The formation of microbial exoskeletons is driven by a controlled calcium-concentrating subcellular niche

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Abstract

In nature, bacteria reside in biofilms - multicellular differentiated communities held together by extracellular matrix. In this work, we identified a novel subpopulation essential for biofilm formation – mineral-forming cells. This subpopulation contains an intracellular calcium-accumulating niche, in which the formation of a calcium carbonate mineral is initiated. As the biofilm colony develops, this mineral grows in a controlled manner, forming a functional macrostructure that serves the entire community.

The molecular mechanisms promoting calcite scaffold formation were conserved between three distant phyla – the Gram-positive *Bacillus subtilis*, Gram-negative *Pseudomonas aeruginosa* and the actinobacterium *Mycobacterium abscessus*. Biofilm development of all three species was similarly impaired by inhibition of calcium uptake and carbonate accumulation. Moreover, chemical inhibition and mutations targeting mineralization both significantly reduced the attachment of *P. aeruginosa* to the lung, as well as the subsequent damage inflicted by biofilms to lung tissues, and restored their sensitivity to antibiotics.

The evolutionary conserved cellular pathway controlling the fundamental feature of biofilm development uncovered in this work offers novel druggable targets for antibiotics to combat otherwise untreatable biofilm infections.

Introduction

While bacteria have been historically studied in homogeneous monocultures, in natural ecosystems and in clinical settings they are typically found in interface-associated multicellular communities called biofilms (Kolter and Greenberg, 2006). It was recently indicated that in soils, oceans, the deep subsurface, phyloshpere and animal microbiomes, 40−80% of bacteria reside in biofilms (Flemming and Wuertz, 2019). Bacterial biofilms are differentiated communities, in which the cells are held together by extracellular matrix. The formation of a biofilm is frequently favored over free-living planktonic life-style, as it increases the fitness of member cells promoting their survival, such as better attachment to hosts, division of metabolic labor and protection from the environment.

Microbial biofilms are of extreme clinical importance, as they are associated with many persistent and chronic infections (Costerton et al., 1999). A prominent feature of biofilms is their inherent resistance to the immune system and to antibiotics (Bryers, 2008; Bucher et al., 2019; Hill et al., 2005) – biofilm cells are up to 1,000 times more tolerant than planktonic bacteria (Bryers, 2008), making the eradication of biofilm infections with currently available antibiotics extremely challenging.

For example, the commensal/aquatic bacterium *Pseudomonas aeruginosa* can cause devastating chronic biofilm infections in compromised hosts, such as cystic fibrosis (CF) patients and those with burn wounds or implanted medical devices (Costerton et al., 1999). Respiratory infections with *P. aeruginosa* are a leading cause of morbidity and mortality in patients with CF (Yoon and Hassett, 2004). Once a chronic infection with *P. aeruginosa* is established, it is almost impossible to eradicate (Cohen-Cymberknoh et al., 2016). Similarly, the biofilms of *Mycobacterium abscessus* cause infections of respiratory tract, skin and central nervous system in patients with CF and chronic obstructive pulmonary disease (Caimmi et al., 2018).

A hallmark of biofilms is their ability to form robust and complex 3D structures, and the distinctive spatial organization provides the residing bacteria with several

benefits. The biofilm architecture was suggested to relieve metabolic stress. For example, channels formed below the ridges and wrinkles within the colony may facilitate diffusion of fluids, nutrients and oxygen (Bloom-Ackermann et al., 2016; Dietrich et al., 2013; Kolodkin-Gal et al., 2013; Wilking et al., 2013). Furthermore, cells located in different areas of the colony are exposed to different levels of oxygen, nutrients and quorum sensing molecules, which affect the genetic programs they express, promoting differentiation (Asally et al., 2012; Hassanov et al., 2018; Liu et al., 2015; Monds and O'Toole, 2009; Serra and Hengge, 2014; Stewart and Franklin, 2008). Thus, community structure allows the genetically identical biofilm cells to display phenotypic heterogeneity and to endure stressful environments. Due to physical protection offered by the biofilm biomass, differentiation and physiological adaptations, cells residing deep within the biofilm are protected from environmental assaults, such as antibiotics. Biofilm colonies are a compelling model system for biofilm development, and many basic biological processes of high relevance to bacterial pathogenicity were first discovered in this well-controlled and robust system (Colvin et al., 2012; Dietrich et al., 2008; El Mammeri et al., 2019; Hufnagel et al., 2018; Jo et al., 2017; Richards et al., 2019; Serra et al., 2013; Steinberg et al., 2020; Wermser and Lopez, 2018).

Until recently, the ability of biofilm-forming bacteria to generate complex architectures was attributed exclusively to self-produced **organic** extracellular matrix (ECM) (Dragos and Kovacs, 2017; Reichhardt and Parsek, 2019; Steinberg and Kolodkin-Gal, 2015), composed of carbohydrate-rich polymers (i.e., lipids or exopolysaccharides), proteins, and nucleic acids (Branda et al., 2005). However, we and others have recently shown that microbial biofilms contain an organized internal mineral structure, composed of crystalline calcium carbonate (calcite), that also contributes to their 3D morphology (Keren-Paz et al., 2018; Keren-Paz and Kolodkin-Gal, 2020; Li et al., 2015).

Formation of minerals is known to be induced in bacterial biofilms, through passive surface-mediated processes promoted by by-products of bacterial metabolism. Biologically induced minerals associated with bacteria include oxides of Fe, Mn,

116 and other metals; metal sulfates and sulfites; phosphates and carbonates; and Fe
117 and Fe-Al silicates (Frankel and Bazylinski, 2003). Of all examples of
118 biomineralization, microbial induced calcium carbonate precipitation (MICCP) is
119 most frequently associated with microbial communities (Weiner and Dove, 2003),
120 (Douglas and Beveridge, 1998). In geological settings, bacterial metabolic
121 processes, such as urea hydrolysis by urease-positive bacteria, increase local
122 concentration of bicarbonate and elevate pH. When enough environmental
123 calcium is present, those changes promote spontaneous precipitation of calcium
124 carbonate, with bacterial envelopes serving as nucleation sites for the growing
125 mineral, leading to formation of crystalline calcium carbonate (Dhami et al.,
126 2013). Similar processes were also suggested to promote biofilm-associated
127 calcification of *Proteus mirabilis* on catheters (Morris and Stickler, 1998), and
128 during dual-species biofilm formation (Li et al., 2016b) and to contribute to *P.*
129 *aeruginosa* virulence in an insect host (Lotlikar et al., 2019). In both clinical and
130 environmental scenarios, calcium deposition in biofilms was mostly seen as an
131 uncontrolled and unintentional byproduct of bacterial metabolic activity.

132 Recently, intracellular amorphous calcium carbonate (ACC) granules were
133 detected in some species of Gram-negative autotropic bacteria (Blondeau et al.,
134 2018). Those intracellular deposits were suggested to contribute to single cell
135 energy metabolism by promoting photosynthesis (Blondeau et al., 2018), or
136 chemolithoautotrophy (Monteil et al., 2020). While the precise molecular
137 mechanisms regulating this process are still unresolved, the presence of a
138 microcompartment dedicated to mineralization raises the possibility that some
139 bacteria might be capable of controlling the formation of biogenic mineral.

140 Our recent discovery of precisely organized mineral macrostructures within
141 biofilms suggests that biomineralization in biofilms is tightly controlled. These
142 calcite ‘skeletons’ contributed to fitness of biofilm colonies in two unrelated soil
143 bacteria: *Bacillus subtilis* and *Mycobacterium smegmatis* (Keren-Paz et al.,
144 2018; Oppenheimer-Shaanan et al., 2016). For both, the mineral structure acted
145 as a structural scaffold supporting the 3D architecture of the colony, and as

diffusion barrier preventing the penetration of solutes into the biofilms. The formation of controlled calcium carbonate macrostructures in phylogenetically distinct heterotrophic biofilms raises the possibility that the function of calcium carbonate is not limited to specific species utilizing it for intracellular chemical energy, but is instead a general phenomenon wide-spread in the bacterial kingdom.

Those reports challenge the current view of biofilm development as a process depending solely on organic ECM production. They raise several fundamental questions regarding the developmental role of mineral skeleton formation within a bacterial community, and the relation between structure and function in microbial biofilms. If indeed mineralization is a controlled process resulting in a structure crucial to biofilm development and function, this is a novel aspect of basic biofilm biology. What is the role of mineral structure in the development of differentiated biofilm community? Are there dedicated ‘osteoblast-like’ cells initiating mineral production? What are the fitness advantages that the mineral exoskeleton confers to the community? Furthermore, organic matrices and their regulation differ between different organisms (Steinberg and Kolodkin-Gal, 2015). However, the formation of calcium carbonate relies on a highly conserved building blocks, calcium and carbonate, generated from carbon dioxide in most heterotopic organisms. Therefore, the generation of mineral scaffolds could be common and conserved across the bacterial domain, contributing to the phenotypical resistance of diverse microbial biofilms. If so, understanding the underlying molecular mechanisms of biofilm mineralization is a crucial step towards our ability to successfully combat biofilm infections.

In this work, we discovered that the calcium-dependent 3D organization of the biofilm colony leads to transcriptional reprogramming of the bacterial community, and was essential for biofilm development. Calcite formation and the assembly of a functional mineralized macro-skeleton supporting the 3D morphology of a biofilm colony was associated with defined intracellular calcium-rich deposits present in a distinct subpopulation of biofilm cells, differing from flagellin and

176 organic ECM producers. Calcite formation turns out to be an extremely
177 conserved process, occurring not only in the beneficial model organism *B.*
178 *subtilis*, but also in two unrelated lung pathogens – *P. aeruginosa* and *M.*
179 *abscessus*. Inhibition of key biomineralization enzymes or calcium uptake
180 prevented biofilm formation by both pathogens. We were able to identify calcite
181 in sputum samples taken from CF patients, suggesting that this conserved
182 process is of clinical importance. Finally, in an *ex vivo* lung model, chemical and
183 genetic inhibition of calcium uptake and of carbonate accumulation blocked
184 biofilm formation and lung colonization, preventing damage inflicted by *P.*
185 *aeruginosa* to lung tissues, and sensitized *P. aeruginosa* to antibiotic treatment.

186 Taken together, our results identify a previously overlooked process essential for
187 bacterial biofilm development – **the tightly regulated formation of mineral**
188 **skeletons by dedicated cells**. Its conservation across the bacterial kingdom
189 highlights the fundamental role it plays in biofilm biology, and could lead to novel
190 therapeutic approaches for combating a broad range of persistent biofilm
191 infections.

192

193 **Results**

194 In a previous work, we demonstrated that *B. subtilis* biofilm morphology is calcium-
195 dependent (Oppenheimer-Shaanan et al., 2016). Removing calcium acetate from
196 the undefined biofilm-promoting B4 medium prevented the development of the
197 complex 3D architecture characteristic of a biofilm colony, without affecting
198 bacterial growth. This 3D structure required calcium (as no equivalent cation could
199 replace it and as it was induced by various calcium salts) and metabolically
200 produced carbonate (as it was eliminated under anaerobic conditions and
201 increased in CO_2-enriched environment). The mineral component was identified
202 as calcite by FTIR and XRD (Supplementary Fig. 1, (Oppenheimer-Shaanan et al.,
203 2016). It spanned the entire colony and was spatially co-localized with colony
204 wrinkles, suggesting it acted as a “scaffold” supporting them (Oppenheimer-
205 Shaanan et al., 2016). The mineral structure developed and increased over time

206 as the colony grew (Keren-Paz et al., 2018). Finally, as the biofilm colonies aged
207 and their 3D spatial structure diminished, the internal and organized mineral
208 deteriorated (potentially due to fermentation and acidification of the medium by
209 oxygen-depleted cells within the inner mass of the aging biofilm), and only large
210 non-structural crystals were left in the colony periphery due to a passive growth of
211 calcite crystals (Oppenheimer-Shaanan et al., 2016). During biomineralization, the
212 formation of exopolysaccharides and the amyloid protein TasA was also essential
213 to form complex morphology, and interactions between the organic and inorganic
214 matrix were evident (Oppenheimer-Shaanan et al., 2016).

215 To better understand the molecular mechanisms underlying the formation of
216 functional mineral macrostructures, we examined the effect of calcium on gene
217 expression in a *B. subtilis* biofilm colony by sequencing the transcriptome of
218 colonies grown either with or without excess calcium (Fig. 1A). The addition of
219 calcium resulted in a dramatic reprogramming of the transcriptional profile of the
220 colony, with 20% of the genome (n=875) significantly changing between the two
221 conditions at all time-points tested (Supplementary File 2). Only in the presence of
222 added calcium, specific developmental processes defining the biofilm state, such
223 as induction of ECM production and sporulation, and the repression of motility,
224 were observed (Fig. 1B). These fundamental changes were mostly sustained over
225 time (Fig. 1C). We next compared the two transcriptional signatures to a collection
226 of published data, containing 269 mRNA profiles of *B. subtilis* grown under over
227 100 different conditions (Nicolas et al., 2012) (Fig. 1D). The transcriptome of cells
228 grown with excess calcium was similar to that of previously analyzed biofilm cells,
229 regardless of the biofilm promoting medium used. On the other hand, the
230 transcription profile of colonies grown without added calcium was similar to that of
231 planktonic cells experiencing nutrient starvation, but was not identified as biofilm
232 in this unbiased analysis.

233 These findings highlight the central role of calcium in biofilm development, and
234 suggest an intimate connection between biofilm structure and function - raising the
235 possibility that microbial cells are actively regulating calcium carbonate
236 biomineralization. Indeed, when a biofilm colony was visualized by scanning

electron microscopy (SEM), we observed a highly mineralized subpopulation of cells, tightly associated with mineral crystals (Fig. 1E, Supplementary Fig. 2). Consistently with our previous characterization of bleached biogenic calcite, the unbleached crystals had rough faces, along with a smooth and flat crystal face and displayed elongated prismatic morphology instead of the rhombohedral morphology of calcite grown in pure solution (Oppenheimer-Shaanan et al., 2016).

The crystals were consistent with biogenic calcite generated by *B. subtilis (Oppenheimer-Shaanan et al., 2016)*, as judged by shape; backscatter mode and energy dispersive X-ray spectroscopy (EDX) (Fig. 1F, Supplementary Fig. 2). In the absence of added calcium, mineral crystals were rarely detected (Supplementary Fig. 3). Mineral producers were not associated with organic ECM, did not carry flagella, and did not sporulate (Figures 1E, Supporting Figures 2 and 3), and therefore morphologically differed from previous subpopulations observed in *B. subtilis* biofilms (Vlamakis et al., 2008). To create the 3D reconstruction, a whole, unfixed bacterial colony was transferred to a plastic slide for a microCT scan, and rotated between the X-ray source and the detector positioned at optimal distances for a voxel size of 0.87 μm. 2D projections were taken at different angles until a full rotation (360˚) was completed. The full set of images was then used to reconstruct the whole volume of the sample by back projection algorithm and thus a high-resolution 3D image was generated (see Supplementary Movie 1). This microCT X-ray scan confirmed that calcite is organized in a macro-structure, generating a non-uniform continuous layer throughout the wrinkles (Fig. 1G).

One possible mechanism linking calcium mineralization and colony structure is that, in the presence of calcium, some kind of specialized cells located in specific regions of the colony, would serve as nucleation sites and promote localized mineral formation. The observed induction of sporulation regulon transcription by calcium (Fig. 1B) raised the possibility that spores could serve this function, possibly due to unique membrane properties of the spore. However, a mutant strain lacking sporulation sigma factor SigF (de Hoon et al., 2010), which does not form spores and is blocked early during sporulation, was able to form highly structured colonies, in a calcium-dependent manner (Supplementary Fig. 4),

268 suggesting that biomineralization promotes biofilm development and sporulation,
269 and not *vice versa*.

270 The tight association of calcium carbonate mineral with the bacterial cells led us to
271 examine the role of intracellular calcium. Disruption of intracellular calcium
272 homeostasis by a chemical inhibitor of P-type ATPases sodium metavanadate
273 (SMV) (Clausen et al., 2016; Guragain et al., 2013; Kuhlbrandt, 2004), prevented
274 the formation of a complex 3D structure even in the presence of excess calcium
275 (Fig. 1H). This morphological defect was not due to inhibition of planktonic growth,
276 which was only mildly affected by SMV (Fig. 1I). This need for controlled
277 intracellular calcium levels raised the possibility that carbonate mineralization
278 starts intracellularly. Therefore, we next examined single cells from biofilms grown
279 with excess calcium.

280 3D reconstructions of cryo-fixed bacteria with scanning transmission electron
281 tomography (CSTET) (Wolf et al., 2014) (Fig. 2A-B) revealed calcium dense foci
282 inside the cells, associated with the membrane. CryoSTEM imaging and cryo-EDX
283 measurements verified excess calcium in these deposits (Supplementary Fig. 5).
284 The appearance of these deposits was calcium dependent, as we could not
285 observe them in cells grown without added calcium (Supplementary Fig. 6). While
286 the mineral producing sub-population was small, it was enriched in the structured
287 zones of the colony (Fig. 2C). Next, we examined the distribution of cellular calcium
288 levels within the biofilm community. We stained live biofilm cells with calcein-AM –
289 a dye that can be used for visualization of intracellular calcium in live cells (Hale et
290 al., 2000). This lipophilic stain can freely enter the cells, but only binds calcium and
291 becomes fluorescent after the acetoxymethyl (AM) groups are removed by cellular
292 esterases. Gated-STED fluorescent microscopy revealed heterogeneity in cellular
293 calcium levels within the biofilm colony, with most cells containing low levels of
294 calcium, while a smaller fraction contained very high levels (Fig. 2D). The number
295 of cells showing a strong calcein signal increased during biofilm development, as
296 the calcified structures matured. While the cytoplasm of most cells was stained
297 evenly, a small fraction of cells (2.6% out of 300 cells examined) displayed
298 concrete, membrane-localized calcium deposits (Fig. 2E), comparable in their

299 frequency, distribution and localization with calcium deposits identified by CSTET
300 (Fig. 2A-C). Consistent with our previous observation, sporulation does not seem
301 to be involved in cellular calcium deposition – as Δ*sigF* cells contained calcium-
302 rich foci similar to those of wild type (Supplementary Fig. 7). Furthermore, we could
303 detect such deposits associated with the cell envelope on the cytoplasm side,
304 embedded within the membrane, and closely associated with the cell from the
305 outside – suggesting different stages of export (Fig. 2F). Similar calcium deposits
306 were also observed by CSTET in close association with the outer membrane of
307 bacterial cells (Fig. 2G). Taken together, those finding support the existence of a
308 calcium-concentrating compartment in a subpopulation of heterotropic biofilm
309 cells, similar to that recently discovered in autotrophic bacteria (Blondeau et al.,
310 2018), and that the mechanism for its formation involves an active and regulated
311 calcium uptake, as well as the export of the stored calcium (Fig.2F).

