Strain-dependent motility defects and suppression by a $flhO$ mutation for $B. subtilis$ bactofilins

Sven Holtrup$^{1,2}$ and Peter L. Graumann$^{1,2}$*

Abstract

Objective: Bactofilins can assemble into polymeric structures and play important roles in cell shape maintenance, chromosome segregation and motility. $Bacillus subtilis$ bactofilins BacE and BacF were shown to be important for swimming motility in strain PY79, and single gene deletions were reported to be lethal, in contrast to a double bacEF deletion.

Results: Extending this work, we show that motility defects vary between different $B. subtilis$ strains, with strain 168 showing no defect in motility, and 3610 showing delayed induction of swimming. Generation of single gene deletions in PY79 was possible by transferring corresponding deletions from 168. In the natural isolate 3610, gene deletions also showed a negative effect on biofilm formation, revealing an additional function for BacE and BacF. A spontaneous arising suppressor mutation in PY79 was mapped to the $flhO$ gene, a constituent of the flagellum, which obtained an 18 amino acid extension at its C-terminus. Our findings show that bactofilin gene deletions lead to different motility phenotypes dependent on the strain background, and affect biofilm formation in the natural isolate 3610. Our data reinforce the idea of a connection between bactofilins and motion via the flagellum, and suggest that they operate in a switch like manner.

Keywords: $Bacillus subtilis$, Bactofilins, Biofilm formation, Flagellum, Motility, Swarming
PG-sacculus and thus enables the typical cork-screw like shape of the bacterium [6].

The gram-positive model organism B. subtilis possesses two bactofilin homologs, BacE (encoded by bacE, earlier named yhbE) and BacF (yhbF) [7]. Using fluorescence-microscopy it was shown that both proteins localize as discrete 60–70 nm assemblies at the cell membrane with BacF assemblies being relatively static and partially co-localizing with the flagella basal-bodies and BacE structures being fewer per cell and highly mobile. Interestingly, the exchange of bacEF by a tetracycline resistance cassette resulted in loss of swimming motility in PY79. Further investigation unveiled that the cells were not able to generate neither the flagellar hook nor the filament [7].

Flagella-mediated motility is a life-saving feature for many bacteria, enabling them to survive in environments that are dominated by nutrient gradients. Due to its drastic influence on the lifestyle of B. subtilis, assembly of functional flagella is a time and energy costly process that involves over 20 structural proteins and underlies a complex assembly-order. It is still unclear how bactofilins may act on the formation of the flagellum, or the regulation thereof. B. subtilis lab strains exhibit two forms of active flagella driven movement, swimming and swarming [8, 9]. Swimming refers to individual cells moving in three-dimensional space through liquid media. In swimming on the other hand, cells form groups to join their flagella forces to move in two dimensional space over solid media [10].

All attempts of creating bacE or bacF single knock-out strains failed in our earlier study. Both genes could only be deleted in the presence of an ectopic copy of both genes, suggesting that the loss of single genes is lethal [7].

To our surprise, we found single bacE and bacF mutants in the Bacillus-genetic-stock-center (BGSC, Columbus, Ohio) deletion collection [11] (strain 168). To clarify if strain-dependent differences exist, we investigated effects of single or double bactofilin deletions in three strains, laboratory strains PY79 and 168, and the undomesticated strain NCIB 3610 carrying comtQ12L mutation [12]. We found motility effects to very different extents in the three strains, and an additional defect in biofilm formation in strain 3610. Interestingly, bacF-mutant colonies in strain PY79 frequently reverted to swarming cells after extended cultivation on solid media. Complementing this work, we created a suppressor mutant of PY79 bacEF::tet that is as motile as its wildtype progenitor and found a single nucleotide deletion in the putative rod-gene flhO as causative reason. This work clarifies and extends results found in El Andari et al. [7]. Results presented here were generated within the duration of around one year.

