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Both clinical and environmental *Caulobacter* species act as opportunistic pathogens

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ABSTRACT

The *Caulobacter* genus, including the widely-studied model organism *Caulobacter crescentus*, has been thought to be non-pathogenic and thus proposed as a bioengineering vector for various environmental remediation and medical purposes. However, *Caulobacter* species have been implicated as the causative agents of several hospital-acquired infections, raising the question of whether these clinical isolates represent an emerging pathogenic species or whether *Caulobacters* on whole possess previously-unappreciated virulence capability. Given the proposed environmental and medical applications for *C. crescentus*, understanding the potential pathogenicity and human health implications of this bacterium is crucial. Consequently, we sequenced a clinical *Caulobacter* isolate to determine if it has acquired novel virulence determinants. We found that the clinical isolate represents a new species, *Caulobacter mirare. C. mirare* phylogenetically resembles both *C. crescentus* and the related *C. segnis,* which was also thought to be non-pathogenic. The similarity to other *Caulobacters* and lack of obvious pathogenesis markers suggested that *C. mirare* is not unique amongst *Caulobacters* and that consequently other *Caulobacters* may also have the potential to be virulent. We tested this hypothesis by characterizing the ability of *Caulobacters* to infect the model animal host *Galleria mellonella*. In this context, two different lab strains of *C. crescentus* proved to be as pathogenic as *C. mirare,* while lab strains of *E. coli* were non-pathogenic. Further characterization showed that *Caulobacter* pathogenesis is mediated by a dose-dependent, cell-associated toxic factor that does not require active bacterial cells or host cellular innate immunity to elicit its toxic effects. Finally, we show that *C. crescentus* does not grow well in standard clinical culture conditions, suggesting that *Caulobacter* infections may be more common than generally appreciated but rarely cultured. Taken together, our findings redefine *Caulobacters* as opportunistic pathogens and highlight the importance of broadening our methods for identifying and characterizing pathogens.

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AUTHOR SUMMARY

Bacterial species have historically been classified as either capable of causing disease in an animal (pathogenic) or not. *Caulobacter* species represent a class of bacteria that were thought to be non-pathogenic. *Caulobacters* have been widely studied and proposed to be used for various industrial and medical applications due to their presumed safety. However, recent reports of human *Caulobacter* infections raised the question of whether disease-causing *Caulobacters* have acquired special factors that help them cause disease or whether the ability to infect is a more general feature of most *Caulobacters.* By combining genomic sequencing and animal infection studies we show that a clinical *Caulobacter* strain is similar to lab *Caulobacters* and that all *Caulobacters* studied can infect a model host. We explore the mechanism of this infectivity and show that it is due to a toxic factor that associates with *Caulobacter* cells. We also provide a possible explanation for why *Caulobacters* have not traditionally been isolated from human patients, owing to their inability to tolerate the salt levels used in most medical culturing systems.

INTRODUCTION

The free-living, gram-negative genus *Caulobacter* was first described and classified as a group of rod-shaped, stalk possessing bacteria in 1935 [1, 2]. Since their identification, *Caulobacter* have been observed in rhizosphere, soil, and aqueous environments, including drinking water reservoirs [3, 4]. Historically, this genus has been considered non-pathogenic due to lack of presence in infection cases, no obvious pathogenicity islands, and increased bacterial mortality at human body temperatures [5]. However, the last two decades has seen several reports of symptomatic infections associated with *Caulobacter* species [6-10]. All reported cases of *Caulobacter* infections appear to be hospital-acquired by

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63 immunocompromised patients, suggesting that these infections are opportunistic. None of the
64 *Caulobacter* isolates associated with human infection have been previously sequenced.
65 Consequently, it remains unclear whether clinical isolates have acquired virulence mechanisms
66 absent from other *Caulobacters,* or if *Caulobacter* species generally have the capacity for
67 human disease in the right context.

68 Among *Caulobacter* species, *Caulobacter crescentus* is the best characterized and most
69 widely studied in laboratory settings [11]. *C. crescentus* has been primarily used as a model
70 organism for understanding bacterial cell-cycle progression due to its highly regulated
71 asymmetrical division and dimorphic lifestyle [12, 13]. Because of its available molecular tools,
72 ability to display proteins in its surface layer (S-layer), and assumed non-toxicity to humans, *C.*
73 *crescentus* has been proposed to be a powerful vector for a wide range of bioengineering
74 applications [14, 15]. For example, *C. crescentus* has been engineered as a biosensor for
75 uranium [16], a bioremediation tool for heavy metals [17], an anti-tumor immunization technique
76 [18], and an anti-viral microbicide in humans [19]. Thus, understanding the potential
77 pathogenicity and human health implications of this bacterium is crucial before its industrial use.

78 Here we obtained and sequenced a *Caulobacter* isolate from a reported human infection
79 (7) to determine if it contains conspicuous virulence determinants or is similar to previously-
80 characterized *Caulobacters.* We found that the clinical isolate represents a new species with
81 similarities to both *C. crescentus* and *C. segnis.* The lack of pathogenicity islands and similarity
82 to lab strains of *C. crescentus* suggested that the potential of this clinical isolate to be an
83 opportunistic pathogen may be a general feature of *Caulobacters.* We confirmed this hypothesis
84 by turning to the *Galleria mellonella* model animal host. The clinical *Caulobacter* isolate and lab
85 strains of *C. crescentus* exhibited similar virulence, which were both significantly higher than
86 non-pathogenic lab strains of *E. coli*. Further characterization revealed that *Caulobacter*
87 pathogenicity is mediated by a toxic cell-associated factor. Our results thus redefine

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Caulobacter as a potential opportunistic pathogen and establish the interaction between *Caulobacter* and *G. mellonella* as a model for host-pathogen interactions.