312 In *B. subtilis*, the transcriptional regulation of calcium uptake is poorly
313 characterized. We therefore examined the potential involvement of several general
314 transcriptional regulators of cation uptake. Non-ferric metal homeostasis in
315 bacteria is regulated by the MntR, MerR and ArsR familes of transcriptional
316 regulators (Moore and Helmann, 2005). Strains lacking *arsR*, *mntR* and *zur*
317 developed robust and structured biofilm colonies. In contrast, *cueR* mutant had a
318 severely altered biofilm phenotype and formed completely flat colonies (Fig. 2H).
319 CSTET analysis was suggestive of diminished calcium-concentrating population
320 (a maximum of 1.3% compared with 4% of the wild type, n=149). CueR is a poorly
321 characterized transcriptional regulator belonging to the metal-responsive MerR
322 family (Moore and Helmann, 2005). CueR binds the promoter of copper
323 transporting P-type ATPase CopA, but a deletion of *copA* had no effect of biofilm
324 morphology (Supplementary Fig. 8). The effect on biofilm morphology was also not
325 due to reduction in growth of *cueR* mutant (Supplementary Fig. 9). Those
326 observations raise the possibility that CueR regulates additional, yet undiscovered,
327 P-type ATPase, involved in calcium uptake. To characterize the regulon of CueR,
328 we repeated the transcriptome analysis of the wild-type strain and of *cueR* mutant,
329 both grown in the presence of calcium. When compared to the wild type colony,

330 the expression of matrix genes was upregulated and motility was inhibited in the
331 *cueR* mutant, suggesting a general dis-regulation of biofilm transcriptional profile
332 (Fig. 2I). Biofilm formation is a tightly regulated developmental process, and both
333 inhibition and overexpression of key components is known to cause severe defects
334 in biofilm development (for example, both deletion and over-activation of the biofilm
335 regulator Spo0A prevent normal biofilm development (Veening et al., 2006). High
336 levels of ECM gene expression further indicates that the featureless morphology
337 of the *cueR* mutant is not a result of the lack of organic ECM components. The
338 subsequent analysis of the expression of divalent cation transporters reveled that
339 their expression is significantly altered in *cueR* mutant, while other transporters are
340 less affected (Fig. 2I). Among those, we identified three calcium transporters with
341 transcriptional levels significantly changed in the mutant: *yloB* (ATP-driven Ca^{2+}
342 pump) (Gupta et al., 2017) *, chaA* (H^+/Ca^{2+} exchanger) (Fujisawa et al., 2009) and
343 *yetJ* (pH-dependent calcium leak channel) (Chang et al., 2014) (Supplementary
344 File 2). Deletion of *yloB* (but not *chaA*, *yetJ*, or both) was sufficient to prevent 3D
345 colony morphology formation, indicating the importance of calcium uptake and
346 homeostasis (Fig. 2J and Supplementary Fig. 10).

347 Taken together, these observations suggest that the first critical step in the
348 formation of calcite mineral is initiated in saturated mineral precursor
349 microenvironments within the cell. After the initial nucleation, the precursor seems
350 to be exported out of the bacterial cell without hampering the membrane integrity
351 of its producer, and to further grow on organic ECM templates in the highly complex
352 biofilm microenvironment, until an organized functional mineral macro-structure is
353 formed.

354 The organic ECM components and the molecular mechanism governing
355 transition from planktonic life style to the biofilm state vary immensely between
356 phylogenetically distant bacteria, making a development of broad range drugs
357 specifically targeting biofilms challenging. In this sense, biomineralization is an
358 extremely appealing target, as it relies on simple and conserved mechanisms
359 allowing accumulation of calcium and carbonate. We have recently reported that,
360 like *B. subtilis*, the biofilm colonies of soil actinobacterium *M. smegmatis* contain

361 functional mineral macro-structures (Oppenheimer-Shaanan et al., 2016). One
362 additional study provided evidence for some spatial organization of calcium
363 carbonate mineral in submerged biofilms formed by the Gram-negative
364 pathogen *P. aeruginosa* (Li et al., 2016a). This led us to explore whether we can
365 detect regulated formation of functional mineral structures in pathogenic biofilm
366 colonies.

367 When grown on biofilm-inducing agar, *P. aeruginosa* PA14 forms complex
368 colonies with a highly characteristic morphology, which were shown to be
369 dependent on polysaccharides encoded by the *pel* operon (Colvin et al., 2011)).
370 However, like in *B. subtilis,* these complex 3D structures were co-localized with
371 spatially organized mineral, as determined by microCT (Fig. 3A), were abolished
372 by the cation chelator EDTA and EGTA, and could be restored by addition of
373 excess calcium (Fig. 3B, C). 3D reconstruction confirmed that the densest mineral
374 areas formed non-continuous layer correlated with colony wrinkles (Fig. 3D),
375 further suggesting that the 3D structure of *P. aeruginosa* biofilms likely relies on
376 the formation of calcite mineral scaffolds. The formation of elaborate calcium-
377 dependent 3D morphology was conserved in an additional strain of *P. aeruginosa*
378 PA01 (Supplementary Fig. 11). The mineral and exopolysaccharides were both
379 essential for structure formation, as a mutant for both *pel* and *psl* operons was flat
380 and featureless, similarly to biofilms grown in the presence of a calcium chelator
381 (Supplementary Fig. 12).

382 To indicate whether the formation of mineral scaffolds is a conserved process of
383 significant clinical potential, we tested whether it occurs in another,
384 phylogenetically distant, drug-resistant pathogen. We therefore examined the
385 biofilms of *M. abscessus,* which are held together by extracellular matrix
386 consisting of mycolic acids (Halloum et al., 2016), which bear little resemblance
387 to EPS components of *B. subtilis* and *P. aeruginosa*. Nevertheless, this
388 bacterium also only formed colonies of complex morphology in the presence of
389 calcium (Fig. 3E). Fourier-transform infrared spectroscopy (FTIR) detected
390 calcium carbonate mineral in the colony (Supplementary Fig. 13), and X-Ray
391 analysis revealed that the densest mineral areas were co-localized with colony

392 wrinkles (Fig. 3F), just like in *B. subtilis* and *P. aeruginosa* colonies. In addition
393 to the organized macro-scale structures present throughout the colony, over
394 time large crystals also formed beneath the colony and in its periphery,
395 reminiscent of crystals passively precipitated in the vicinity of *B. subtilis* colonies
396 at later stages. All the bacteria we tested formed mineral macrostructures in a
397 slightly basic-neutral macro pH (Supplementary Fig. 14). Under such conditions,
398 passive mineral precipitation is unlikely, supporting a potential biological
399 regulation of mineralization.

400 The cellular pathways of biomineralization present in all three bacteria are
401 strikingly conserved (Fig. 4A), and can be inhibited at several points. The role of
402 urease and carbonic anhydrase (CA) in biomineralization is well established
403 (Jansson and Northen, 2010; Phoenix and Konhauser, 2008). The consequent
404 action of those enzymes increases local bicarbonate levels, and at the same
405 time increases the pH, creating an environment favorable to mineral
406 precipitation. In the section above, we have shown that intracellular calcium was
407 needed for calcite formation. Consistent with the central role of calcite scaffolds
408 in supporting biofilm morphology, inhibition of urease, CA, and especially P-type
409 ATPases, disrupted *P. aeruginosa* biofilm morphology (Fig. 4B) and abolished
410 the mineral macrostructures as judged by microCT X-Ray (Supplementary Fig.
411 15). This effect could not be explained by inhibition of planktonic growth, which
412 was mostly unaffected (Fig. 4C, Supplementary Fig. 16). Similarly, reducing self-
413 produced and atmospheric carbon dioxide reduced the mineral macrostructure
414 (Supplementary Fig. 17).

415 Inhibition of complex calcium-dependent colony formation, but not planktonic
416 growth, was also observed in mutants lacking two or more carbonic anhydrase
417 genes or a calcium transporter CalC (Fig. 4D). Biofilm formation on polystyrene,
418 as quantified by crystal violet staining, was also severely compromised by either
419 chemically or genetically impairing carbonate formation or calcium uptake (Fig.
420 4E-F). The fact that SMV addition or CalC deletion could prevent 3D morphology
421 development and biofilm formation, but had little effect on planktonic growth, is

422 consistent with our view that carbonate mineralization in biofilms relies on
423 regulation of intracellular calcium homeostasis.

424 Potentially broad clinical implication of these findings was supported by testing
425 the same chemical inhibitors on *M. abscessus*. Colony morphology could be
426 compromised by inhibiting biomineralization enzymes (Fig. 4G and
427 Supplementary Fig. 19). Morphology defects could not be explained by inhibition
428 of cell growth, as most potent morphology inhibitors AHA and SMV had the
429 smallest effect on planktonic growth (Fig. 4G). We could therefore conclude that
430 biomineralization is indeed highly conserved, and is mediated by a previously
431 untargeted druggable pathway.

432 Biofilm mineralization was not previously demonstrated in the lung
433 microenvironment. To test the ability of lung pathogens to form structured
434 communities in such conditions, we grew *M. abscessus* and *P. aeruginosa* on
435 synthetic CF sputum medium (SCFM). Both species were capable to form highly
436 organized 3D colonies on SCFM (Supplementary Fig. 20). For *P. aeruginosa*, this
437 robust colony architecture was enhanced by addition of calcium to the growth
438 medium, and diminished with the addition of calcium chelator EGTA (Fig. 4H);
439 suggesting that biofilms may rely on crystalline calcium carbonate scaffolds to
440 support the community architecture in the lung. Therefore, we examined whether
441 calcite formation by lung pathogens could be also observed in clinical samples.
442 We collected sputum samples from CF patients chronically infected with *P.*
443 *aeruginosa* and removed the organic matter by bleach. Insoluble material
444 indicating the presence of a mineral was found in 31 out of 50 samples examined
445 (Supplementary Table 3). These putatively positive patients were further examined
446 by FTIR for residual pure minerals, and spectra typical of calcite, lacking a v1 peak
447 and containing a v_2 peak at 875 cm^{-1}, v_3 peak at 1425 cm^{-1} , and v_4 peak at 713 cm^{-}
448 ^{1} (Supplementary Figure 21), were identified in 7 samples.

449 To further explore the role of biomineralization during lung infection by *P.*
450 *aeruginosa*, we set up a lung *ex vivo* model. Organ cultures derived from lung and
451 colon tissues have been applied previously for the study of biofilm infections and

452 viral tropism (Kolodkin-Gal et al., 2008; Kolodkin-Gal et al., 2007; Massler et al.,
453 2011; Wu et al., 2021), and thus can potentially be used to study lung infections.
454 We cultured mice lungs and then infected them with *P. aeruginosa* constitutively
455 expressing GFP. Fluorescent microscopy revealed biofilms forming on the tissue
456 prior to its destruction (Supplementary Fig. 22). Using this system, we then tested
457 the effect of chemically blocking biomineralization during initial infection stages.
458 When left untreated, *P. aeruginosa* infected tissue (labeled by DAPI for
459 visualization) was completely deteriorated. Infection in the presence of urease
460 inhibitor AHA prevented biofilm formation and rescued the lung tissue (Fig. 5A).
461 While the tissue was rescued, the bacterial load in the growth media remained
462 similar (Fig. 5B). This is consistent with the *in vitro* results presented above – as in
463 all cases inhibition of biomineralization prevented biofilm formation, but had little
464 effect on planktonic growth. This further strengthens our proposed hypothesis,
465 suggesting biomineralization is a specific and controlled developmental feature of
466 bacterial biofilms. To quantify the effects of urease inhibition during lung tissue
467 infection, the tissue was stained with H&A and the nuclei were counted (Fig. 5C-
468 D). We observed a significant rescue of the infected tissue by AHA, in a dose-
469 dependent manner. In agreement with these findings, the growth of bacteria in the
470 medium was not reduced by AHA addition, but their colonization of the lung tissue
471 was largely compromised (Fig. 5E and 5F).

472 Significant reduction of lung cell death during infection by *P. aeruginosa* was also
473 obtained with CA and calcium uptake inhibitors (Fig. 6A-C), and with mutants
474 lacking CAs (Fig. 6D). Consistent with our observation that both Diamox and AHA
475 inhibited lung colonization and subsequent cell death, the combination of both was
476 additive (Figure 6E). At the concentrations used here, AHA and DTNB were slightly
477 toxic to the uninfected lung tissue, while SMV and Diamox had no adverse effect
478 (Supplementary Fig. 23). We can therefore conclude that the entire
479 biomineralization pathway, rather than a specific enzyme, is of crucial importance
480 to host infection.

481 Finally, while the ability to rescue host tissue by inhibition of biomineralization is
482 encouraging, in clinical settings the eradication of bacteria is essential. We

483 therefore tested whether inhibition of mineralization during lung infection of *P.*
484 *aeruginosa* could increase its sensitivity to antibiotics. While neither
485 biomineralization inhibitors nor quinolone antibiotics (a first-line antibiotic for
486 treating *P. aeruginosa* infections) (Hewer and Smyth, 2017) could effectively
487 eradicate established biofilms, the combination of both classes of drugs
488 significantly increased the sensitivity of biofilms to treatment (Fig. 6F) – highlighting
489 the potential clinical applications of biomineralization inhibition.

490

491 **Discussion**

492 Bacterial biofilms are widespread in nature, forming multicellular colonies on
493 various surfaces. The molecular mechanisms guiding the complex network of
494 events leading to the transition from a free-living planktonic bacterium to the
495 differentiated community are well characterized. Much is known about the
496 production of organic matrix, the division of labor between the different cell
497 types, the metabolic adaptations, the methods of communication between the
498 community members – and the fitness advantages of such a communal life style
499 are evident.

500 In addition, bacterial biofilms were extensively shown to promote biomineralization
501 of metals (such as gold and iron), phosphate and carbonate (Phoenix and
502 Konhauser, 2008). In this work, we uncover a previously overlooked, but
503 fundamental, connection between the highly regulated biofilm development and
504 the seemingly random biomineralization occurring within biofilms. We propose that
505 bacterial communities actively control the formation of mineral macrostructures
506 and that those "skeletons" play an important role in biofilm development and
507 fitness.

508 Moreover, the conserved and wide-spread nature of biomineralization offers
509 novel therapeutic approaches to target one of the most challenging global
510 threats to human health – drug-resistant chronic biofilm infections.

511 The first clues to the presence of a structural mineral component in a biofilm
512 came when we demonstrated that a mature 3D structure of biofilm colonies of *B.*
513 *subtilis* depends on their ability to precipitate precisely organized patterns of
514 calcium carbonate (Oppenheimer-Shaanan et al., 2016). A controlled deposition
515 of a calcium carbonate scaffold structurally supports the complex architecture of
516 biofilm colonies and protects them from the environment by limiting solute diffusion
517 (Keren-Paz et al., 2018).

518 In this work, we build on those preliminary observations to show that crystalline
519 calcium carbonate production is a controlled process fundamental to biofilm
520 development. Only when calcification occurs, *B. subtilis* can successfully activate
521 and maintain the characteristic biofilm transcriptional program, activating matrix
522 production and sporulation while repressing motility (Fig. 1).

523 Mechanistically, the formation of mineral macro-structures is initiated within
524 intracellular compartments of a small subpopulation of cells, which secrete small
525 calcium-rich granules, while remaining intact (Fig. 2). The process is likely actively
526 controlled, as the membrane integrity of the exporting cells is not compromised.
527 Initiating mineral formation within the controlled cellular environment allows the
528 organism to regulate the composition, structure, and physical properties of the
529 mineral – therefore controlling the final function.

530 The best known example of bacteria producing functional minerals formed in a
531 defined intracellular compartment by a highly regulated cellular pathway is the
532 magnetotactic bacteria, forming invagination of membranes to generate
533 magnetosomes (Komeili et al., 2006). Recently, intracellular calcium carbonate
534 mineralization was reported in cyanobacteria (Blondeau et al., 2018) and in a
535 subgroup of poorly characterized magnetotactic bacteria (Monteil et al., 2020).
536 Unlike the above examples, which occur in a specific group of phylogenetically
537 related planktonic aquatic bacteria, the formation of mineralized macrostructures
538 in biofilms is conserved across the microbial domain, highlighting their potential
539 importance for general microbial fitness, unrelated to specific adaptations of single-
540 cell microbial metabolism.

541 Regulated calcification was found to mediate complex 3D biofilm structure in three
542 phyla – the Gram-positive spore former *B. subtilis* and actinobacterium *M.*
543 *abscessus*, and the Gram-negative *P. aeruginosa* (Fig. 3). The conserved nature
544 of cellular pathways promoting biomineralization allowed us to prevent
545 biomineralization in phylogenetically distinct bacteria by chemically and genetically
546 inhibiting carbonate accumulation and calcium uptake (Fig. 4). These results
547 support the notion that mineralization in biofilms is a well-defined developmental
548 process, initiated by a dramatic change in the structure and function at the single
549 cell level within a subpopulation of cells in bacterial biofilms. Similar to the
550 formation of bones initiated by the osteoblast cells within higher animals, the
551 presence of mineral-producing cells within a biofilm leads to the formation of a
552 macrostructure serving specific functions and increasing the fitness of the
553 microbial community.