Main text

Results

Single and double bacEF mutants cause different motility defects in different strain backgrounds

As reported earlier [7], a double yhbEF (from now on called bacEF) deletion resulted in cells of the lab-strain PY79 lacking filaments and hooks, and the construction of single bactofilin knock-out mutants failed. Motility in the bacEF double mutant strain could not be complemented by ectopically expressing either of these genes, only the expression of both proteins was able to restore motility. However, within the B. subtilis mutant strain collection [11], we found single knock-out strains of these genes, albeit in the background of the other commonly utilized lab-strain 168. By using total DNA isolated from this strain (also referred to as genomic DNA or gDNA) for transformation, we were able to obtain single knock-outs also in PY79. These experiments show that lethality of the double deletion was an artefact of the DNA construct that was chosen to create the deletion of both genes, which had been analogous to constructs for single gene deletions. We next created a bacEF double mutant in 168 by using genomic-DNA from PY79 bacEF::tet, and analogously, single and double gene deletion strains in the undomesticated strain 3610 (strains are listed in Table 1).

All strains were verified by PCR and were assayed on 0.3% soft agar plates (Fig. 1A) (primer sequences are given in Additional file 1). As reported earlier, surface movement under these conditions is due to flagella-based motility. In agreement with our earlier study, bacE mutant cells showed a motility defect in strain PY79, while bacF mutants were able to spread over the surface similar to wild type cells. Curiously, double mutant cells lacked any detectable motility in strain PY79 (Fig. 1A). Interestingly, in strain 168, neither the single nor the double deletions resulted in a loss of motility (Fig. 1A). These results show that PY79 cells show strikingly different requirements for motility than 168 cells. In the background of 3610, we did not observe any effect of single or double deletions
Fig. 1 (See legend on previous page.)
on motility 24 h after incubation (Fig. 1A), suggesting that defects found in PY79 are laboratory artefacts. However, 6 h after incubation, 3610 bacF mutant cells did not show any spreading across the surface, in contrast to 3610 wild type cells, which showed spreading including visible threads of cells showing high motility (Fig. 1B). Also, bacE mutant cells showed a phenotype of apparent swimming activity, but these cells lacked structured thread-like structures (Fig. 1B). Surprisingly, bacEF double mutant cells showed wild type-like behavior, showing that a deletion of both genes suppresses single deletion phenotypes. These findings show that bactofilin deletions do impact motility in natural isolate B. subtilis cells, but seemingly in a switch-like manner rather than in a structural manner, because induction of swarming appears to be delayed, rather than swimming disturbed per se.

As an extension of these analyses, we found that a majority of bacF mutant cells of strain 3610 developed suppressor mutations occurring as early as 8 to 24 h (Fig. 1C), much earlier than we observed for similar mutant cells of strain PY79 (not shown). While cells in the center of the colony continued to lack motility after restreaking (Fig. 1C, right panel), cells from the outer areas continued to swim. We were curious to investigate the nature of such regaining of motility (see further below).

Bactofilin deletions influence biofilm formation

In addition to a delay in swimming activity, we found that single bactofilin gene deletions in strain 3610 were not able to form biofilms on soft agar (0.3%), unlike wild type cells, which formed structured colonies (Fig. 1B). Different from its domesticated derivatives PY79 and 168, strain 3610 creates a thick biofilm when grown on solid medium, including many differently differentiated areas continued to swim. We were curious to investigate the nature of such regaining of motility (see further below).

PY79 bactofilin motility defects can be suppressed by an extension of the flhO open reading frame

In order to gain further insight into the mechanisms underlying the flagellar defect of a bactofilin double deletion of PY79, we generated suppressor mutant colonies. To this end, we dropped multiple spots onto 0.3% soft agar plates and incubated them for several days (note the difference to the single bacF deletion in 3610, Fig. 1C) until we observed motile cells that were swimming out from one of the spots (Fig. 2A). We compared surface swarming of the suppressor mutant to the PY79 double mutant strain and the PY79 wild type by measuring spot diameter over an 8 h duration. As illustrated in Fig. 2B, mutant cells that had regained motility exhibited surface spreading comparable to that of wild type cells. Both strains, PY79 bacEF::tet<sup>sup</sup> and its motile derivative, were sent for whole-genome sequencing. Comparing both datasets, we found a single point mutation within the reading frame of the flagellar rod protein FlhO, where a thymidine close to the stop codon had been deleted, resulting in a frameshift. There was no other mutation in the genome of the suppressor strain, clearly identifying the mutation as the relevant change for regaining of motility. The new ORF of flhO encodes for an 18 aa extension at its C-terminus (FKKTEEKNGGHQLCS-GQC). We used the alpha-fold algorithm [15] for modeling the native B. subtilis FlhO structure (Fig. 2D) and its extended counterpart, labeled FlhO<sup>+</sup> (Fig. 2E). The predicted local superposition-free score (pLDDT) suggest a good confidence for FlhO<sup>+</sup> except for a small area around residue 60 where the pLDDT is only at around 60 and the extended C-terminal sequence that gives a low confidence. As a consequence, the peptide sequence added to the C-terminal FlhO alpha-helix is predicted as unstructured, but does not appear to change the overall fold of
FlhO. It is unclear, how the FlhO extension can compensate for the lack of bactofilins.