RESULTS

Clinical *Caulobacter sp. SSI4214* shares homology with soil- and freshwater-associated species of *Caulobacter*

To genomically characterize a clinical *Cauloacter* isolate, we obtained a clinical strain of *Caulobacter* species isolated from the dialysis fluid of a 64-year-old man in Denmark with peritonitis [7]. There was only one bacterial species that could be cultured from the peritoneal fluid using Danish blood agar medium, and the infection responded to gentamycin treatment suggesting that this species was the likely cause of the infection [7]. Imaging of the cultured bacteria revealed a crescent-shaped morphology similar to that of *Caulobacter crescentus* and 16S ribosomal profiling showed 99.5% homology between the clinical isolate (*Caulobacter sp.* SSI4214) to a common laboratory *C. crescentus* strain CB15 [7]. We performed next-generation Illumina sequencing on the *Caulobacter sp.* SSI4214 strain and created a draft genome assembly to understand the isolate's relationship to other *Caulobacter* species. Analysis of the 16S rRNA gene obtained from Illumina sequencing confirmed the initial report, with 99.5% similarity to *C. crescentus.* However, phylogenetic reconstruction comparing the 16S sequences of all available whole-genome *Caulobacter* species revealed that *Caulobacter sp.* SSI4214 resides in a separate clade within the *Caulobacter* genus, between *Caulobacter crescentus* and *Caulobacter segnis* (Fig 1A). SSI4214 was also similar to both *C. crescentus* and *C. segnis* with respect to overall GC content and two-way average nucleotide identity (Table 1). Given the convention that isolates of the same bacterial species should have at least 95% average nucleotide identity, our results indicate that SSI4214 represents a distinct species in the

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Caulobacter genus, which we have named *Caulobacter mirare,* as "mirare" is the Latin root for "mirror" and this species mirrors previously-characterized *Caulobacters* (Table 1, S1 Fig) [20].

Annotation of the *C. mirare* genome allowed us to compare homology of its genes to those of *C. crescentus* and *C. segnis*, including both broadly-conserved and *Caulobacter*-specific genes [21]. Overall, *C. mirare* is predicted to encode 4,329 protein-encoding genes. This number is similar to that of the *C. segnis* genome (4,330 genes), and larger than *C. crescentus* (3,819) (Table 1) [22, 23]. Among broadly-conserved genes, subunits of DNA polymerase, RNA polymerase, and ribosomes all exhibited at least 86% sequence similarity to both *C. crescentus* and *C. segnis*. *C. mirare* also possesses clear homologs of many *Caulobacter*-specific genes including the cell-cycle regulator *ctrA*, the curvature determinant *creS*, the S-layer secretion protein *rseE*, and the holdfast attachment protein *hfaA* (Fig 1B). We note that *C. segnis* does not possess a majority of the holdfast synthesis genes, including *hfaA* (Fig 1B) [22]. The observations that *C. mirare* is more similar to *C. segnis* with respect to gene number but more similar to *C. crescentus* with respect to holdfast gene content supports its placement as an independent clade in between the two related species. Importantly, like *C. crescentus* and *C. segnis,* no known annotated virulence factor homologues or pathogen-associated genes are predicted to be present in *C. mirare* [24]. Thus, genome sequencing suggests that the pathogenicity of *C. mirare* is not the result of acquisition of a significant pathogenicity island, and that this clinical isolate broadly resembles environmental *Caulobacter* isolates that were previously considered non-pathogenic.

Both *C. mirare* and *C. crescentus* are pathogenic towards *Galleria mellonella*

Given the genomic similarity between *C. mirare* and *C. crescentus* we sought to directly compare their pathogenic potential in an *in vivo* host model. *Galleria mellonella*, the greater wax moth, has emerged as a useful system for assessing infection potential due to its relatively short

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lifespan and ability to inject a defined inoculum of bacteria [25]. Additionally, *Galleria* produces melanin upon infection as part of its immune response, providing a robust visual readout for host health. The process of melanization is irreversible such that even if *Galleria* successfully eliminates the cause of infection, it maintains a dark coloration that corresponds to the degree of its immune response [26, 27].

To quantitatively assay bacterial virulence, we injected *Galleria* with similar numbers of exponentially growing bacteria and monitored melanization after 24 hours, which included fatal events (Fig 2A). As a negative control, we confirmed that mock injections of *Galleria* with water had no effect on melanization. Injection with a lab *E. coli* MG1655 strain also had no effect on *Galleria* melanization, indicating that not all bacteria are pathogenic towards *Galleria* (Fig 2B) [28]. In contrast, injection with *C. mirare* resulted in significant melanization within 24 hours (Fig 2B), suggesting that *Galleria* is a useful model for studying *C. mirare* pathogenesis. Interestingly, injection with two different lab strains of *C. crescentus,* CB15 and NA1000, also resulted in significant *Galleria* melanization within 24 hours (Fig 2B) [29]. The extent of the pathogenesis of the lab *C. crescentus* strains towards *Galleria* was comparable to that of the clinical *C. mirare* strain.

To follow the dynamics of pathogenesis we performed a healthspan assay by monitoring melanization as a function of time after injecting *E. coli* (MG1655)*, C. mirare* (SSI4214)*,* and *C. crescentus* (CB15 and NA1000). Even five days post injection, no melanization was observed with *E. coli*, validating its use as a non-pathogenic control (Fig 2C). Meanwhile, significant melanization was observed within 1 day of injecting any of the *Caulobacter* strains and increased as a function of time (Fig 2C). *Galleria* injected with the clinical *C. mirare* and lab *C. crescentus* strains displayed similar healthspans (Fig 2C). Together these data suggest that *C. mirare* can function as an opportunistic pathogen, consistent with its clinical isolation and pathology. However, *C. mirare* pathogenesis is not unique, but rather a feature it shares with environmental isolates of *C. crescentus.*

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163

164 **A cell-associated toxic factor facilitates the pathogenesis of *Caulobacter* in *Galleria***

165 Since *C. crescentus* infected *Galleria* as well as *C. mirare* but is more experimentally
166 tractable, we focused our efforts on characterizing the mechanism of *Caulobacter* pathogenesis
167 on *C. crescentus.* We first determined whether *Galleria* melanization requires *C. crescentus*
168 growth within the host by heat-killing exponentially-growing bacterial cells prior to injection.
169 Using the same starting number of bacterial cells, heat-killed *C. crescentus* induced similar
170 melanization to living cells (Fig 3A). Thus, the melanization of *Galleria* by *C. crescentus* is not
171 merely a secondary consequence of bacterial growth within the host or outcompeting the host
172 for nutrients.