554 In this work, we demonstrate that, in a subpopulation of cells, calcium uptake and
555 functionally conserved pathways of carbonate production alter the intracellular
556 microenvironment, promoting mineral nucleation and leading to formation of a
557 mineral macro-structure. Numerous studies of *B. subtilis, P. aeruginosa* and
558 additional biofilm formers, established that complex colony architecture is
559 dependent on the organic extracellular matrix (Branda et al., 2005; Colvin et al.,
560 2012; Jones and Wozniak, 2017; Kobayashi and Iwano, 2012; Romero et al., 2010;
561 Vlamakis et al., 2013), and thus hypothesized that the presence of the organic
562 ECM components is sufficient for bacterial biofilm morphogenesis. We found that
563 non-organic ECM components are also necessary for the development of
564 structured bacterial communities. While our results indicate that mineral formation
565 initiates within the cells and requires a controlled intracellular environment, the final
566 mineral macro-structure assembly has to occur in the extracellular
567 microenvironment, guided by complex interactions with the extracellular organic
568 matrix (Keren-Paz and Kolodkin-Gal, 2020), as indicated by the scanning electron
569 microscopy (Figure 4C, (Oppenheimer-Shaanan et al., 2016)). Therefore, both
570 organic ECM components and biogenic minerals are essential but not sufficient for
571 the formation of complex structures.

572 While in our settings bacteria relied on calcite to generate functional scaffolds,
573 other minerals may serve similar functions. For example, deposition of calcium
574 phosphate crystals in *P. mirabilis, P. vulgaris* and *Providencia rettgeri* biofilms
575 blocks catheters in infected patients (Broomfield et al., 2009; Mathur et al., 2006;
576 Tan et al., 2018), and urease-dependent calcium phosphate mineralization was
577 shown to increase *P. mirabilis* resistance to ciprofloxacin (Li et al., 2016c) and
578 increase its survival in dual-species biofilms (Li et al., 2016b). Importantly, the
579 physiological role of calcium phosphate deposits within these medical biofilms and
580 whether dedicated cells facilitate this process remains to be determined.

581 Intriguingly, we could observe the presence of bacterially produced calcite
582 minerals in the sputum of some CF patients infected with *P. aeruginosa*. This
583 observation raises the possibility that lung pathogens can generate functional
584 minerals within the host. While biofilm colonies formed on top of biofilm-inducing
585 agar are a convenient model system mimicking many of the environmental and
586 physical conditions of the host, it lacks the host itself. While several murine models
587 are available for the study of CF (such as *Cftr-/-* knockout mice), they all fail to
588 replicate the acute lung disease and chronic bacterial infections characteristic of
589 CF patients (Semaniakou et al., 2018). *Ex vivo* organ cultures are an experimental
590 system that recapitulates the three-dimensional structure of the tissue and includes
591 several cell types and a variety of extracellular matrix components. On the other
592 hand, organ culture is a controlled microenvironment lacking compounding factors
593 that play a role in any infection, such as efficiency of drug delivery and the immune
594 system (Kolodkin-Gal et al., 2007).

595 Using chemical inhibitors and mutants, we demonstrated that biomineralization by
596 *P. aeruginosa* was necessary for lung deterioration, and that preventing it could
597 rescue the infected tissue. The fact the free-living bacteria do not seem to be
598 affected by inhibiting biomineralization, and remain in the culture, while the tissue
599 itself is rescued, highlights the importance of host attachment during infection. It is
600 also possible that the restrictive properties of crystalline minerals on the diffusivity
601 and viscoelasticity could be deleterious to the host tissue. Moreover, consistent
602 with the protective role of the mineral for the bacterial biofilm, chemical or genetic

603 inhibition of mineralization restored the sensitivity the *P. aeruginosa* to antibiotic
604 treatment (Fig. 5 and Fig. 6).

605 Our work suggests that it is time to reconsider the old definition of bacterial
606 mineralization solely as a passive, unintended byproduct of environmental
607 bacterial activity. Instead, here we provide evidence that it can also be a regulated
608 developmental process originating from a dedicated, previously uncharacterized
609 microbial compartment, conferring clear benefits to biofilm-residing bacteria.
610 Biomineralization is highly conserved, and thus is of enormous clinical significance,
611 as it can yield completely novel classes of broad-spectrum drugs to combat
612 emerging biofilm infections.

613

614 **Methods**

615 **Strains and media**

616 All strains used in this work were either wild type or derivatives from *Bacillus*
617 *subtilis* NCIB 3610, *Pseudomonas aeruginosa* PA14, PA01 and *Mycobacterium*
618 *abscessus* ATCC 19977 (recently renamed *Mycobacteroides abscessus*). A list of
619 strains used in this study can be found in Supplementary Table 1. Deletions of
620 *arsR*, *mntR*, *zur*, *cueR*, *copA* and *chaA* were generated by transforming *B. subtilis*
621 NCIB 3610 with genomic DNA isolated from *B. subtilis* 168 deletion library {Koo,
622 2017 #6} (Addgene, Cat#1000000115) , and verified by PCR. Deletion of *yetJ* and
623 *yloB* was done as previously described, and double and triple mutants were
624 generated by transformation with genomic DNA, and verified by PCR. *P.*
625 *aureginosa* PA01 single *psCA1* (CA1), double *psCA1-psCA2* (CA1-2), and triple
626 *psCA1-psCA2-psCA3* (CA1-2-3) mutants were reported earlier (Lotlikar et al.,
627 2019). Deletion of *calC* was generated as described in (Hoang et al., 2000).

628

629 **Biofilm assays**

630 *B. subtilis* and *M. abscessus* biofilms were grown on B4 biofilm-promoting solid
631 medium (0.4% yeast extract, 0.5% glucose, and 1.5% agar) (Barabesi et al., 2007)

632 supplemented with calcium acetate at 0.25% v/v, incubated at 30°C and 37°C
633 respectively, in a sealed box for enriched CO_2 environment achieved by using the
634 candle jar method (Oppenheimer-Shaanan et al., 2016). *P. aeruginosa* biofilms
635 were grown on TB medium, as described previously (Dietrich et al., 2013) or on
636 Brain Heart Infusion Broth (BHI, Sigma-Aldrich) supplemented with 1% agar.
637 SCFM medium was prepared as in (Palmer et al., 2005) and supplemented with
638 1.5% agar. Crystal-violet biofilm assay was carried out as previously described
639 (O'Toole, 2011).

640 When indicated, the medium was supplemented with the following inhibitors
641 purchased from Sigma-Aldrich: acetohydroxamic acid (AHA) (Cat. #159034); 5,5'-
642 Dithiobis(2-nitrobenzoic acid) (DTNB) (Cat. #D8130); acetazolamide (Diamox)
643 (Cat. #A6011) and sodium metavanadate (SMV) (Cat. #590088). The
644 concentrations used are indicated in the text.

645

646 **Phase microscopy**

647 Biofilm colonies were observed using a Nikon D3 camera or a Stereo Discovery
648 V20" microscope (Tochigi, Japan) with objectives Plan Apo S × 0.5 FWD 134 mm
649 or Apo S × 1.0 FWD 60 mm (Zeiss, Goettingen, Germany) attached to Axiocam
650 camera, as required. Data were captured using Axiovision suite software (Zeiss).

651

652 **Planktonic growth assays**

653 All strains were grown from a single colony isolated over lysogeny broth (LB) plates
654 to a mid-logarithmic phase of growth (4 h at 37°C with shaking). Cells were diluted
655 1:100 in 150 µl liquid B4 medium in 96-well microplate (Thermo Scientific). Cells
656 were grown at 30°C for 20 h in a microplate reader (Synergy 2, BioTek), and the
657 optical density at 600 nm (OD_{600}) was measured every 15 min. Three independent
658 experiments were conducted, with three technical repeats per plate.

659 CFU assay was carried out as previously described in detail (Bucher et al., 2016).

660

661 **RNA extraction and library preparation**

662 Biofilm colonies were grown on biofilm-promoting B4 solid medium with and
663 without calcium for 1, 2, 3 and 6 days. Three independent experiments were
664 conducted, with three colonies from each treatment combined for RNA extraction
665 in each experiment. The samples were frozen in liquid nitrogen and stored until
666 extraction. Frozen bacterial pellets were lysed using the Fastprep homogenizer
667 (MP Biomedicals) and RNA was extracted with the FastRNA PROT blue kit (MP
668 Biomedicals, 116025050) according to the manufacturer's instructions. RNA levels
669 and integrity were determined by Qubit RNA BR Assay Kit (Life Technologies,
670 Q10210) and TapeStation, respectively. All RNA samples were treated with
671 TURBO DNase (Life Technologies, AM2238).

672 A total of 5 ug RNA from each sample was subjected to rRNA depletion using the
673 Illumina Ribo-Zero rRNA Removal Kit (Bacteria, MRZB12424), according to the
674 manufacturers' protocols. RNA quantity and quality post-depletion was assessed
675 as above. RNA-seq libraries were contracted with NEBNext® Ultra™ Directional
676 RNA Library Prep Kit (NEB, E7420) according to the manufacturer's instructions.
677 Libraries concentrations and sizes were evaluated as above, and were sequenced
678 as multiplex indexes in one lane using the Illumina HighSeq2500 platform.

679

680 **RNAseq processing**

681 Reads were trimmed from their adapter with cutadapt and aligned to the *B. subtilis*
682 genome (subsp. *subtilis* str. NCIB 3610, NZ_CM000488.1) with Bowtie2 version
683 2.3.4.1 (Langmead and Salzberg, 2012). The number of uniquely mapped reads
684 per gene was calculated with HT-seq (Anders et al., 2015). Normalization and
685 testing for differential expression was performed with DESeq2 v1.16. A gene was
686 considered to be differentially expressed using the following criteria: normalized
687 mean read count ≥ 30, fold change ≥ 3, and adjusted p value < 0.05. First, we
688 tested for differential expression between samples grown with and without calcium
689 separately for each time point; however, since the results for days 1, 2 and 3 were
690 very similar, we joined days 1-3. The crude read count, normalized read count,

691 and the result of the differentially expression tests are available in Supplementary
692 File 1.

693

694 **Comparison between growth conditions**

695 We compared our RNAseq expression data to publically available transcriptomes
696 representing 269 different growth conditions (Nicolas et al., 2012). Because that
697 study used microarray platform and not RNAseq, the comparison was performed
698 using the top 10% genes with the highest expression level of every condition and
699 every replicate (383 genes per sample). We than used Jaccard index to measure
700 the overlap between the conditions of the two platforms (i.e. the current study and
701 (Nicolas et al., 2012)). Prior to the analysis, we removed 152 genes that appear
702 among the top 10% in more than 80% of the conditions.

703

704 **Scanning electron microscopy and EDX**

705 Biofilm colonies were grown for 1, 3, 6, 10 and 15 days at 30°C on biofilm-
706 promoting B4 solid medium, with or without calcium. The colonies were fixed
707 overnight at 4°C with 2% glutaraldehyde, 3% paraformaldehyde, 0.1 M sodium
708 cacodylate (pH 7.4) and 5 mM $CaCl_2$, dehydrated and dried as in (Bucher et al.,
709 2016; Bucher et al., 2015b). Clinical samples were first partially or completely
710 bleached to remove the organic material by 1 hour of incubation in either 3% or
711 6% sodium hypochlorite, respectively. The insoluble material was collected,
712 washed three times in PBS, three times in acetone and air-dried for 16 hours.

713 Mounted samples were coated with 15 nm thick carbon layer in carbon coater
714 (EDWARDS). The imaging by secondary electron (SE) or back scattered electron
715 (BSE) detectors and the Energy Dispersive X-ray Spectroscopy (EDS, Bruker)
716 were performed using Carl Zeiss Ultra 55 or Supra scanning electron microscopes.

717

718 **Cryo-STEM analysis**

719 Bacterial colonies grown as described were suspended in PBS buffer. Quantifoil
720 TEM grids were glow-discharged with an Evactron Combi-Clean glow-discharge
721 device, and 5 microliters of suspended cells were deposited onto the glow-
722 discharged grids. Ten nm-diameter gold fiducials (Duchesne et al., 2008) were
723 applied before blotting and verification using a Leica EM-GP automated plunging
724 device (Leica). No chemical fixation was used to ovoid artifacts

725 Vitrified samples were observed with a Tecnai F20 S/TEM instrument (Thermo
726 Fisher Scientific) at 200 kV, with Gatan 805 brightfield and Fischione HAADF
727 detectors. Microscope conditions: extraction voltage = 4300 V, gun lens = 3 or 6,
728 and spot size = 5 or 6 with 10 micrometer condenser apertures, yielding probe
729 diameters of 1-2 nm and semi-convergence angles of ~ 1.3- 2.7 mrad. Images of
730 2048 X 2048 pixels were recorded with probe dwell times of 8-18 microseconds.
731 Spatial sampling was set between 1 and 4 nm/pixel. Electron doses were 1–3
732 electrons/A^2 per image. Single-axis tilt series were recorded using SerialEM
733 (Kremer et al., 1996). EDX was performed in STEM mode on vitrified cell samples
734 with the same electron microscope set-up as used for STEM imaging, using a
735 liquid N_2 cooled Si(Li) detector (EDAX). Vitrified grids contacting 100-200 cells per
736 grid were automatically imaged on a Talos Arctica (Thermo Fisher Scientific)
737 microscope in STEM mode, and mapped at intermediate magnifications (10-13k)
738 using SerialEM software to obtain large fields of view for quantification of data
739 presented in Fig. 2C. The quantification was done by manually counting the cells
740 with or without dense deposits.

741

742 **Tomography reconstructions and visualization**

743 The CSTET tomographic tilt series were aligned using fiducial markers and
744 reconstructed using weighted back projection (Frangakis and Hegerl, 2001) as
745 implemented in the IMOD software suite (Kremer et al., 1996). Reconstructions
746 are displayed after non-linear anisotropic diffusion filtering within IMOD.
747 Segmentation and volume rendering were performed using Amira 6.3 (FEI
748 Visualization Sciences Group).

749 **STED image acquisition**

750 Cells were isolated from 3-6 day old biofilm colonies and stained with Calcein-AM
751 (20 µM) and NileRed (100 µg/ml). Immediately after the staining procedure, the
752 cells were mounted on a coverslip and immobilized by an agarose pad (1%
753 agarose, $S7_{50}$ minimal media). STED microscopy was performed using a Leica
754 SP8 confocal microscope with a 100x oil immersion objective (NA: 1.4). A 552 nm
755 laser line was used for confocal detection of NileRed, a 494 nm excitation laser
756 and a 592 nm depletion laser were used for G-STED detection of Calcein-AM.
757 Channels were acquired with 200 Hz (4 x line averaging) and the appropriately set
758 HyD hybrid detectors. Images processing was performed using Leica LAS X and
759 the deconvolution of the respective channels was performed using the Huygens
760 algorithm.

761

762 **Micro-CT X-ray analysis**

763 Images of indicated magnification were taken using a Zeiss micro XCT 400
764 instrument (Pleasanton, CA, USA). Tomography was carried out using a micro-
765 focused source set at 20 kV and 100 µA. 1200 separate 2D images were taken
766 with a pixel size of 0.87 mm over 1800, exposure time of 30 sec. Image analysis
767 was carried out with Avizo software (VSG, Hillsboro, OR, USA).

768

769 **FTIR spectrophotometer analysis**

770 Calcite was collected as described by Mahamid *et al*., with some modifications
771 (Mahamid et al., 2008): agar samples were slightly bleached with 6% sodium
772 hypochlorite for 1 min to remove organic matter, washed with Milli-Q water twice
773 and dehydrated in acetone.

774 FTIR spectra of the bleached samples were acquired in KBr pellets by using a
775 NICOLET iS5 spectrometer (Thermo Scientific, Pittsburgh, PA, USA). The
776 samples were homogenized in an agate mortar and pestle with about 40 mg of

KBr, and pressed into a 7 mm pellet using a manual hydraulic press (Specac, Orpington, UK). Infrared spectra were obtained at 4 cm−1 resolution for 32 scans.

The infrared calcite spectrum has three characteristic peaks, designated v_2, v_3, and v_4. Calcium carbonate v_3 peak is expected at 1425 cm^{-1} for calcite, 1490 cm^{-1} for vaterite and 1475 cm-1 for aragonite. The calcium carbonate v_2 peak is expected at 875 cm^{-1} for calcite, 850 cm^{-1} for vaterite and 855 cm^{-1} for aragonite. Finally, the calcium carbonate v_4 peak is expected at 713 cm^{-1} for calcite, 750 cm^{-1} for vaterite and 715 cm^{-1} for aragonite (Politi et al., 2004).

Patient's samples

Sputum was collected from adult patients as published previously (Schiller and Millard, 1983) and stored in 4ºC degrees. All patients positive for calcite were carrying chronic *pseudomonas* infections. Specific patient information can be found in Supplementary Table 2. The samples were collected under Helskinki approval to Prof. Eitan Keren 0456-17-HMO. For FTIR analysis, organic matter was removed as described above.

***Ex vivo* lung infection system**

Animal work was carried out under ethical approval MD-16-15035-1. Lungs were harvested from 2 mice (one month old) and placed in petri dishes containing DMEM 5% FCS. We divided the tissue into circular pieces 3 mm in diameter with a biopsy punch and transferred to a 24 well plate (4-5 explants/well) with 450 µl DMEM. DMEM was applied with carbenicillin 100 µg/ml (Sigma-Aldrich) and chemical inhibitors, as indicated. To each respective well, either 50 µl DMEM (control) or *P. aeruginosa* (pretreated with chemical inhibitors when indicated and diluted within DMEM to attain OD_{600} 0.4) was added – with three technical repeats for each condition. The plates were incubated at 37ºC for ~2 days (52 hours), washed twice with PBS, fixed with PFA 4% for 10 minutes, and imbedded in either cryosection (OCT) compound or paraffin. Paraffin samples were cut into 7 micron

806 slices and stained with H&E; whereas cryosections were cut into 10 micron slices
807 and placed on superfrost plus slides.

808

809 **Cultivable Cell Quantification (CFU)**

810 CFU quantification was done as previously described (Bucher et al., 2015a). For
811 CFU of bacteria infecting *ex vivo* tissue culture: individual punches or their growth
812 media were collected, Punches were resuspended in 1 ml PBS (Biological
813 Industries), and free-living bacteria were pelleted and resuspended in PBS at the
814 same volume. Samples were thoroughly vortexed (5 min). The samples were then
815 mildly sonicated (BRANSON digital sonifier, Model 250, Microtip, amplitude 30%,
816 pulse 2 × 5 s). In all cases, to determine the number of viable cells, samples were
817 serially diluted in PBS, plated on LB plates, and colonies were counted after
818 incubation at 30°C overnight. For

819

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1101 **Figure Legends:**

1102 **Figure 1: Calcium is necessary for normal biofilm development and structure**

1103 A. Light microscopy images of 6-day-old *B. subtilis* NCIB 3610 biofilm colonies
1104 grown on B4 agar, showing the effect of addition of calcium (0.25% v/v calcium
1105 acetate) on colony architecture. Scale bar – 2 mm. A representative image (out
1106 of n = 3 experiments) is shown.

