle formation in AR3110 on Muller Hinton broth. Note that identical results were observed on YESCA, LB Lennox, and LBON; (C) DPH fluorescence anisotropy was used to measure membrane fluidity (fluidity is inversely proportional to fluorescence); (D) Ectopic expression of FabB (pSU2718 vector, induced with 250 μM IPTG) restored pellicle

formation inhibited by cerulenin treatment (E) Overexpression of FabB blocked pellicle formation in AR3110, but this defect was alleviated by cerulenin treatment. (F) Ectopic expression of FabB significantly increased membrane fluidity. Note that the increased fluidity can be ameliorated with cerulenin treatment. Panels A, B, D and F were representative of at least three independent observations. Data in Panels C and F were obtained from three independent repeats. Error bar is indicative of standard deviation values.