173 We next interrogated whether *Galleria* melanization by *C. crescentus* is due to a
174 hyperactivated immune response. Dexamethasone 21-phosphate was previously shown to
175 suppress immune function in *Galleria* by inhibiting macrophage-like haemocyte cells that are
176 responsible for cellular and humoral immunity [30]. Because suppression via dexamethasome
177 would limit activation of the humoral response, decreased melanization upon infection and
178 dexamethasome treatment would indicate that the response is due to immune hyperactivation
179 while increased melanization would indicate that the response is due to bacterial-associated
180 cytotoxicity. We thus co-injected *Galleria* with each of our bacterial strains and dexamethasone.
181 For MG1655 *E. coli* we found that dexamethasone treatment significantly increased
182 melanization (Fig 3B). This finding is consistent with a previous study suggesting that *E. coli* are
183 capable of virulence towards *Galleria* when immunosuppressed by dexamethasone but that the
184 *Galleria* immune system is normally sufficient to prevent *E. coli* pathogenesis [30]. For CB15 *C.*
185 *crescentus* we found that dexamethasone treatment did not reduce melanization. However, *C.*
186 *crescentus*-induced melanization is so robust even in the absence of immunosuppression that
187 any such increase is difficult to detect (Fig 3B). These results suggest that active haemocytes

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188 prevent *E. coli* from being deleterious in *Galleria* while *C. crescentus* possesses an additional
189 feature that contributes to its ability to infect immunocompetent worms.

190 Given that the dexamethasone treatment suggested that *C. crescentus* is directly
191 cytotoxic towards *Galleria*, we hypothesized that symptomatic infection is induced via a toxic
192 factor. A hallmark of toxin-associated pathogenesis is quantitative dependence on bacterial
193 load. To assess the bacterial load required to cause an infection phenotype, we injected *Galleria*
194 with four-fold serial dilutions of overnight cultures of CB15 and MG1655 and performed
195 healthspan assays. For both *C. crescentus* and *E. coli,* we observed the expected dose-
196 dependence of infection, with increased melanization as a function of increased numbers of
197 bacteria injected (Fig 3C). This experiment also reinforced the difference in pathogenic potential
198 of the two bacterial species, as the lowest number of *C. crescentus* injected, (~10^3), caused
199 more melanization than even the highest number of *E. coli* injected (~10^7).

200 To determine whether the cytotoxicity of *C. crescentus* is due to a secreted or cell-
201 associated factor, we injected *Galleria* with *C. crescentus*-conditioned media. Specifically, we
202 centrifuged an overnight *C. crescentus* culture capable of inducing *Galleria* melanization at low
203 speeds (5700 g) to remove bacterial cells and cell-associated factors and injected the
204 supernatant that retains secreted factors. CB15 and MG1655 conditioned media did not induce
205 *Galleria* melanization (Fig 3D). As a positive control to confirm that it is possible to induce
206 melanization with secreted toxins we also isolated conditioned media from *Pseudomonas*
207 *aeruginosa* strain PA14, which is known to secrete exotoxins (Fig 3D) [31]. We confirmed that
208 PA14-conditioned media induced *Galleria* melanization, suggesting that the *C. crescentus* toxic
209 factor is not secreted.

210

211 **_Caulobacter_ detection in the clinic may be limited due to culturing requirements**

212 *Caulobacters* are ubiquitously present in water systems and our results suggest that they can
213 function as pathogens in some contexts, raising the question of why *Caulobacters* have not

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been more commonly associated with human infections. One possibility is that *Caulobacter* infections are more common than currently appreciated but that *Caulobacters* are not readily isolated by traditional clinical methods. Because most classical identification methods rely on culturing, we compared the culturing requirements of *C. crescentus* and *C. mirare*. *C. mirare* was isolated using Danish blood agar plates [7], and we confirmed that the SSI4214 strain indeed grows on sheep's blood agar (Fig 4A). In contrast, CB15 *C. crescentus* was unable to grow on sheep's blood agar (Fig 4A). To determine the root cause of this difference, we compared *C. crescentus* and *C. mirare* growth on several complex media. Both species grew robustly on peptone-yeast extract agar, the standard culturing medium for CB15, and nutrient agar. Media with higher salt concentrations, such as Luria broth and terrific broth, did not allow for growth of either *Caulobacter* species. Meanwhile, lower salt-containing media such as tryptic soy agar and super optimal broth, promoted the growth of *C. mirare* but not *C. crescentus* (Fig 4A).

To directly determine if salt content is the relevant growth-determining difference in these media we plated both *Caulobacter* species on PYE in which we replaced the normal $MgSO_4$ salt with varying amounts of NaCl. Both species still grew on modified PYE with no salt added (Fig 4B). We then increased the NaCl content of the modified PYE and found that while both *Caulobacter* species grew well at 8.6 mM NaCl, *C. mirare* continued to grow well at 86 mM and 171 mM NaCl, while *C. crescentus* grew poorly at 86 mM NaCl and failed to grow at all at 171 mM NaCl (Fig 4B). These data suggest that the increased salt tolerance of *C. mirare*, while not necessary for *Galleria* pathogenesis, may explain why this strain could be cultured from an infected patient.

DISCUSSION

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Our work demonstrates that both the clinical *C. mirare* and lab *C. crescentus* species can function as pathogens of *Galleria* with similar degrees of virulence. Not all bacteria can perturb *Galleria* healthspan, as lab strains of *E. coli* proved non-pathogenic in this context (Fig 2). Furthermore, sequencing and analysis of the *C. mirare* genome indicated that this clinical isolate is similar to *C. crescentus* and related *Caulobacters* that were also considered to be non-pathogenic like *C. segnis* (Fig 1). *C. mirare* does not appear to have acquired any clear pathogenicity islands or virulence factors [24]. Coupled with its similar extent of pathogenicity as *C. crescentus*, our findings thus suggest that *C. mirare* is not unique in its ability to cause human disease but that the capacity for virulence may be a general yet previously-unappreciated feature of *Caulobacters*.