1107 B. Volcano-plot depicting calcium-dependent changes in the transcriptome in *B.*
1108 *subtilis* biofilm colonies. Significance (P values) vs difference (fold changes)
1109 are plotted. Functional categories were determined by DAVID analysis.

1110 C. A heatmap with all differentially expressed genes (n = 876), scaled to the mean
1111 expression level of each gene. The map is ordered by age of biofilm colonies,
1112 as shown by the left colored bar, where green are genes that are differentially
1113 expressed in days 1-3, and day 6; purple genes that are differentially expressed
1114 in days 1-3 and yellow genes that are differentially expressed in day 6.

1115 D. The top 20 conditions (Nicolas et al., 2012), showing the highest similarity
1116 (expressed as Jaccard index) to the transcriptome of *B. subtilis* 6-day-old
1117 biofilm colonies grown with or without calcium.

1118 E. Biofilm cells closely associated with prismatic mineral structures, as visualized
1119 by Scanning Electron Microscopy (SEM) image of 10-day-old *B. subtilis* biofilm
1120 colony grown with calcium. Upper panel - secondary mode; lower panel -
1121 backscattering mode. Magnification – X25000, scale bar – 1 μm. A
1122 representative field (out of n=5 fields, from 6 experiments) is shown.

1123 F. EDX analysis of areas indicated in (E), indicating that mineral structures are
1124 rich in calcium. Ca – calcium-rich, Cnt – control.

1125 G. 3D re-construction of microCT X-Ray analysis revealing 3D mineral distribution
1126 in a 5-day old *B. subtilis* colony grown with calcium. Color indicates intensity,
1127 with green representing the densest mineral. Scale bar – 0.2 mm. A
1128 representative image (out of n = 3 experiments) is shown.

1129 H. Light microscopy images of 5-day-old *B. subtilis* biofilm colonies grown with
1130 calcium, and supplemented with calcium uptake inhibitor (0.1 mg/ml sodium
1131 metavanadate (SMV)), as indicated. NT indicated untreated culture. Scale bar
1132 – 2 mm. A representative image (out of n = 3 experiments) is shown.

1133 I. The effect of inhibiting calcium uptake on planktonic growth. *B. subtilis* was
1134 grown shaking in liquid LB medium with calcium, supplemented with 0.1 mg/ml
1135 SMV, as indicated. Results are averages of nine wells, bars represent standard
1136 deviations. A representative experiment (out of n=3 experiments) is shown.

1137

1138 **Figure 2: Mineralization is initiated intracellularly**

1139 A. A 30-nm thick virtual slice through a CSTET 3D reconstruction of *B. subtilis*
1140 cells showing intracellular calcium-rich deposits (arrows). Scale bar – 400 nm.
1141 A representative field (out of n=20 fields, from 3 experiments) is shown.

1142 B. Volume rendering (orange) and a single orthoslice (greyscale) through the
1143 center of the volume, from a 3D CSTET reconstruction. The calcium-rich
1144 deposits are artificially colored (blue).

1145 C. Quantification of different subpopulations within a 6-day old *B. subtilis* biofilm
1146 colony, showing enrichment of cells with mineral foci in the structured areas
1147 (wrinkles) of the colony.

1148 D. Intracellular calcium levels of bacterial cells (n=150) stained with calcein-AM, a
1149 calcium-specific fluorescent dye. Cells were isolated from *B. subtilis* biofilm
1150 colonies (day 3 and 6) grown with calcium. Inset – a representative image of
1151 stained cells isolated from a 6 day-old biofilm. Green – calcein-AM, red –
1152 NileRed membrane stain, gray – DIC. Scale bar – 2 μm.

1153 E. and F. G-STED images *B. subtilis* cells isolated from 6 day-old biofilm colonies
1154 grown with calcium. Green – calcein-AM, red – NileRed membrane stain, gray
1155 – DIC. Scale bar – 2 μm.

1156 G. 30-nm thick virtual slice through a CSTET 3D reconstruction. Arrows indicate
1157 calcium-rich deposits associated with a membrane from the outside of the cell.
1158 Scale bar – 400 nm.

1159 H. Light microscopy images of 3-day-old biofilm colonies of wild-type *B. subtilis*
1160 and its mutant derivatives (see text for details) grown with calcium. Scale bar –
1161 2 mm. A representative experiment (out of n=3 experiments) is shown.

1162 I. Volcano-plot depicting CueR-dependent changes in expression of genes in 3-
1163 day old *B. subtilis* biofilm colony. Significance (P values) vs difference (fold
1164 changes) are plotted. Functional categories were determined by DAVID
1165 analysis.

1166 J. Light microscopy images of 2-day-old biofilm colonies of wild-type *B. subtilis*
1167 and its mutant derivatives (see text for details) grown with calcium. Scale bar –
1168 2 mm. A representative experiment (out of n=3 experiments) is shown.

1169

1170 **Figure 3: The role of calcium in the biofilm structure is conserved across**
1171 **bacterial species**

1172 A. Light microscopy (left) and microCT-X-ray (right) images of 3-day-old *P.*
1173 *aeruginosa* PA14 biofilm colonies grown on TB agar. In the X-Ray images, the
1174 bright contrast appears white, and indicates the location of dense mineral, while
1175 the organic matter appears dark. Scale bar – 1 mm. A representative image
1176 (out of n = 3 experiments) is shown.

1177 B. Light microscopy images of 3-day-old *P. aeruginosa* PA14 of biofilm colonies
1178 grown on TB agar supplemented with 1 mg/ml EDTA (chelating agent), either
1179 with or without the addition of 700 mM $CaCl_2$. Scale bar – 1 mm. A
1180 representative experiment (out of n=3 experiments) is shown.

1181 C. Light microscopy images of 3-day-old *P. aeruginosa* PA14 biofilm colonies,
1182 grown on TB agar either untreated (NT), or supplemented with 700 mM $CaCl_2$
1183 and EGTA (calcium chelator), as indicated. Scale bar – 5 mm. A representative
1184 image (out of n = 3 experiments) is shown.

1185 D. 3D re-construction of microCT X-Ray analysis revealing mineral distribution in
1186 a 5-day old *P. aeruginosa* PA14 colony. Color indicates intensity, with green
1187 representing the densest mineral. Scale bar – 0.2 mm. A representative image
1188 (out of n = 3 experiments) is shown.

1189 E. Light microscopy images of 5 day-old *M. abscessus* biofilm colonies, grown on
1190 B4 agar, supplemented with either calcium acetate (0.25% v/v), or 1.5 mM
1191 EDTA, as indicated. Scale bar – 5 mm. A representative image (out of n = 3
1192 experiments) is shown.

1193 F. 3D re-construction of microCT X-Ray analysis revealing mineral distribution in
1194 a 5-day old *M. abscessus* colony grown on B4 agar with calcium. Color
1195 indicates intensity, with green representing the densest mineral. Scale bar –
1196 0.1 mm. A representative image (out of n = 3 experiments) is shown.

1197

1198 **Figure 4: Inhibition of cellular pathways leading to calcium carbonate**
1199 **production disrupts biofilm development in *P. aeruginosa* and *M. abscessus*.**

1200 A. A schematic representation of the chemical reaction leading to calcium
1201 carbonate production, with the inhibitors used in this study indicated in
1202 parenthesis next to the step they inhibit.

1203 B. Light microscopy images of 3-day-old *P. aeruginosa* PA14 biofilm colonies
1204 grown on TB agar containing the inhibitors indicated in (A) as follows: untreated
1205 (NT), 1.5 mg/ml DTNB, 1.75 mg/ml AHA, 2.5 mg/ml Diamox and 0.01 mg/ml
1206 SMV. Scale bar – 2 mm. A representative image (out of n = 3 experiments) is
1207 shown.

1208 C. The effect of inhibiting calcium carbonate production on planktonic growth of *P.*
1209 *aeruginosa* PA14. Bacteria were grown shaking in liquid TB medium,
1210 supplemented with: 1.5 mg/ml DTNB, 1.75 mg/ml AHA, 2.5 mg/ml Diamox and
1211 0.01, 0.05 and 0.01 mg/ml SMV, or untreated (NT). Growth was monitored by
1212 measuring OD600 in a microplate reader every 30 min. Results are averages

1213 of six wells, bars represent standard deviations. A representative experiment
1214 (out of n= 3 experiments) is shown.

1215 D. Light microscopy images of 4-day-old biofilm colonies of wild type *P.*
1216 *aeruginosa PA01* and indicated mutants grown on BHI agar: carbonic
1217 anhydrases (*psCA1* – PA0102, *psCA2* - PA2053, *psCA3* – PA4676) and
1218 calcium channel (Δ*calC*). A representative experiment (out of n= 3
1219 experiments) is shown. Insert – the indicated mutant strains were grown
1220 planktonically in shaking liquid BHI medium. Growth was monitored by
1221 measuring OD600 in a spectrophotometer at indicated times. Results are
1222 averages of 4 wells, bars represent standard deviations. Scale bar – 2 mm. A
1223 representative experiment (out of n= 3 experiments) is shown.

1224 E. Crystal violet assay quantifying biofilm formation of *P. aeruginosa* PA14, grown
1225 in BHI supplemented with the indicated inhibitors. Results are averages and
1226 standard deviation of three independent experiments. P values, as determined
1227 by student's t-test are indicated (* pVal <0.01, ** pVal <0.001, when compared
1228 to the untreated control).

1229 F. Crystal violet assay quantifying biofilm formation of wild type and indicated
1230 mutant derivates of *P. aeruginosa* PA01, grown with in liquid BHI medium.
1231 Results are averages and standard deviation of three independent
1232 experiments. P values, as determined by student's t-test are indicated (* pVal
1233 <0.01, when compared to the untreated control).

1234 G. Light microscopy images of 5 day-old *M. abscessus* biofilm colonies, grown on
1235 B4 agar supplemented with calcium and 2.5 mM of the indicated inhibitors or
1236 untreated (NT). A representative experiment (out of n = 3) experiments is
1237 shown. Insert – *M. abscessus* was grown planktonically in liquid B4 medium.
1238 Growth was monitored by measuring OD_{600} in a spectrophotometer at indicated
1239 times. Scale bar – 2 mm. Results are averages of three independent repeats,
1240 bars represent standard deviations.

1241 H. Light microscopy images of 3-day-old *P. aeruginosa* PA14 biofilm colonies
1242 grown on SCFM agar, either untreated (NT) or suplemented with 700 mM $CaCl_2$

1243 and EGTA, as indicated. Scale bar – 5 mm. A representative experiment (out
1244 of n = 3) experiments is shown.

1245

1246 **Figure 5: Inhibition of urease prevents biofilm formation and tissue**
1247 **deterioration in an *ex-vivo* lunge model**

1248 A. and B. Fluorescent microscopy images of 10 micron slices of lung tissues,
1249 infected with *P. aeruginosa* PA14 and treated with AHA, as indicated. (A) Blue
1250 – lung cell nuclei stained by DAPI, (magnification x20). (B) Green - GFP
1251 expressing *P. aeruginosa*, (magnification x40). A representative experiment
1252 (out of n = 3) experiments is shown.

1253 C. Light microscopy images of 7 micron slices of lung tissue. Samples were
1254 treated as in (A), and stained with hematoxylin and eosin (H&E). Blue – nuclei,
1255 red – extracellular matrix and cytoplasm, (magnification x40). A representative
1256 experiment (out of n = 3) experiments is shown.

1257 D. Quantification of lung viability. Lung tissue cultures were infected with *P.*
1258 *aeruginosa* PA14 and treated with AHA, as indicated. ImageJ 1.51g software
1259 was used to automatically count lung cell nuclei in 4 randomly chosen fields for
1260 each treatment. P values, as determined by student's t-test are indicated (**
1261 pVal <0.001).

1262 E. CFU analysis of *P. aeruginosa* PA14 infecting lung tissue and surrounding
1263 media, with and without treatment. P values, as determined by student's t-test
1264 are indicated (* pVal <0.01, ** pVal <0.001, when compared to the untreated
1265 control).

1266 F. Quantification representing the ratios of tissue to medium CFU in (E).

1267

1268 **Figure 6: Inhibition of calcium carbonate production prevents biofilm**
1269 **formation and tissue deterioration in an *ex-vivo* lunge model**

1270 A. Light microscopy images of 7 micron slices of lung tissues infected with *P.*
1271 *aeruginosa* PA14 and treated with biomineralization inhibitors as indicated.
1272 Samples were stained with H&E. Blue – nuclei, red – extracellular matrix and
1273 cytoplasm, (magnification x40). A representative experiment (out of n = 3)
1274 experiments is shown.

1275 B. CFU of *P. aeruginosa* PA14 infecting lung tissue culture and surrounding media
1276 with and without indicated inhibitors (as in A). P values, as determined by
1277 student's t-test are indicated (* pVal <0.01, ** pVal <0.001, when compared to
1278 the untreated control).

1279 C. Quantification of lung viability. Lung tissue cultures were infected with *P.*
1280 *aeruginosa* PA14 and treated with biomineralization inhibitors as indicated.
1281 ImageJ 1.51g software was used to automatically count lung cell nuclei in 4
1282 randomly chosen fields for each treatment. P values, as determined by
1283 student's t-test are indicated (** pVal <0.001).

1284 D. CFU analysis of wild type *P. aeruginosa* PA01 and the indicated mutants
1285 infecting lung tissue culture and surrounding media. Diamox was applied at 1.1
1286 mg/ml, when indicated. P values, as determined by student's t-test are
1287 indicated (* pVal <0.01, ** pVal <0.001, when compared to the untreated
1288 control).

1289 E. Quantification of lung viability. Lung tissue cultures were infected with *P.*
1290 *aeruginosa* PA14 and treated with biomineralization inhibitors, as indicated
1291 (Diamox and AHA applied at 1.1 mg/m). ImageJ 1.51g software was used to
1292 automatically count lung cell nuclei in 4 randomly chosen fields for each
1293 treatment. P values, as determined by student's t-test are indicated (** pVal
1294 <0.001).

1295 F. *P. aeruginosa* PA14 biofilms were grown for 72 h on TB agar. The colonies
1296 were physically divided into equal parts, harvested, and half was treated for 2
1297 h, as indicated. CIP – Ciprofloxacin, 3 mg/ml; GC – gentamycin, 10 mg/ml; AHA
1298 – 2.5 mg/ml; DTNB – 2 mg/ml. Viability is expressed as the ratio of CFU
1299 between the treated and the untreated parts of a colony. Results are averages

1300 and standard deviation of three independent experiments. P values, as
1301 determined by student's t-test are indicated (** pVal <0.001).

1302

Figure 1

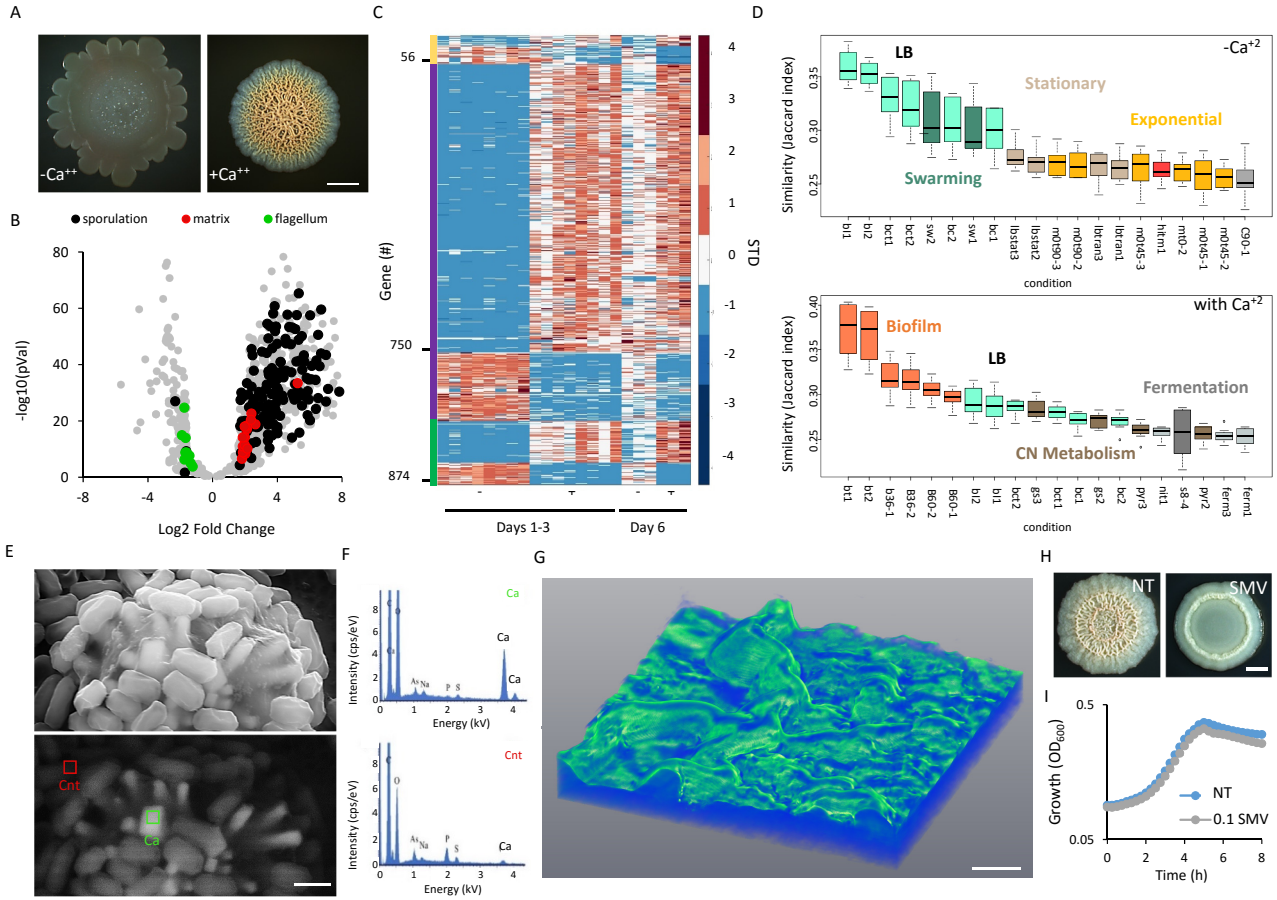
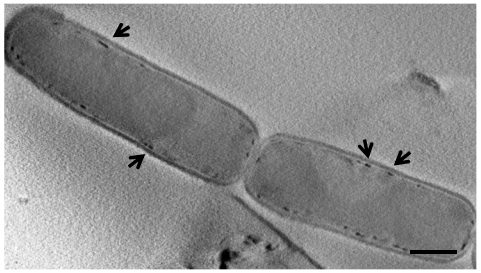
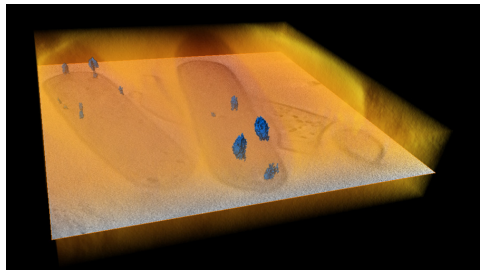