FlhO is a structural protein in the Rod-complex of the flagellum, and cells lacking FlhO are deficient in synthesizing a filament. FlhO, together with FlhP is encoded outside of the 27 kb che/fla operon under the control of the SigD-dependend flhO-promotor. Assembly of the B. subtilis rod-complex follows a distinct assembly order, starting from the distal FliE over FlgB, FlgC and FlhO to the peripheral FlhP. Intermediates were found to be metastable or subject to proteolysis [16]. Because bactofilin mutants have been shown to be deficient in the hook structure, arising from the rod complex, our finding of FlhO acting as a suppressor mutation represents another hint on the role of Bacillus bactofilins on flagella assembly. Evidently, the requirement for bactofilin activity is different for different B. subtilis strains, and our finding that single bactofilin deletions can lead to motility defects, while double deletions in strain 3610 show no defects suggests that bactofilins play a regulatory role and possible set up a negative feed-back loop (Table 1).

**Limitations**

The identification of a single suppressor (flhO*) for a bacEF deletion in PY79 does not mean that there could be other mutations, which might compensate for the lack of BacF. These could also appear in structures other than the flagellum. Delay of swimming activity seen in bacF mutant cells in strain 3610, and arising of suppressors is highly stochastic, so this effect is highly variable and
difficult to (Additional file 1) quantify, also the number of colonies developing suppressor mutations.

Abbreviations
PG: Peptidoglycan; LDDT: Local superposition-free score; Wt: Wild type; BGSC: Bacillus Genetic Stock Centre; gDNA: Genomic DNA.

Supplementary Information
The online version contains supplementary material available at https://doi.org/10.1186/s13104-022-06048-6.

Table 1 List of strains used in this study

| Strain          | Description                  | Resistance-marker | References |
|-----------------|------------------------------|-------------------|------------|
| PY79 (wild type)| B. subtilis lab-strain PY79   | –                 | [17]       |
| PY79 bacEF::tet| bacEF replaced by tetracycline-resistance cassette | Tetracycline | [7] |
| PY79 bacEF::tet_mot Suppr | suppressor mutant, generated from PY79 bacEF::tet [7] | Tetracycline | This study |
| PY79 ΔbacE    | ΔbacE, generated using gDNA from 168 ΔbacE [12] | Kanamycin | This study |
| PY79 ΔbacF    | ΔbacF, generated using gDNA from 168 ΔbacF [12] | Kanamycin | This study |
| 168 (wild type)| B. subtilis lab-strain 168    | –                 | [18]       |
| 168 ΔbacE     | ΔbacE-strain                 | Kanamycin         | [11]       |
| 168 ΔbacF     | ΔbacF-strain                 | Kanamycin         | [11]       |
| 168 bacEF::tet| ΔbacEF strain, generated by using gDNA from PY79 bacEF::tet [7] | Tetracycline | This study |
| 3610 (wild type)| NCBI_3610, comIQ12L  | –                 | [12]       |
| 3610 ΔbacE    | ΔbacE strain, generated using gDNA from 168 ΔbacE | Kanamycin | This study |
| 3610 ΔbacF    | ΔbacF strain, generated using gDNA from 168 ΔbacF | Kanamycin | This study |
| 3610 bacEF::tet| ΔbacEF strain, generated by using gDNA from PY79 bacEF::tet | Tetracycline | This study |

Competing interests
The authors declare that they have no competing interests.

Author details
1 SYNMIKRO, Zentrum Für Synthetische Mikrobiologie, Karl-von-Frisch-Str. 14, 35043 Marburg, Germany. 2 Fachbereich Chemie, Hans-Meerwein-Straße 4, 35032 Marburg, Germany.