In addition to showing that both *C. mirare* and *C. crescentus* can be pathogenic, establishing *Galleria* as a model host for *Caulobacter* provided us with a tractable system for probing the mechanism of pathogenesis. Specifically, we showed that *C. crescentus* pathogenesis persisted in immunocompromised worms, suggesting that *Caulobacter* directly damages the worm as opposed to overactivating the immune system (Fig 3B). Consistent with this finding, all clinical reports of human *Caulobacter* infections occurred in hospital settings with patients who are likely immunocompromised [7-10]. Additional characterization suggested that *C. crescentus* has a cell-associated toxin responsible for its pathogenesis, as *C. crescentus* virulence is dose-dependent, heat-tolerant, and not secreted (Fig 3). One class of cell-associated factors are immunogenic factors such as lipopolysaccharide (LPS) or peptidoglycan (PG) [32, 33]. However, our immunosuppression results suggest that *C. crescentus* pathogenesis is not due to immune hyperactivation and the avirulence of *E. coli* (which has both LPS and PG) indicates that the *C. crescentus* toxic factor is not ubiquitous among Gram-negative bacteria. Alternatively, *C. crescentus* could express a cytotoxic product that actively targets eukaryotic cells. Supporting this model, a survey of bacteria from various aquatic

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263 sources demonstrated that *Caulobacter segnis* (the only *Caulobacter* species present in this
264 survey) can directly lyse amoebae [34]. In the future, identification and characterization of the
265 toxin responsible for *Caulobacter* pathogenesis would enable the engineering of *Caulobacter*
266 strains that are less toxic and thus more attractive as vectors for bioengineering or medical
267 applications.

268 If *Caulobacter* species can generally function as opportunistic pathogens, perhaps even
269 of humans, why is the isolation of *Caulobacters* as human pathogens so rare? Typically,
270 successful pathogens need to survive in the environment of their hosts [35]. *Caulobacter* is
271 often described as an oligotroph since it is found in nutrient-poor environments such as fresh-
272 water lakes and drinking water [3, 36]. However, both our work and previous studies show that
273 *Caulobacter* can also thrive in nutrient-rich culturing conditions (Fig 4). Metabolomic studies of
274 the fluids from common infection sites such as peritoneal fluid, cerebral spinal fluid, and plasma
275 show that these fluids contain metabolites and salt concentrations similar to those in media that
276 support *Caulobacter* growth [37-39]. Thus, it is possible that *Caulobacter* species can grow in
277 human hosts and that the reason they are not often detected is that they are not readily
278 culturable on the media commonly used for clinical microbiology [40, 41]. Consistent with this
279 hypothesis, we showed that *C. mirare* can be cultured on blood agar while *C. crescentus*
280 cannot, likely due to the increased salt tolerance of *C. mirare* (Fig 4). This difference could
281 explain why *C. mirare* could be isolated from a patient and suggests *Caulobacter* infections may
282 be more widespread nosocomial pathogens than previously appreciated. Assessing the true
283 extent of *Caulobacter* as a human pathogen will be aided by implementation of culture-
284 independent pathogen identification method like those based on mass spectrometry or
285 metagenomics [42, 43].

286 The ability of a classically defined “non-pathogen” like *C. crescentus* to cause disease in
287 the *Galleria* animal model raises the question of what defines a pathogen and are there really

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non-pathogenic bacteria? Combining our findings with previous work on *C. crescentus* suggests that *Caulobacter* can carry out many of the processes typical of other pathogens, including biofilm formation, antibiotic resistance, killing of non-self bacteria, and toxin production [7, 44, 45]. Unlike the patient-isolated *C. mirare,* the CB15 *C. crescentus* strain studied here is an environmental isolate from a freshwater lake [1]. The ability of this environmental isolate to retain pathogenesis towards an animal host suggests that *Caulobacters* can survive in multiple niches [3, 35]. Both *C. crescentus* and *C. segnis* lack obvious host invasion factors, suggesting that their pathogenesis requires a compromised host and explaining why they are opportunistic pathogens. A recent opinion article suggested that pathogenesis should be viewed as a spectrum and that most bacteria will be pathogenic if they can grow to a sufficient concentration within a host [46]. Our study supports this perspective, suggesting that broadening how we identify and isolate pathogens in clinical settings will allow us to better understand the spectrum of pathogens that actually infect humans. Elucidating the pathogenic potential of more bacteria and the mechanisms by which they cause disease may thus ultimately help combat the challenge of undiagnosed infections.

MATERIALS AND METHODS

Bacterial strains and growth conditions

For this study, an overnight culture is defined as a single colony inoculated in 5 ml tubes and grown for 16 hours. Exponential phase cultures were obtained by a 20-fold back dilution of overnight culture in fresh media and grown to an OD_{660} of ~0.5. *Caulobacter crescentus* laboratory strains (CB15 and NA1000) were grown in shaking culture at 30^{o}C in PYE media on platform shakers. *Caulobacter mirare* (SSI4214) was grown in nutrient broth (NB) at 37^{o}C in shaking culture. *E. coli* MG1655 and *Pseudomonas aeruginosa* PA14 were grown at 37^{o}C in LB

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312 medium either in shaking culture or roller drum, respectively. Components of organisms'
313 respective growth media as well as other medias for agar plating have been described
314 previously.

315 **Genomic analysis and phylogeny construction**

316 Paired-end 150 nt Illumina MiSeq sequencing was performed on all samples at
317 Princeton University's Genomics Core. Scaffolds were generated from reads using UniCycler
318 default settings on "normal mode," and assembly metrics were compiled using QUAST [47].
319 Annotation of the genome was accomplished via DFast with default settings [21]. For
320 phylogenetic construction, an online pipeline (www.phylogeny.fr) was used with default settings.
321 Alignment via MUSCLE was run on "full mode" and phylogeny was determined by bootstrapping
322 with 100 runs. Visualization of the tree was created using TreeDyn [48].

323 ***Galleria mellonella* healthspan assay**

324 All *Galleria mellonella* larvae were Vita-Bugs© distributed through PetCo© (San Diego, CA) and
325 kept in a 20^{o}C chamber. Larvae were used for healthspan assays within three days of receipt of
326 package. Worms which were not already melanized were assigned randomly to infection or
327 control cohorts. All inoculums were administered using a sterile 1 ml syringe attached to a KD
328 Scientific pump. Same volume injections (5 µL) were delivered at a rate of 250 µl/min to the
329 fourth leg of the worm, which was sterilized with ethanol. Melanization phenotype was
330 determined by observation of a solid black line along the dorsal midline of the larva (Fig 3A).
331 Each figure graph is a representative cohort (n = 10-15 per treatment) from a biological
332 triplicate, except for PA14 which was performed separately (Fig 4D). Mantel-cox statistics for
333 the cohort were calculated using PRISM, and the pooled results are presented in the
334 supplement (S2 Fig).