A
-Ca++
+Ca++
B
sporulation
matrix
flagellum
-log10(pVal)
Log2 Fold Change
C
Gene (#)
STD
Days 1-3
Day 6
D
-Ca+2
LB
Stationary
Exponential
Swarming
Similarity (Jaccard index)
condition
with Ca+2
Biofilm
LB
Fermentation
CN Metabolism
E
Cnt
Ca
F
Intensity (cps/eV)
Energy (kV)
G
H
NT
SMV
I
Growth (OD600)
NT
0.1 SMV
Time (h)

Figure 2

A



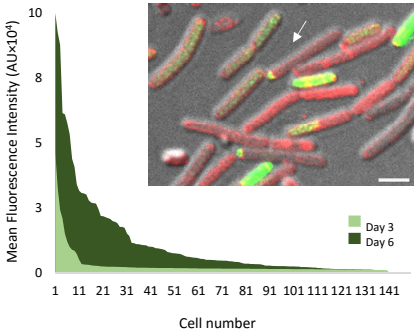
B



C

	Dead	Spore	Mineral producer
Structured (n=510)	12.5%	12.7%	4%
Total colony (n=308)	7.5%	7.5%	1.6%

D



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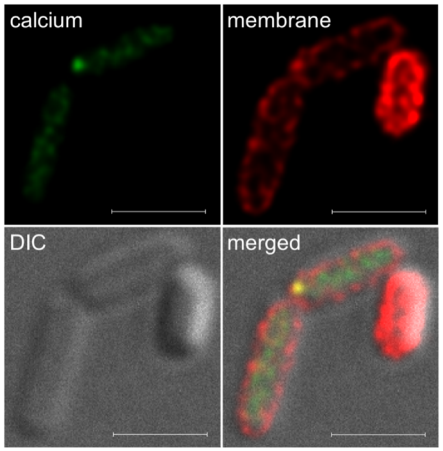

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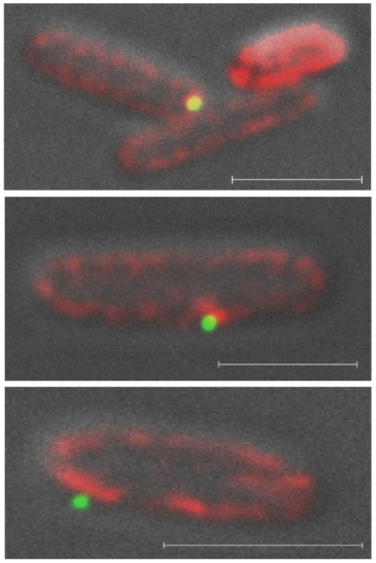
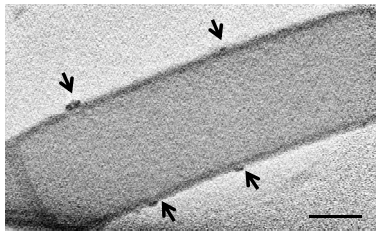


G



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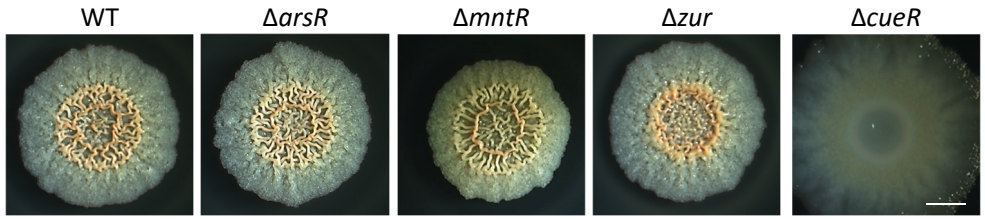

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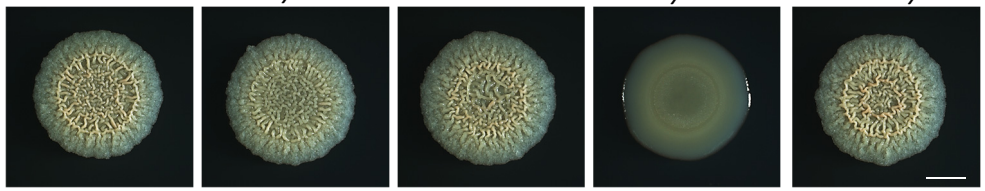

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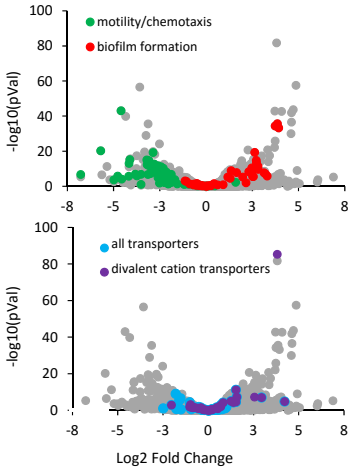


Figure 3

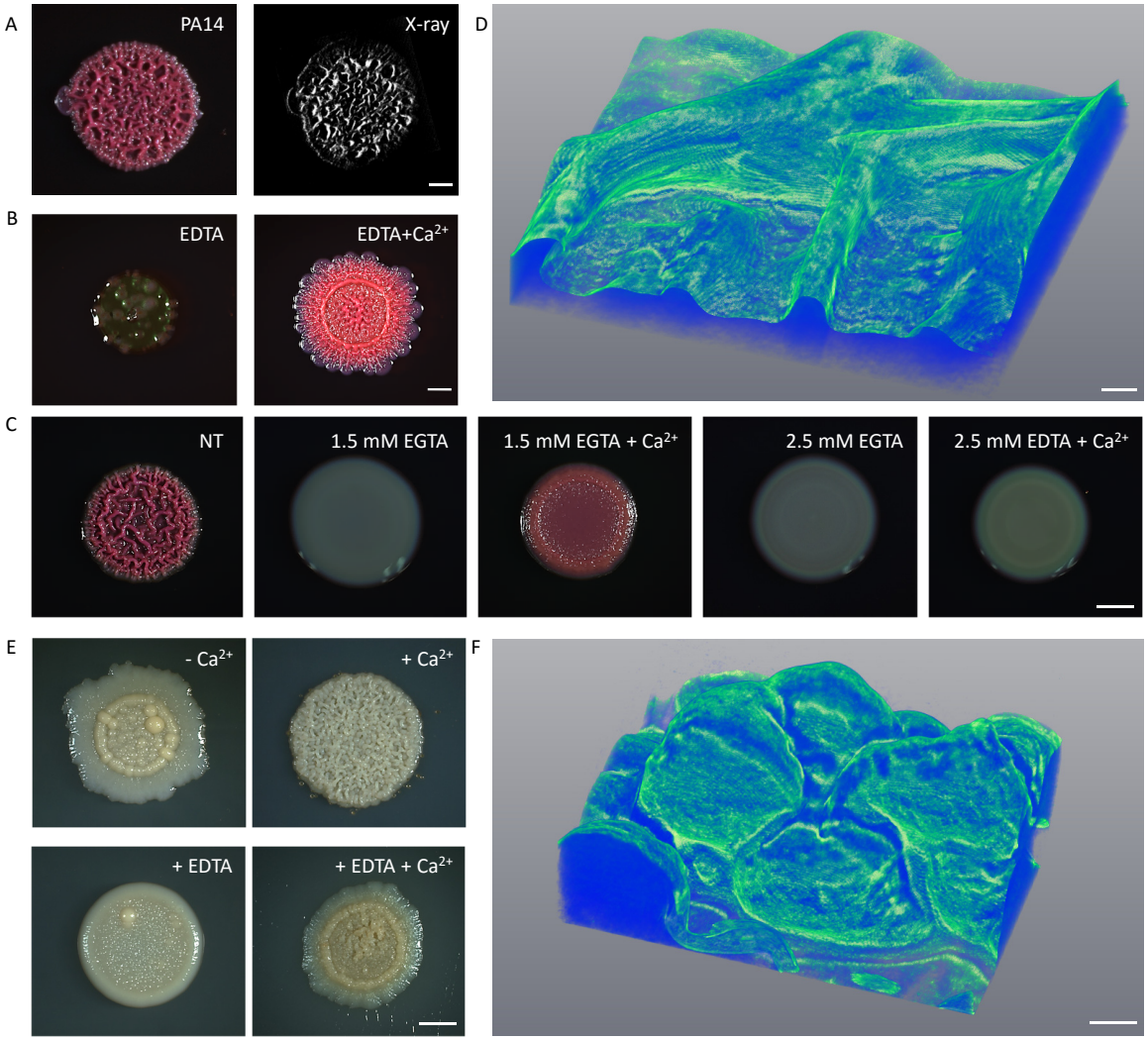

A
PA14
X-ray
B
EDTA
EDTA+Ca2+
C
NT
1.5 mM EGTA
1.5 mM EGTA + Ca2+
2.5 mM EGTA
2.5 mM EDTA + Ca2+
D
E
- Ca2+
+ Ca2+
+ EDTA
+ EDTA + Ca2+
F

Figure 4

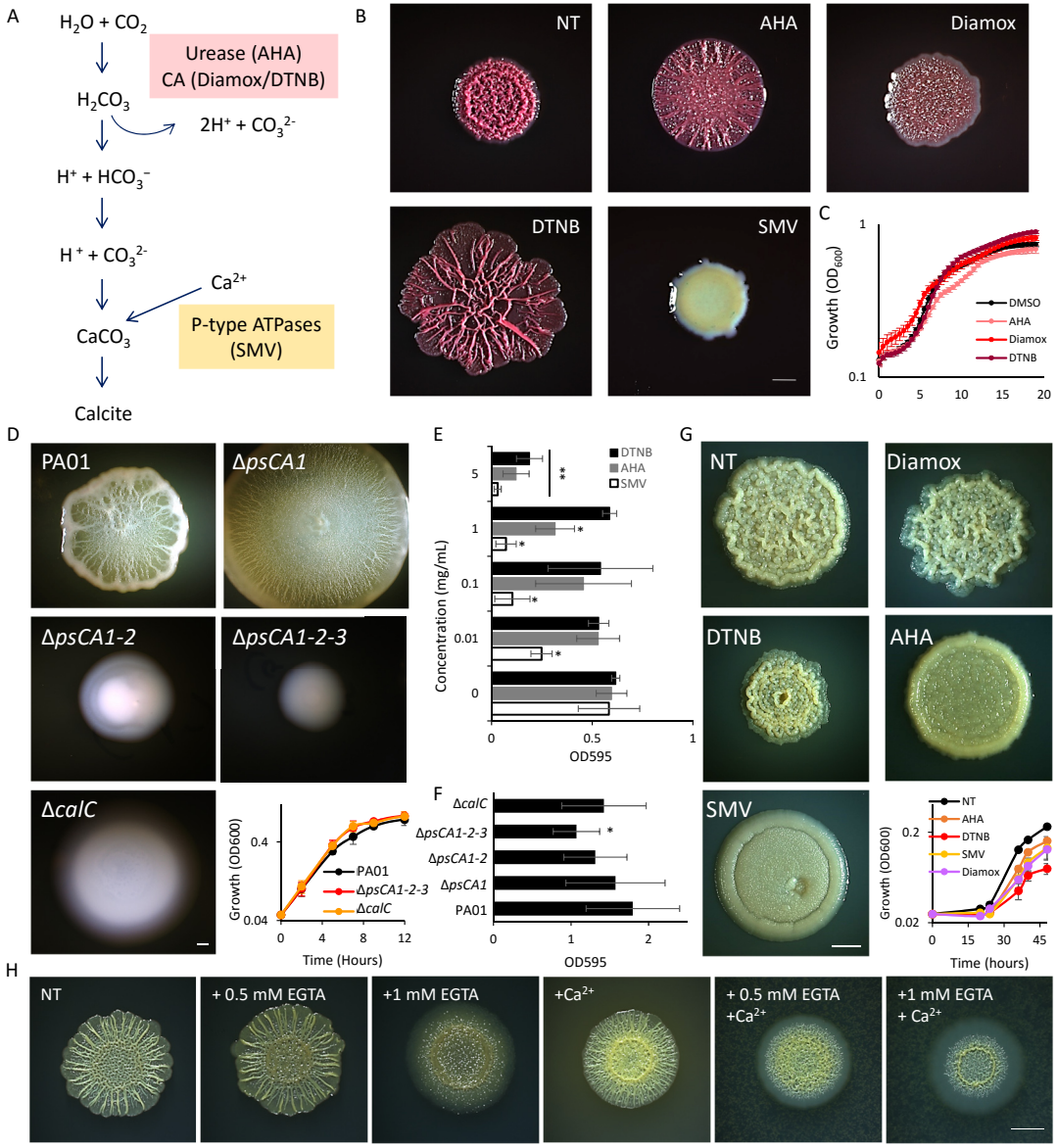

A
$H_2O + CO_2$
Urease (AHA)
CA (Diamox/DTNB)
H_2CO_3
$2H^+ + CO_3^{2-}$
$H^+ + HCO_3^-$
$H^+ + CO_3^{2-}$
Ca^{2+}
P-type ATPases (SMV)
$CaCO_3$
Calcite
B
NT
AHA
Diamox
DTNB
SMV
C
Growth (OD_{600})
DMSO
AHA
Diamox
DTNB
D
PA01
ΔpsCA1
ΔpsCA1-2
ΔpsCA1-2-3
ΔcalC
Growth (OD600)
Time (Hours)
E
DTNB
AHA
SMV
Concentration (mg/mL)
OD595
F
ΔcalC
ΔpsCA1-2-3
ΔpsCA1-2
ΔpsCA1
PA01
OD595
G
NT
Diamox
DTNB
AHA
SMV
Growth (OD600)
Time (hours)
H
NT
+ 0.5 mM EGTA
+1 mM EGTA
+Ca^{2+}
+ 0.5 mM EGTA +Ca^{2+}
+1 mM EGTA + Ca^{2+}

Figure 5

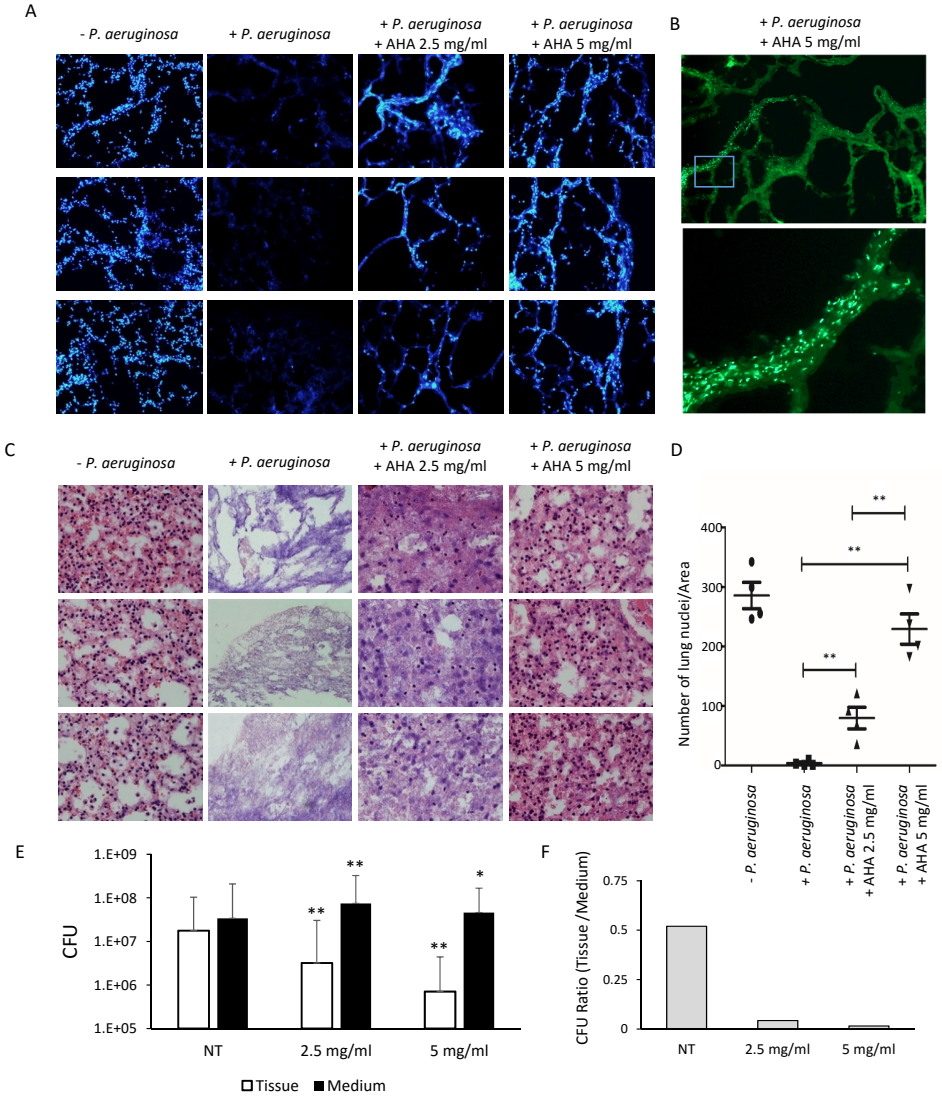
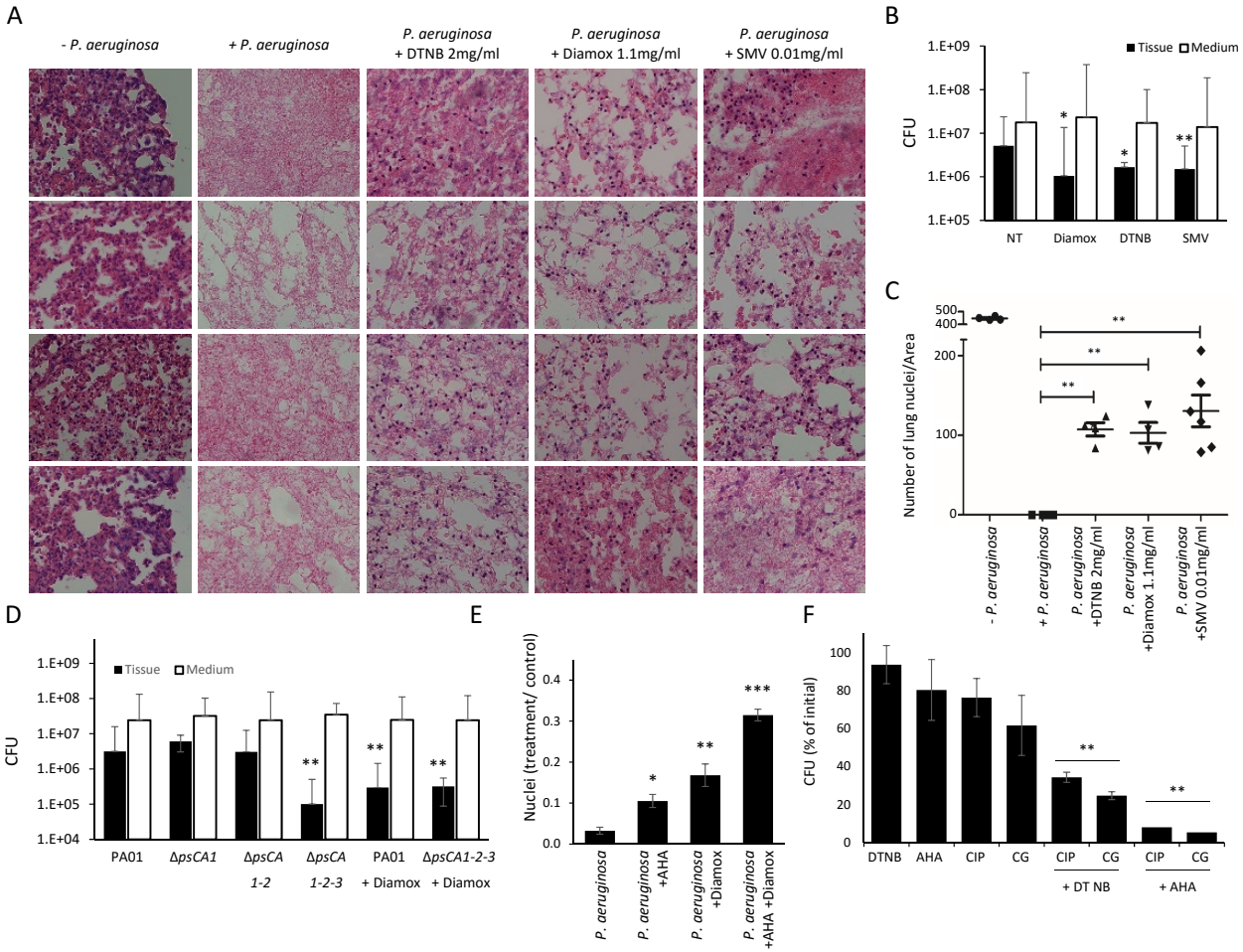