Received: 9 December 2021   Accepted: 24 April 2022
Published online: 13 May 2022

References
1. Kühn J, Briegel A, Morschel E, Kahnt J, Leser K, Wick S, Jensen GJ, Thanbichler M. Bactofilins, a ubiquitous class of cytoskeletal proteins mediating polar localization of a cell wall synthase in Caulobacter crescentus. EMBO J. 2010;29(2):327–39.
2. Deng X, Llamazares AG, Wagstaff JM, Hale VL, Cannone G, McLaughlin SH, Kureisaite-Ciziene D, Lowe J. The structure of bactofilin filaments reveals their mode of membrane binding and lack of polarity. Nat Microbiol. 2019;4(12):2357–68.
3. Shi C, Fricke P, Lin L, Chevelkov V, Wegstroth M, Giller K, Becker S, Thanbichler M, Lange A. Atomic-resolution structure of cytoskeletal bactofilin by solid-state NMR. Sci Adv. 2015;1(11):e1501087–e1501087.
4. Holtrup S, Heimerl T, Linne U, Altegoer F, Noll F, Waidner B. Biochemical characterization of the Helicobacter pylori bactofilin-homolog HP1542. PLoS ONE. 2019;14(6):e0218474–e0218474.
5. Koch MK, McHugh CA, Hoiczyk E. BacM, an N-terminally processed bactofilin of Myxococcus xanthus, is crucial for proper cell shape. Mol Microbiol. 2011;80(4):1021–51.
6. Sycuro LK, Pincus Z, Gutierrez KD, Riboy J, Stern CA, Vollmer W, Salama NR. Peptidoglycan crosslinking relaxation promotes Helicobacter pylori helical shape and stomach colonization. Cell. 2010;141(5):822–33.
7. El Andari J, Altegoer F, Bange G, Graumann PL. B. subtilis bactofilins are essential for flagellar hook-and filament assembly and dynamically localize into structures of less than 100 nm diameter underneath the cell membrane. PLoS ONE. 2015;10(10):e0141546–e0141546.
8. Keams DB, Losick R. Swarming motility in undomesticated Bacillus subtilis. Mol Microbiol. 2003;49(9):811–90.
9. Nishihara T, Freese E. Motility of Bacillus subtilis during growth and sporulation. J Bacteriol. 1975;123(1):366–71.
10. Keams DB. A field guide to bacterial swarming motility. Nat Rev Microbiol. 2010;8(9):634–44.

Additional file 1 Table S1: List of primers used in this study
11. Koo B-M, Kritikos G, Farelli JD, Todor H, Tong K, Kimsey H, Wapinski I, Galardini M, Cabal A, Peters JM, et al. Construction and analysis of two genome-scale deletion libraries for Bacillus subtilis. Cell Syst. 2017;4(3):291–305.

12. Konkol MA, Blair KM, Kearns DB. Plasmid-encoded ComI inhibits competence in the ancestral 3610 strain of Bacillus subtilis. J Bacteriol. 2013;195(18):4085–93.

13. Arnaouteli S, Bamford NC, Stanley-Wall NR, Kovács ÁT. Bacillus subtilis biofilm formation and social interactions. Nat Rev Microbiol. 2021. https://doi.org/10.1038/s41579-021-00540-9.

14. Patrick JE, Kearns DB. Laboratory strains of Bacillus subtilis do not exhibit swarming motility. J Bacteriol. 2009;191(22):7129–33.

15. Jumper J, Evans R, Pritzel A, Green T, Figurnov M, Ronneberger O, Tuyyasuvunakool K, Bates R, Žídek A, Potapenko A, et al. Highly accurate protein structure prediction with AlphaFold. Nature. 2021;596(7873):583–9.

16. Burrage AM, Vanderpool E, Kearns DB. Assembly order of flagellar rod subunits in Bacillus subtilis. J Bacteriol. 2018;200(22):e00425-00418.

17. Youngman P, Perkins JB, Losick R. Construction of a cloning site near one end of Tn917 into which foreign DNA may be inserted without affecting transposition in Bacillus subtilis or expression of the transposon-borne =erm gene. Plasmid. 1984;12(1):1–9.

18. Spizizen J. Transformation of biochemically deficient strains of Bacillus subtilis by deoxyribonucleate. Proc Natl Acad Sci USA. 1958;44(10):1072–1072.

Publisher's Note
Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.