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For co-injection immunosuppression experiments, a dexamethasone 21-phosphate disodium stock solution was made (solubilizing 50 mg/ml in H_20) and injected at 200 µg/larva as previously described [29]. Worms not co-injected with dexamethasone-21 were mock injected with sterile H_20. For serial dilution experiments, overnight cultures were diluted 4-fold in their respective medium. CFUs were determined by plating overnight cultures on agar plates. For conditioned media experiments, overnight cultures were centrifuged at 5700xg for 3 minutes and the resulting supernatant was injected into the worms. For heat-killing experiments, exponentially growing bacteria were held at 100°C for 10 minutes.

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FIGURE LEGENDS

Figure 1: Genomic comparison of *Caulobacter mirare* SSI4214 to related *Caulobacter* species. (A) Phylogenetic tree containing SSI4214 along with closely related species. Numbers indicate bootstrapping confidence values for nodes after 100 replicates. Bar represents average nucleotide substitution/site (B) BLAST values of conserved and *Caulobacter* genus-specific genes.

Table 1: Genomic features of *Caulobacter mirare* draft genome assembly compared to *C. crescentus* and *C. segnis*.

Figure 2: *Galleria mellonella* healthspan decreases upon *Caulobacter* infection. (A) Example images of phenotypes considered for scoring in healthspan assay. (B) Percentage of worms scored as healthy or melanated 24 hours post-inoculation. Error bars represent standard error for three biological replicates. (C) Kaplan-Meier survival analysis for *C. crescentus* strains CB15 and NA1000, *E. coli* strain MG1655 and *C. mirare* strain SSI4214. Survival curve shown is one representative cohort (n=15) of three biological replicates (Mantel-Cox test for statistics, ***P < .001).

Figure 3: *Caulobacter* pathogenesis is induced by a toxic cell-associated factor. (A) Healthspan of *Galleria* upon injection of 5 µL of OD_{660} ~ 0.5 (exponentially-growing) live and heat-killed CB15 and MG1655 (B). Healthspan of *Galleria* upon injection with serial dilutions of