A
- P. aeruginosa
+ P. aeruginosa
+ P. aeruginosa + AHA 2.5 mg/ml
+ P. aeruginosa + AHA 5 mg/ml
B
+ P. aeruginosa + AHA 5 mg/ml
C
- P. aeruginosa
+ P. aeruginosa
+ P. aeruginosa + AHA 2.5 mg/ml
+ P. aeruginosa + AHA 5 mg/ml
D
Number of lung nuclei/Area
400
300
200
100
0
**
**
**
- P. aeruginosa
+ P. aeruginosa
+ P. aeruginosa + AHA 2.5 mg/ml
+ P. aeruginosa + AHA 5 mg/ml
E
CFU
1.E+09
1.E+08
1.E+07
1.E+06
1.E+05
**
**
**
*
NT
2.5 mg/ml
5 mg/ml
Tissue
Medium
F
CFU Ratio (Tissue /Medium)
0.75
0.5
0.25
0
NT
2.5 mg/ml
5 mg/ml

Figure 6


A
- P. aeruginosa
+ P. aeruginosa
P. aeruginosa + DTNB 2mg/ml
P. aeruginosa + Diamox 1.1mg/ml
P. aeruginosa + SMV 0.01mg/ml
B
Tissue
Medium
CFU
1.E+09
1.E+08
1.E+07
1.E+06
1.E+05
NT
Diamox
DTNB
SMV
C
Number of lung nuclei/Area
500
400
200
100
0
- P. aeruginosa
+ P. aeruginosa
P. aeruginosa +DTNB 2mg/ml
P. aeruginosa +Diamox 1.1mg/ml
P. aeruginosa +SMV 0.01mg/ml
D
Tissue
Medium
CFU
1.E+09
1.E+08
1.E+07
1.E+06
1.E+05
1.E+04
PA01
ΔpsCA1
ΔpsCA 1-2
ΔpsCA 1-2-3
PA01 + Diamox
ΔpsCA1-2-3 + Diamox
E
Nuclei (treatment/ control)
0.4
0.3
0.2
0.1
0.0
P. aeruginosa
P. aeruginosa +AHA
P. aeruginosa +Diamox
P. aeruginosa +AHA +Diamox
F
CFU (% of initial)
100
80
60
40
20
0
DTNB
AHA
CIP
CG
CIP
CG
CIP
CG
+ DT NB
+ AHA

The formation of microbial exoskeletons is driven by a controlled calcium-concentrating subcellular niche

Supplementary Information

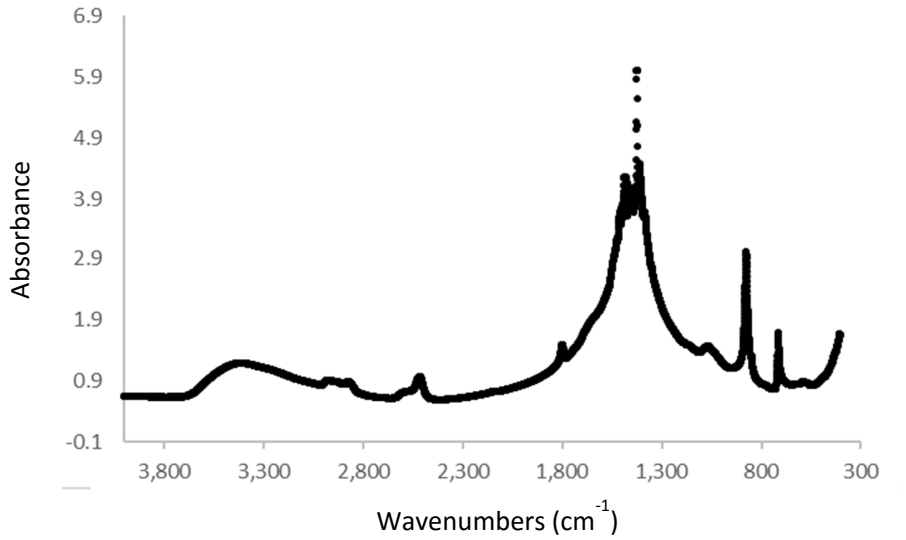
Content

Supplementary Figures

Supplementary Files and Movies Legends

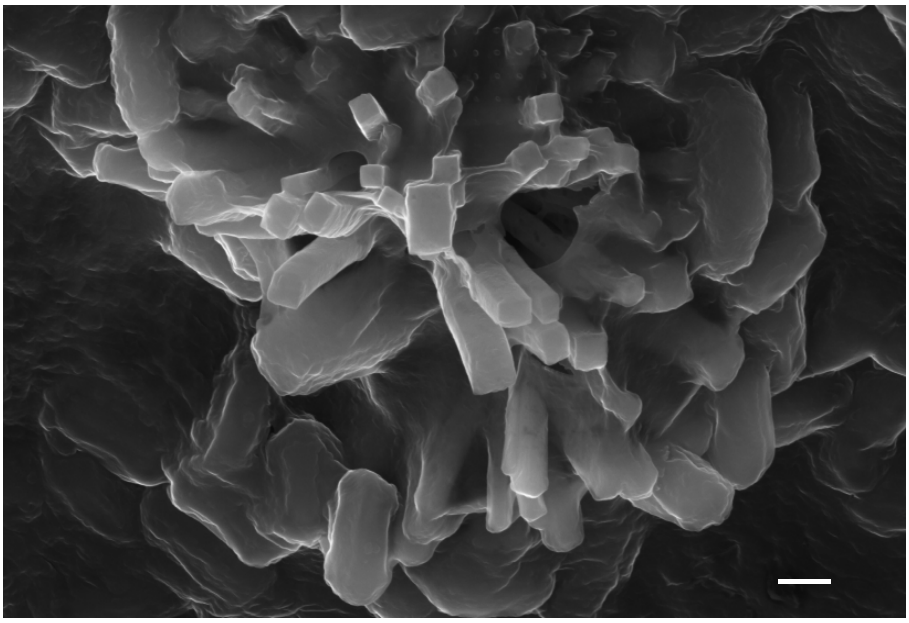
Supplementary Tables

Supplementary figures



Supplementary Figure 1. FTIR spectra of bleached *B. subtilis* biofilm colonies, displaying vibrations characteristic of calcite (crystalline calcium carbonate).

A



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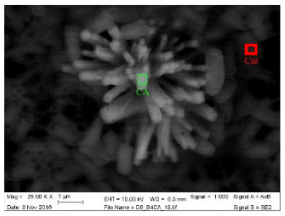
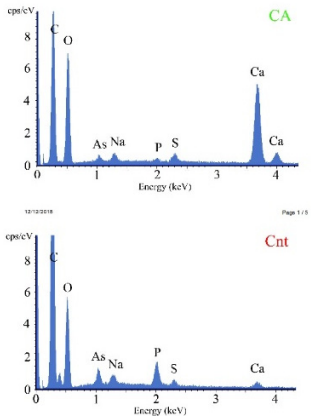
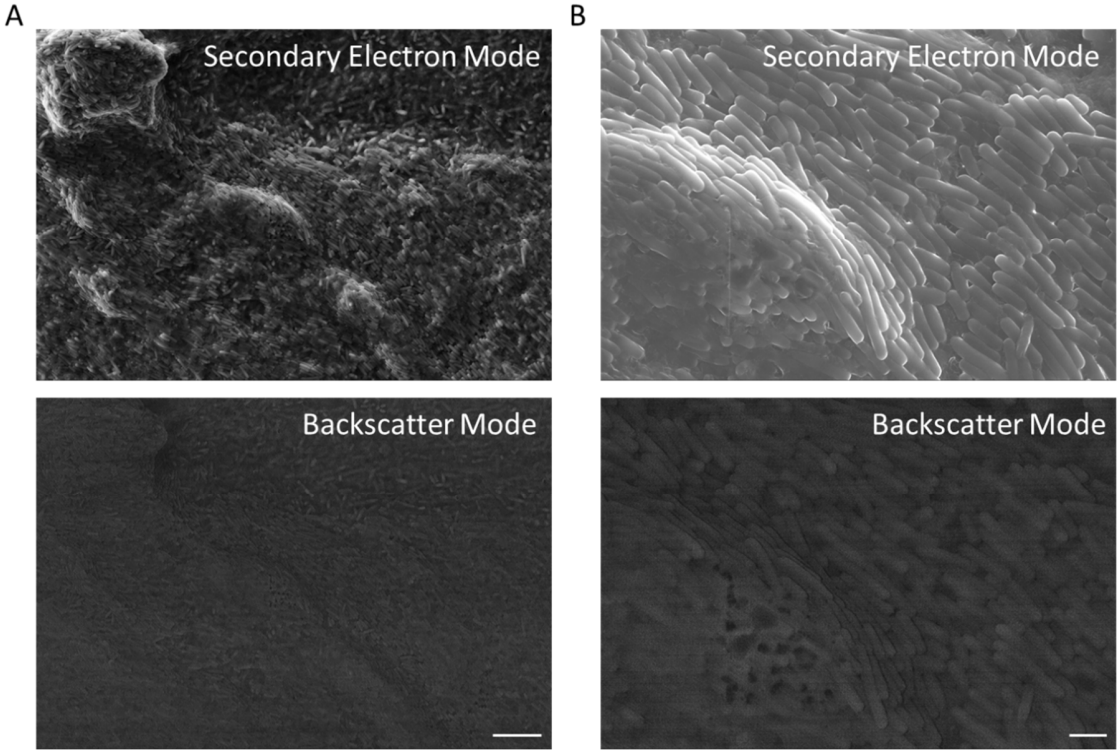


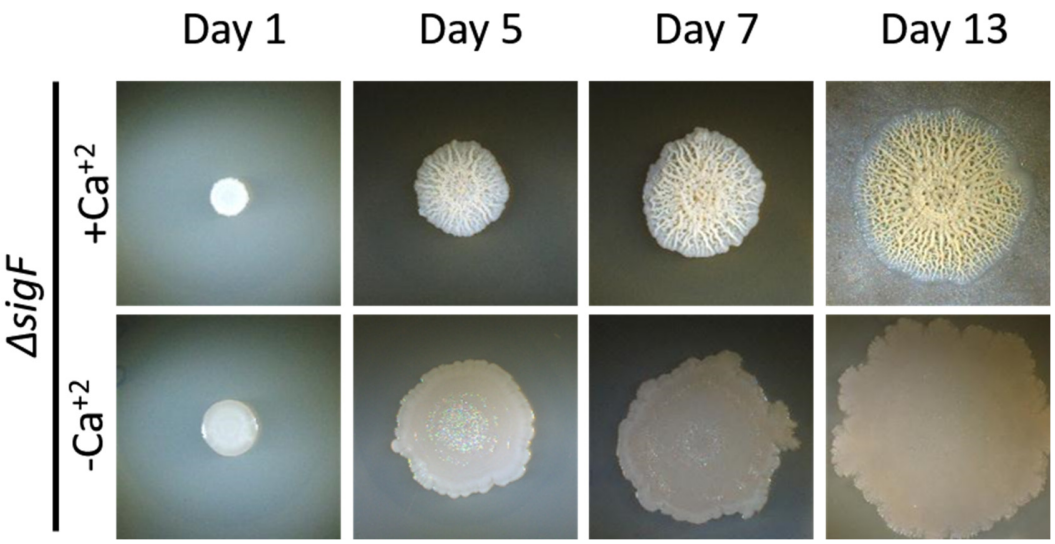
C



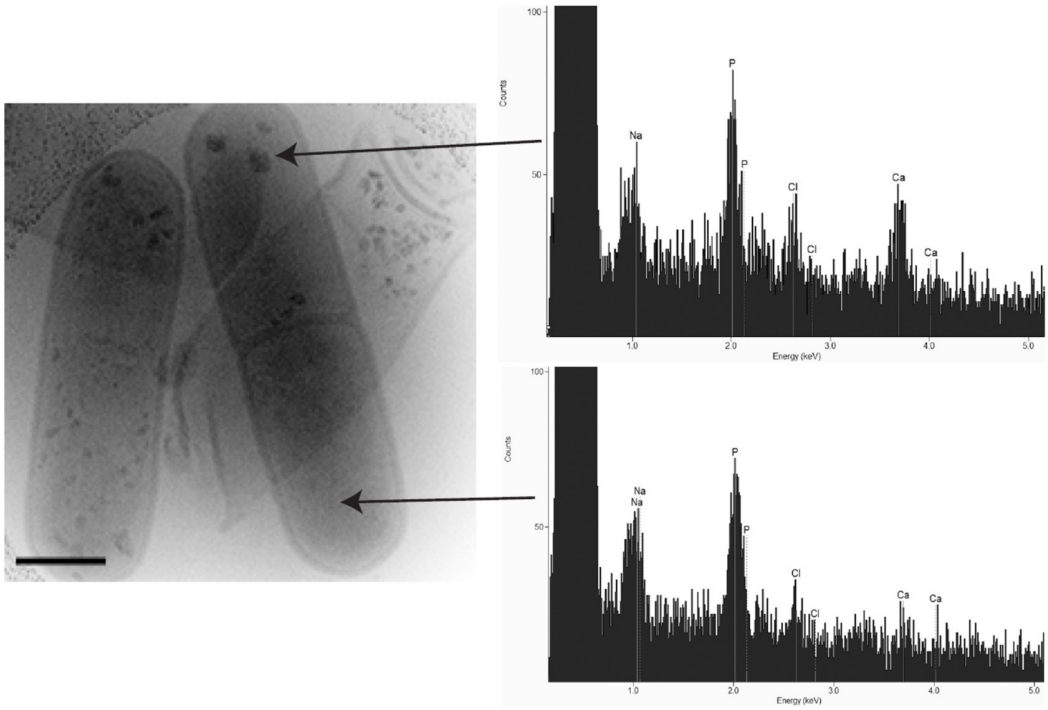
Supplementary Figure 2. (A). Scanning Electron Microscopy (SEM) image of *B. subtilis* biofilm colony grown for 6 days on a biofilm-inducing B4-Ca^{2+} agar. Shown are the surface of the biofilm and rhombohedral mineral structures in secondary electron mode. Magnification – X25000, scale bar – 1 µm. **(B).** Backscattered mode SEM image of the area shown in (**A**). **(C)** EDX spectra of the area shown in (**A**). CA – calcium rich area; Cnt – control area. A representative field (out of n=4 fields, from 8 independent experiments) is shown.



Supplementary Figure 3. Scanning Electron Microscopy (SEM) image of *B. subtilis* biofilm colony grown for 6 days on a B4 agar (without excess calcium). Shown are the surface of the biofilm in secondary electron and in backscattered mode of a representative field. **(A)** Magnification – X2500, scale bar – 10 μm. **(B).** Magnification – X10000, scale bar – 2 μm. Representative fields (out of n=4 fields, from 8 independent experiments) are shown.



Supplementary Figure 4. Light microscopy images of Δ*sigF* mutant strain. Biofilm colonies were grown on B4-Ca^{2+} agar for the indicated time at 30°C. The experiment was repeated 3 times, in a technical quadruplicate – and representative images are shown.