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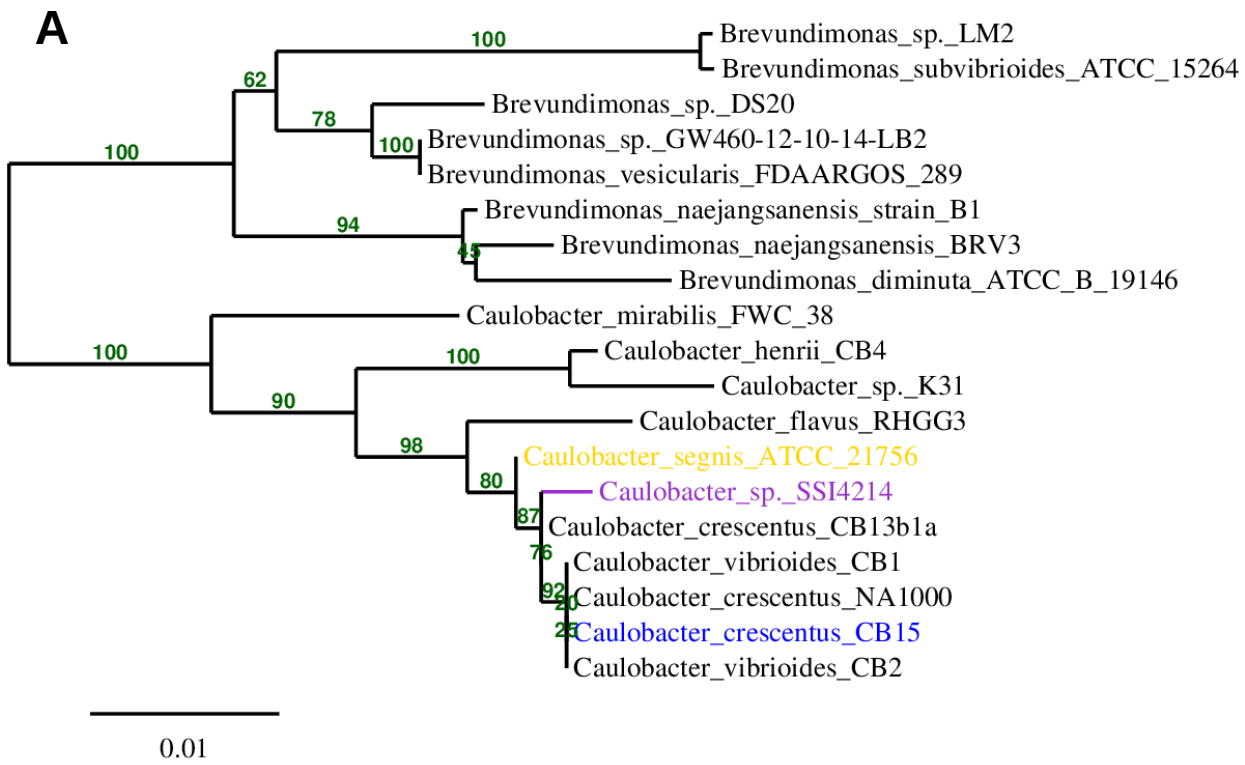
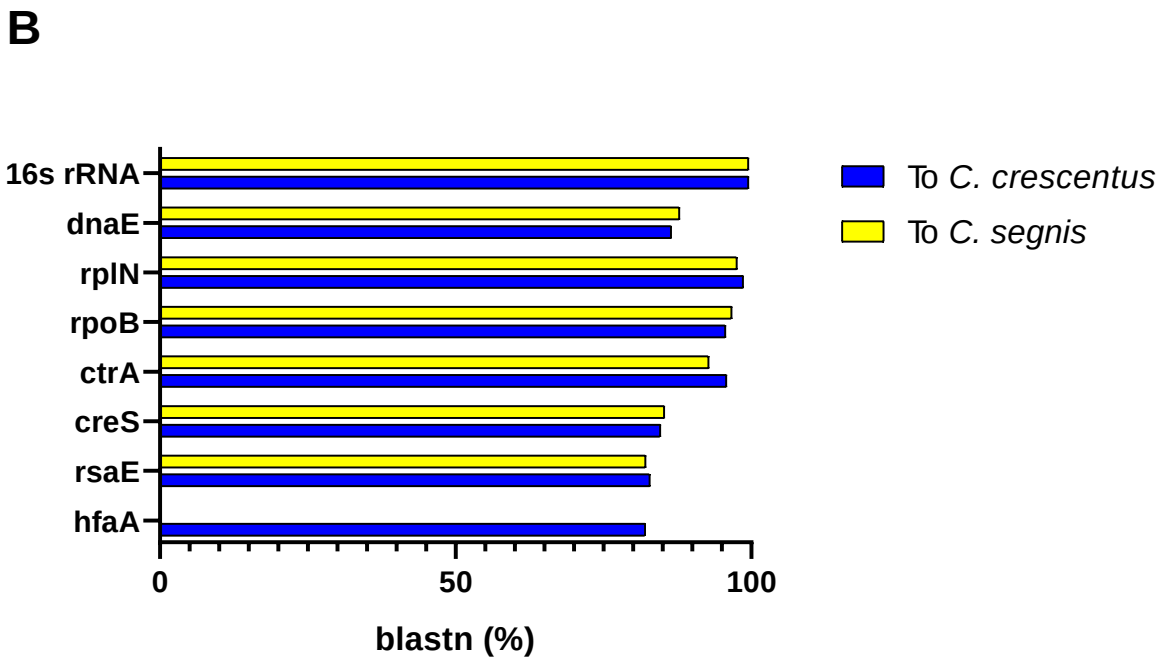
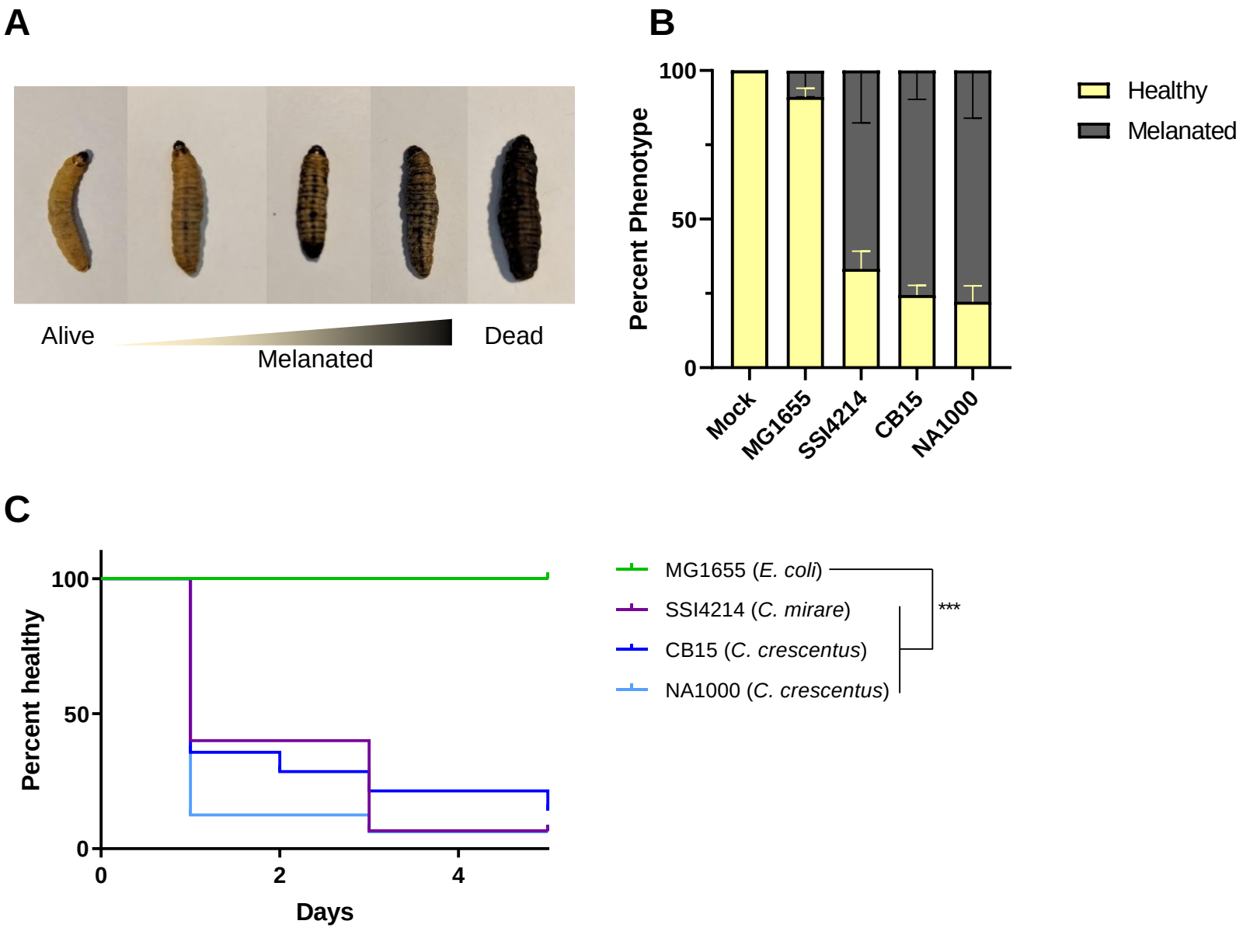
overnight CB15 or MG1655. (C) Healthspan of *Galleria* upon co-injections of exponentially growing CB15 or MG1655 and 200 ug/larva dexamethasone 21-phosphate (+Dex). (D) Healthspan of *Galleria* upon injection of supernatant derived from overnight cultures of CB15, MG1655, or *Pseudomonas aeruginosa* strain PA14. All survival curves are a representative cohort (n = 10-15) of three biological replicates (Mantel-Cox test for statistics, *P < 0.05, **P < 0.01, ***P < .001).

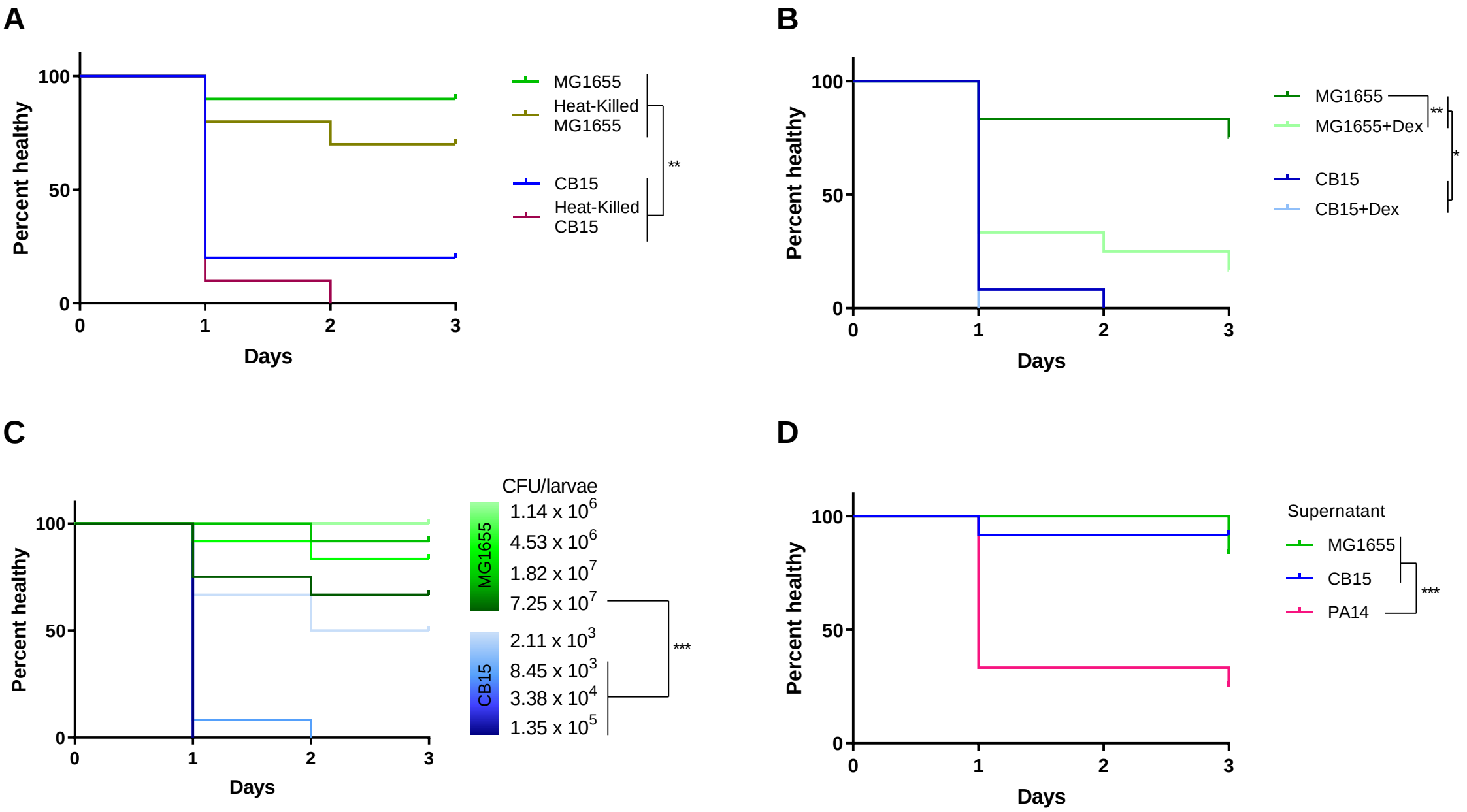
Figure 4: Culturability of *Caulobacter crescentus* (CB15) and *Caulobacter mirare* (SSI4214). (A-B) Three replicates each of 10^{-3}-diluted overnight culture of CB15 (left) or SSI4214 (right) on various media. (A) PYE = peptone-yeast extract, NB = nutrient broth, LB = luria broth, TB = terrific broth, TSA = tryptic soy agar, TSA+SB = tryptic soy agar + 5% sheep blood, SOB = super optimal broth. (B) PYE-ns = PYE without added salts, +8.6mM NaCl = PYE-ns with addition of 8.6 mM NaCl, +86mM NaCl = PYE-ns with addition of 86 mM NaCl, +171 mM NaCl = PYE-ns with addition of 171 mM NaCl.


A
100
62
78
100
100
94
45
100
100
90
98
80
87
76
92
20
25
Brevundimonas_sp._LM2
Brevundimonas_subvibrioides_ATCC_15264
Brevundimonas_sp._DS20
Brevundimonas_sp._GW460-12-10-14-LB2
Brevundimonas_vesicularis_FDAARGOS_289
Brevundimonas_naejangsanensis_strain_B1
Brevundimonas_naejangsanensis_BRV3
Brevundimonas_diminuta_ATCC_B_19146
Caulobacter_mirabilis_FWC_38
Caulobacter_henrii_CB4
Caulobacter_sp._K31
Caulobacter_flavus_RHGG3
Caulobacter_segnis_ATCC_21756
Caulobacter_sp._SSI4214
Caulobacter_crescentus_CB13b1a
Caulobacter_vibrioides_CB1
Caulobacter_crescentus_NA1000
Caulobacter_crescentus_CB15
Caulobacter_vibrioides_CB2
0.01