Supplementary Figure 5. Left panel - Bright-field STEM image of representative *B. subtilis* biofilm cells from a showing cellular calcium deposits, from a colony grown 10 days on B4-Ca^{2+} agar. The black arrow indicates the cell used for EDX analysis in panels (B-C). Right panel – EDX analysis of the calcium deposits. A representative mineralizing cell imaged (out of n=50 cells, from 4 independent experiments performed with EDX is shown.

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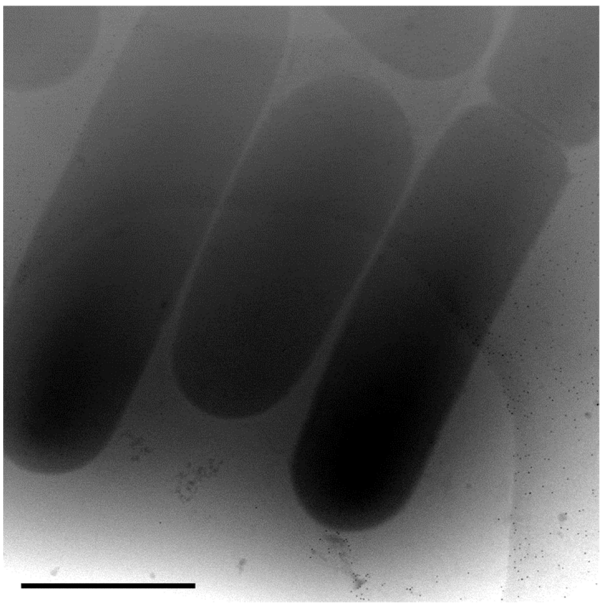
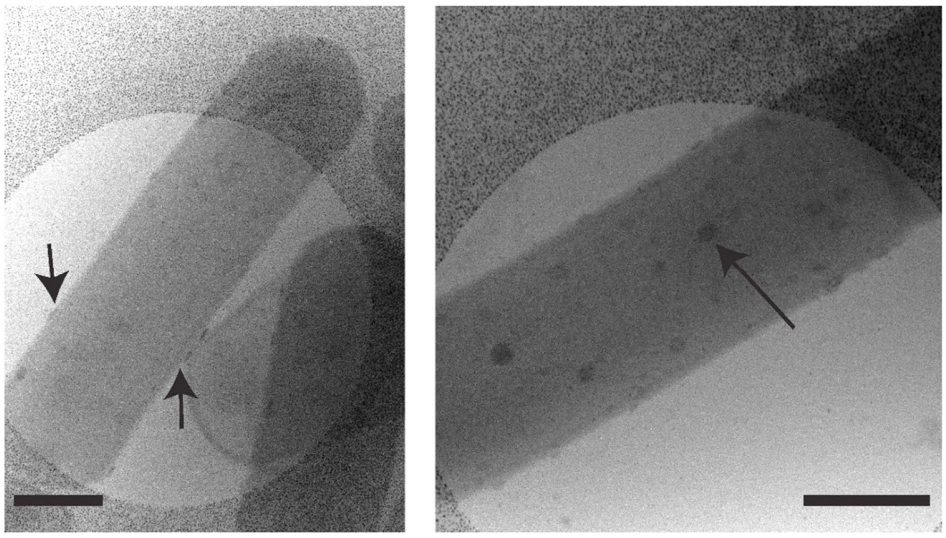
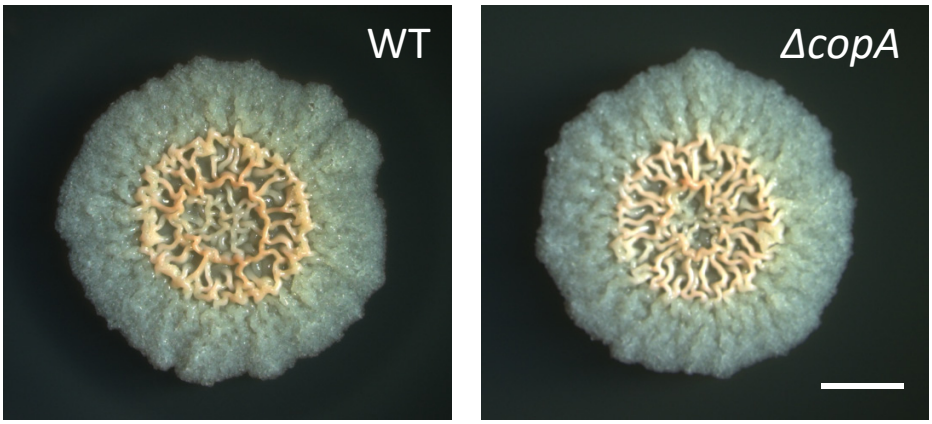


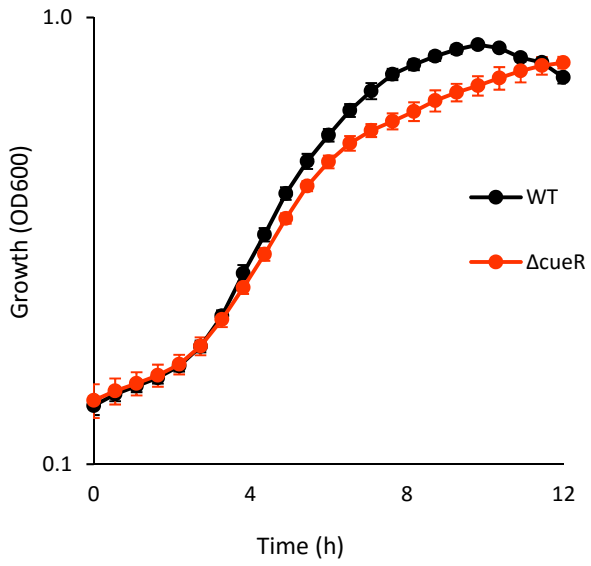
Supplementary Figure 6. Cryo-STEM bright-field images of representative biofilm cells from a *B. subtilis* colony grown for 10 days on B4 agar (without calcium). Scale bar 1 μm. A representative field imaged (out of n=30 cells, from 4 independent experiments is shown.



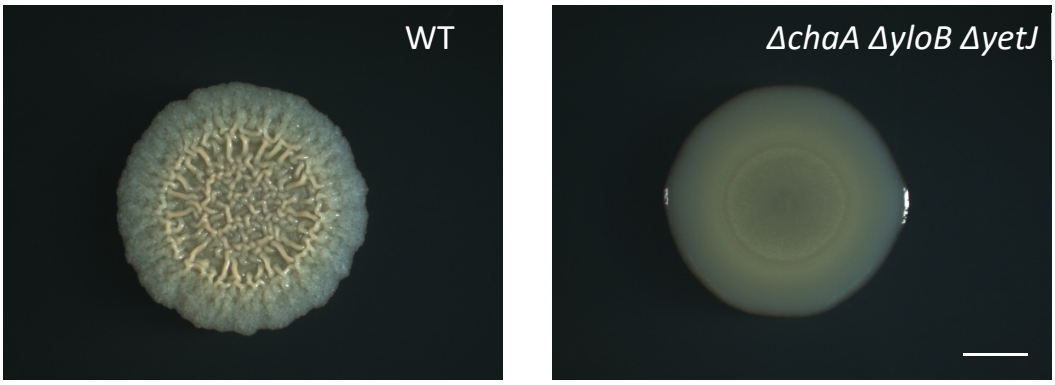
Supplementary Figure 7. Cryo-STEM bright-field images of representative biofilm cells from a Δ*sigF* colony grown for 10 days on B4 agar. Black arrows point to aggregates with edge-on views (left panel) and top views (right panel). Scale bars 500 nm. A representative field imaged (out of n=30 cells), from 4 independent experiments is shown.



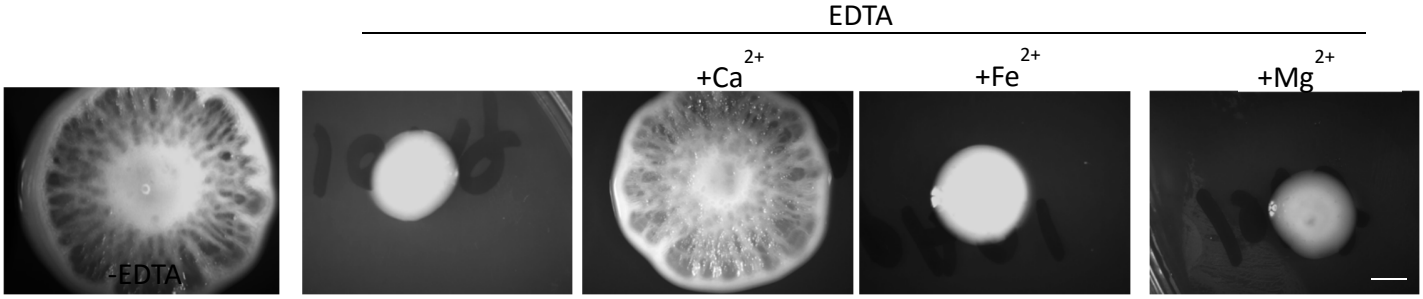
Supplementary Figure 8. Light microscopy images of wild type *B. subtilis* (WT) and Δ*copA* mutant. Biofilm colonies were grown on B4-Ca^{2+} agar for 3 days at 30°C. Scale bar – 2 mm. The experiment was repeated 3 times, in a technical quadruplicate – and representative images are shown.



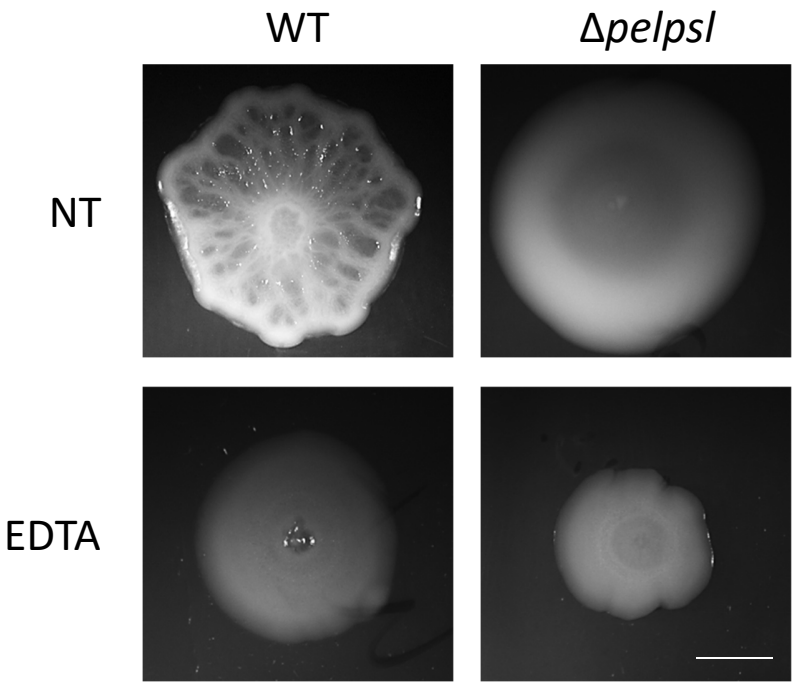
Supplementary Figure 9. Planktonic growth assay. Wild type *B. subtilis* (ET) and *ΔcuerR* mutant were grown at 30°C with shaking in liquid LB, and growth was monitored by measuring OD_{600} in a microplate reader every 30 min. Results are averages of six wells, bars represent standard deviations. A representative experiment out of at least 3 independent experiments is shown.



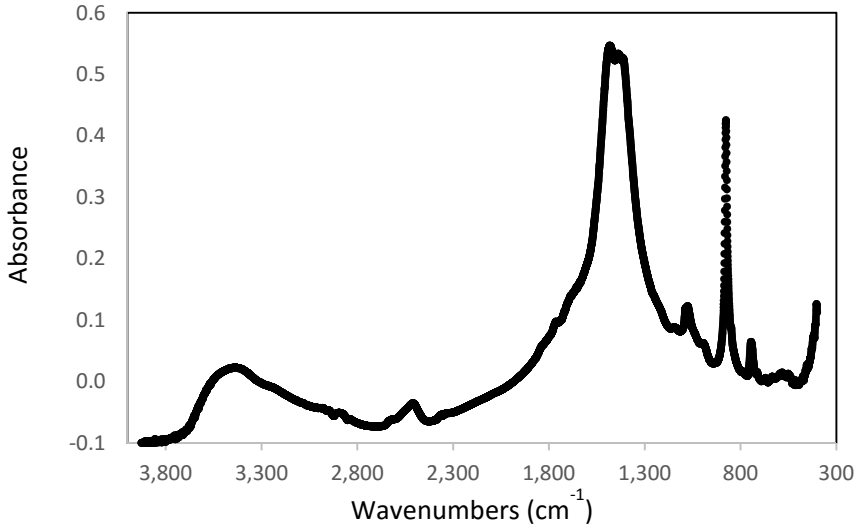
Supplementary Figure 10. Light microscopy images of wild type (WT) and triple *ΔchaA ΔyloB ΔyetJ* mutant. Biofilm colonies grown on solid B4-Ca^{2+} agar for 3 days at 30°C. Scale bar – 2 mm. The experiment was repeated 3 times, in a technical quadruplicate – and representative images are shown.



Supplementary Figure 11. Light microscopy of biofilm colonies of *P. aeruginosa* PA01 supplemented with EDTA (0.1mg/ml), and divalent ions (1mM), as indicated. Colonies were grown on BHI solid medium for 4 days at 23ºC. Scale bar – 5 mm. The experiment was repeated 3 times, in a technical 3 repeats – and representative images are shown.



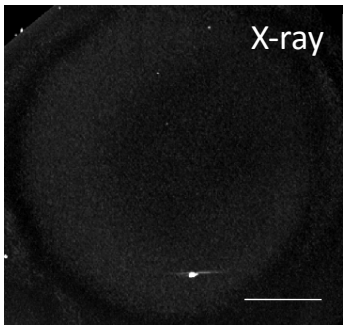
Supplementary figure 12: Light microscopy of biofilm colonies of *P. aeruginosa* PA01 either untreated or supplemented with EDTA (0.1mg/ml) as indicated. Colonies were grown on BHI solid medium for 4 days at 23ºC. Scale bar = 5 mm. The experiment was repeated 2 times, in a technical 3 repeats – and representative images are shown.



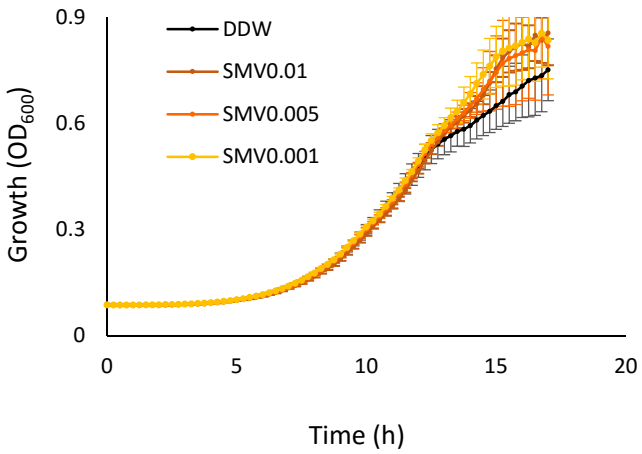
Supplementary Figure 13. FTIR spectra of bleached *M. abscessus* biofilm colonies, displaying vibrations characteristic of varterite (crystalline calcium carbonate).

Bacteria	Medium	pH					
		medium control	far from colony	close to colony	below the colony	colony center	Medium Langelier Saturation index (LSI)
B. subtilis	B4Ca^{2+}	6	7.5	7.75	7.75	8	0.51-0.015
P. aeruginosa	TB	6	8	8	8	8	2.4—1.4
M. abscessus	B4Ca^{2+}	6	8.5	8.5	8	8	0.51-0.015

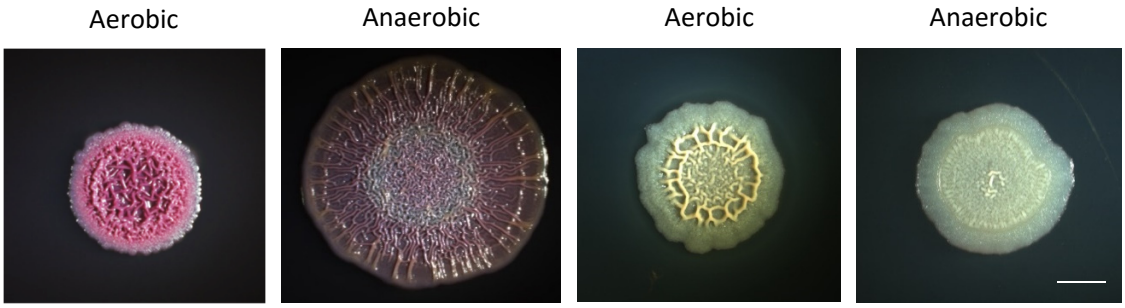
Supplementary Figure 14. pH of colonies and the agar medium they were grown on was assessed using pH-indicator strips (MColorpHast™). The experiment was conducted at least twice for each medium, with at least 4 technical repeats. LSI calculation refers to the indicated growth media. ND – undetermined.



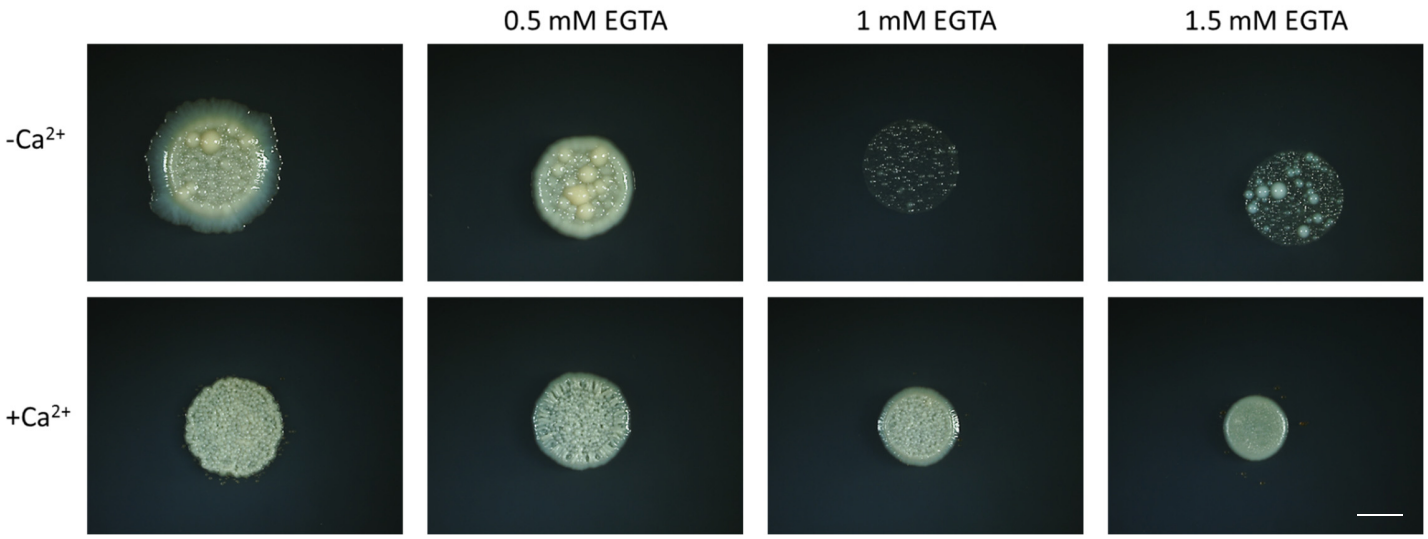
Supplementary Figure 15. MicroCT-XRay image of a wild type *P. aeruginosa* PAO14 colony grown for 3 days at 23°C on TB agar containing SMV (0.01 mg/ml). The absence of visible bright areas indicates lack contrast, indicating no dense mineral (compare to the control colony shown in Fig. 3A). Scale bar – 2 mm. The experiment was repeated twice with similar results.



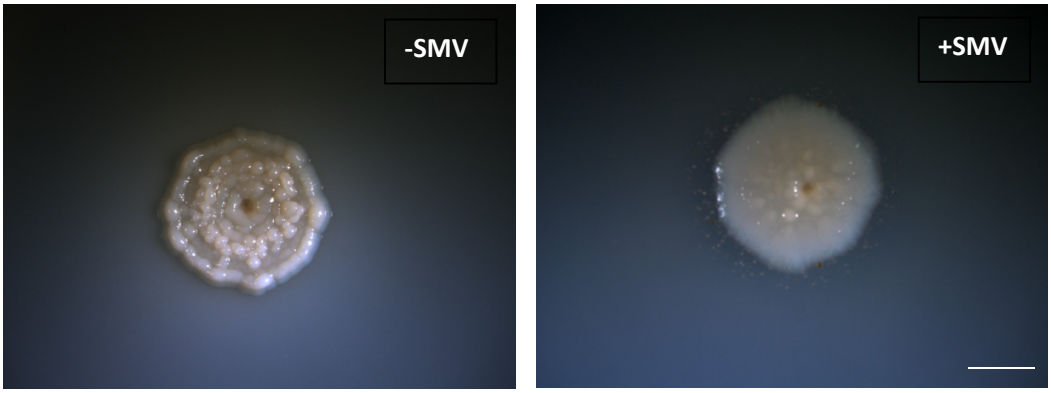
Supplementary Figure 16. Planktonic growth assay. *P. aeruginosa* PA14 was grown at 37°C with shaking in liquid TB medium, supplemented with: 1.5 mg/ml DTNB, 1.75 mg/ml AHA, 2.5 mg/ml Diamox and 0.01, 0.05 and 0.01 mg/ml SMV. Growth was monitored by measuring OD600 in a microplate reader every 30 min. Results are averages of six wells, bars represent standard deviations. A representative experiment out of at least 3 independent experiments is shown.



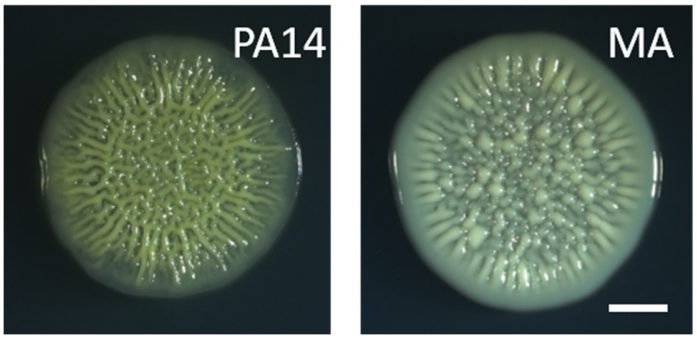
Supplementary Figure 17. Light microscopy of biofilm colonies formed by *P. aeruginosa* PA14 (left) and *B. subtilis* (right). The biofilms were grown TB and B4-Ca^{2+} agar, respectively, with nitrate source (0.5% $KN0^3$). Under aerobic conditions, CO_2 is available from the atmosphere and also produces as a byproduct of aerobic respiration. Under anaerobic conditions of nitrate reduction (as used here - anaerobic growth chamber, a mix of H_2 and N_2 (5/95%), CO_2 is absent from the atmosphere, and aerobic respiration (and subsequent CO_2 emission) is not feasible due to the lack of oxygen. The experiment was repeated 4 times, in a technical 3 repeats– and representative images are shown.



Supplementary Figure 18. Light microscopy of 3-day-old biofilm colonies formed by *M. abscessus* on B4 agar supplemented with calcium acetate (0.25% v/v) and EGTA, as indicated. Scale bar = 5 mm. The experiment was repeated 3 times, in a technical 3 repeats– and representative images are shown.

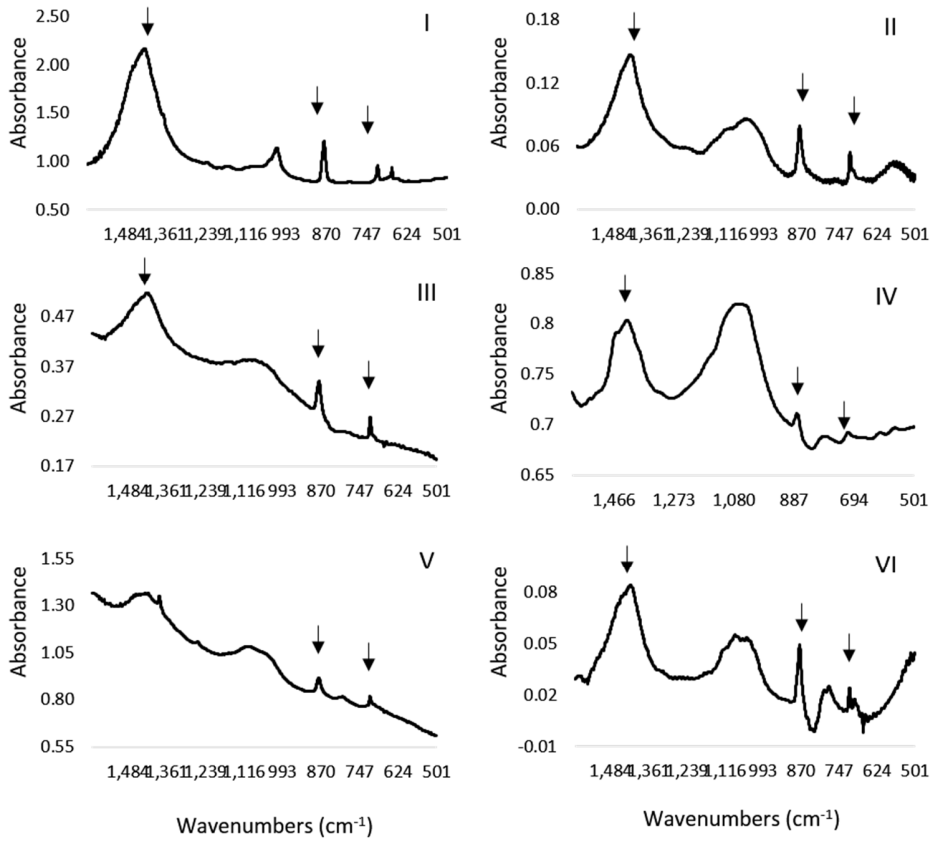


Supplementary Figure 19. Light microscopy images of biofilm colonies formed by *M. abscessus*, supplemented with SMV (0.025mg/ml) as indicated. Colonies were grown on B4-Ca^{2+} medium for 5 days, at 37°C. Scale bar = 5 mm. The experiment was repeated 2 times, in a technical 3 repeats – and representative images are shown.

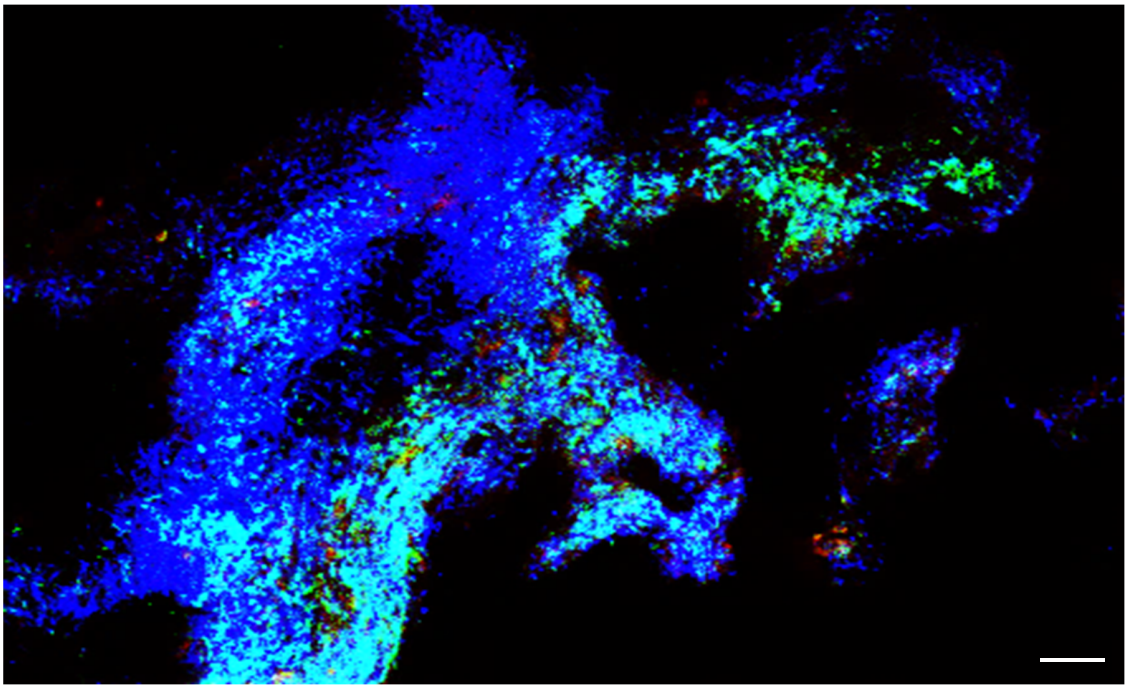


Supplementary Figure 20.

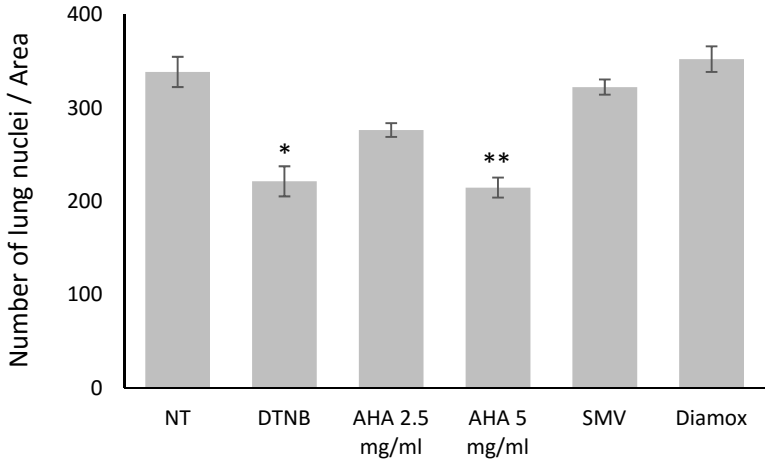
P. aeruginosa and *M. abscessus* biofilm colonies grown on solid SCFM medium for 4 days at 23ºC (*P. aeruginosa*) and 30ºC (*M. abscessus* and *S. aureus*). Scale bar – 2 mm. The experiment was repeated 3 times, in a technical 3 – and representative images are shown.



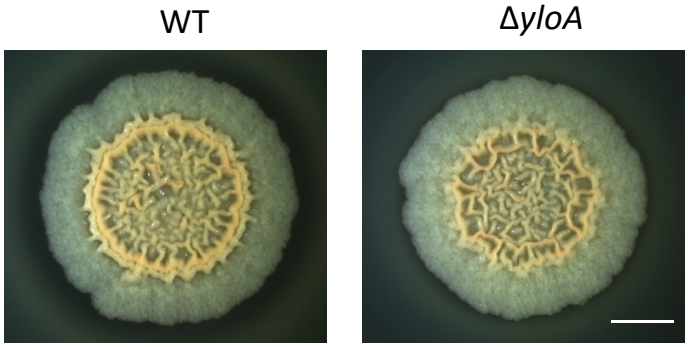
Supplementary Figure 21. FTIR spectra of bleached sputum samples of *P. aeruginosa* positive CF patients (I-VI). Arrows indicate vibrations characteristic of calcite. For patient details, see Supplementary table 2.



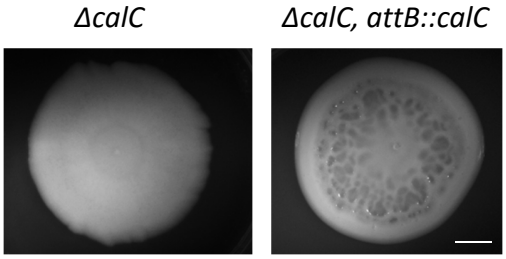
Supplementary Figure 22. Confocal image of *ex-vivo* lung model. Lungs were harvested from one-month-old mice, cultured in DMEM containing carbenicillin 100 µg/ml, and infected with *P. aeruginosa* PA14 constitutively expressing GFP. Green – bacterial cells. Blue - nuclei of lung cells stained with DAPI. Scale bar – 50 µm. The experiment was repeated 3 times, in a technical 4 repeats – and representative image is shown.



Supporting Figure 23. Quantification of lung viability. Lung tissue cultures were treated with biomineralization inhibitors DTNB (2 mg/ml), SMV (0.01 mg/ml), Diamox (1.1 mg/ml) and AHA, as indicated. ImageJ 1.51g software was used to automatically count lung cell nuclei in 4 randomly chosen fields for each treatment. P values, as determined by student's t-test, are indicated (* pVal <0.01, ** pVal <0.001).



Supporting Figure 24. Light microscopy images of wild type (WT) and *ΔyloA* mutant. Biofilm colonies grown on solid B4-Ca^{2+} agar for 3 days at 30°C. Scale bar – 2 mm. The experiment was repeated 3 times, in a technical quadruplicate – and representative images are shown.



Supporting Figure 25. Light microscopy of biofilm colonies of *P. aeruginosa* PA01 indicated strains. Colonies were grown on BHI solid medium for 4 days at 23ºC. Scale bar = 5 mm. The experiment was repeated 3 times, in a technical 2 or more repeats – and representative images are shown.

Supplementary Files:

Supplementary File 1: Differentially expressed genes in *B. subtilis* NCIB 3610, + vs - calcium.

Supplementary File 2: Differentially expressed genes *B. subtilis* NCIB 3610, WT vs *cueR*.

Supplementary File 3: Patients information. Clinical parameters of patients analyzed for presence of calcite in sputum samples.

Supplementary Movies:

Supplementary Movie 1: *B. subtilis* colony

Supplementary Movie 2: *P. aeruginosa* PA14 colony

Supplementary Movie 3: *M. abscessus* colony

The intact colonies were visualized under X-ray, and the obtained 2D images were used to generate high-resolution 3D image (see Keren-Paz *et al.,* 2018 for details). Rotation of 360° was taken around an axis perpendicular to the biofilm surface. Results are of a representative experiment out of three independent repeats.

Supplementary Tables:

Supplementary Table 1: Strains used in this study

Genotype	Reference
Bacillus subtilis NCIB 3610	Branda *et al* (2001)
ΔarsR (kan)	This work
ΔmntR (kan)	This work
Δzur (kan)	This work
ΔcueR (kan)	This work
ΔcopA (kan)	This work
ΔyetJ (spec)	This work
ΔchaA (kan)	This work
ΔyloB (mls)	This work, the lack of polar effect was confirmed by the deletion of *yloA* (Figure S24)
ΔchaA (kan) *ΔyetJ* (spec)	This work
ΔchaA (kan) *ΔyetJ* (spec) *ΔyloB* (mls)	This work
Pseudomonas aeruginosa PA14	Lab collection
Pseudomonas aeruginosa PA14, Ppuc-GFP (carb)	[1]
Pseudomonas aeruginosa PA01	Lab collection
Pseudomonas aeruginosa PA01 Δ*psCA1*	[1]
Pseudomonas aeruginosa PA01 Δ*psCA1-2*	[1]
Pseudomonas aeruginosa PA01 Δ*psCA1-2-3*	[1]

Pseudomonas aeruginosa PA01 Δ*calC*	This work, This work, the lack of polar effect was confirmed by complementation of *calC* from an ectopic chromosomal loci (Figure S25), ΔcalC attB::calC
Mycobacterium abscessus ATCC 19977	A kind gift from Daniel Barkan

1 Lotlikar, S. R. *et al.* Pseudomonas aeruginosa beta-carbonic anhydrase, psCA1, is required for calcium deposition and contributes to virulence. *Cell Calcium* **84**, 102080, doi:10.1016/j.ceca.2019.102080 (2019).

Supplementary Table 2:

Patient	Medical	Gender	CFTR mutations	Infection
I 463116	Carmel	M	W1282X/F508	PA+MA
II A19	Hadassah	M	G542X/F508	PA
III 463125	Carmel	F	T360K, Q359K	PA
IV B9	Hadassah	F	W1282X/W1282X	PA
V A21	Hadassah	F	W1282X/F508	PA
VI A32	Hadassah	M	W1282X/W1282X	PA

The details of patients included Supplementary Figure 15 and their respected panels are indicated.