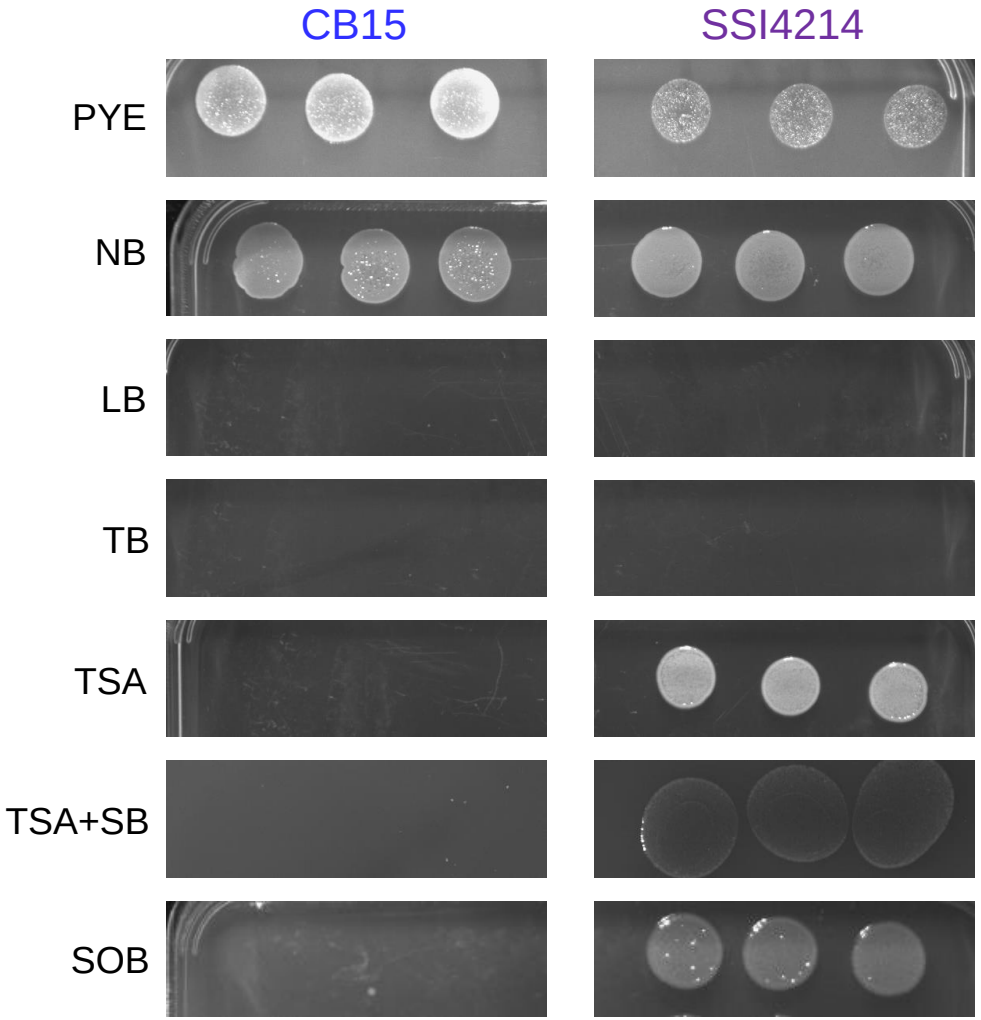

B
16s rRNA
dnaE
rplN
rpoB
ctrA
creS
rsaE
hfaA
0
50
100
blastn (%)
To C. crescentus
To C. segnis

	C. crescentus CB15 (complete)	*C. mirare* SSI4214 (draft genome)	*C. segnis* TK0059 (complete)
Genome Size (bps)	4,016,947	4,789,750	4,655,622
GC Content (%)	67.21	67.51	67.67
Predicted-Coding Genes	3,819	4,329	4,330
Pathogenicity Islands	0	0	0
Average Nucleotide Identity (%) of SSI4214 to	83.88		84.75

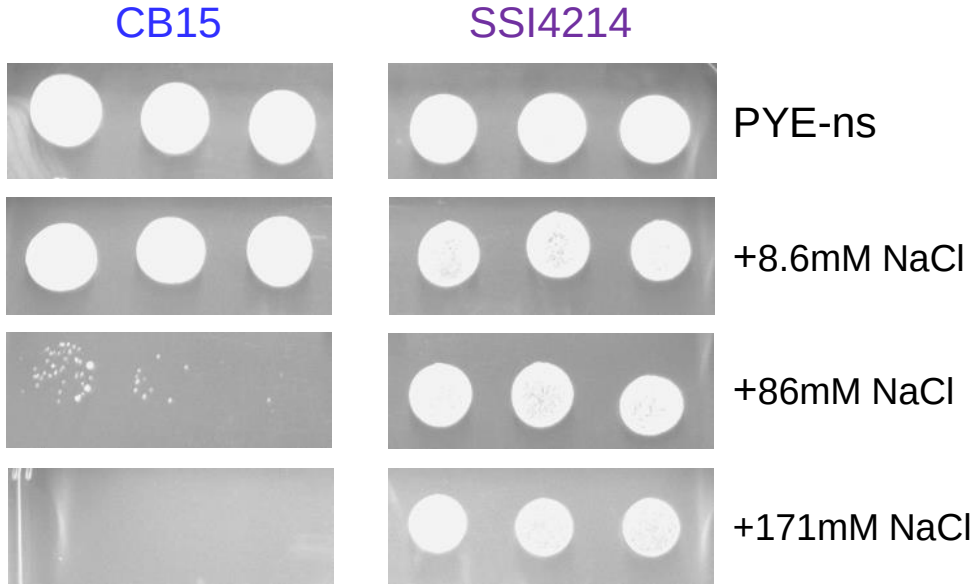

A
Alive
Dead
Melanated
B
Percent Phenotype
100
50
0
Mock
MG1655
SSI4214
CB15
NA1000
Healthy
Melanated
C
Percent healthy
100
50
0
0
2
4
Days
MG1655 (E. coli)
SSI4214 (C. mirare)
CB15 (C. crescentus)
NA1000 (C. crescentus)
***


A
Percent healthy
100
50
0
0
1
2
3
Days
MG1655
Heat-Killed MG1655
CB15
Heat-Killed CB15
**
B
Percent healthy
100
50
0
0
1
2
3
Days
MG1655
MG1655+Dex
CB15
CB15+Dex
**
*
C
Percent healthy
100
50
0
0
1
2
3
Days
CFU/larvae
MG1655
1.14 x 10^6
4.53 x 10^6
1.82 x 10^7
7.25 x 10^7
CB15
2.11 x 10^3
8.45 x 10^3
3.38 x 10^4
1.35 x 10^5
***
D
Percent healthy
100
50
0
0
1
2
3
Days
Supernatant
MG1655
CB15
PA14
***

A


CB15
SSI4214
PYE
NB
LB
TB
TSA
TSA+SB
SOB

B


CB15
SSI4214
PYE-ns
+8.6mM NaCl
+86mM NaCl
+171mM NaCl